ELUCIDATING THE EFFECT OF SEX ON THE METABOLIC AND VASCULAR PERTURBATIONS INDUCED BY THE ABSENCE OF ADIPONECTIN

by

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A thesis submitted to the Faculty of Graduate Studies,
University of Manitoba
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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University of Manitoba
2016

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ABSTRACT

Adiponectin is an abundant hormone secreted by adipocytes that exhibits anti-diabetic, anti-inflammatory and anti-atherogenic properties. However, in obesity, as adipocytes enlarge, adiponectin secretion declines. A sex dimorphism is observed in adiponectin levels with women having higher levels than men. We hypothesized that a lack of adiponectin negatively affects both insulin sensitivity and adipose physiology in a sex-dependent manner. In this study, male and female adiponectin knockout or wild-type mice were fed with either a low fat or high fat diet. Male knockout animals were more glucose intolerant and had elevated fasting glucose levels. In contrast, both adiponectin knockout and wild-type females showed decreased hepatic lipid accumulation on a high fat diet. In both sexes, lean adiponectin knockout mice had significantly smaller fat depot weights and lesser hepatic lipid accumulation than the lean wild-type mice. However, on high fat diet, only male adiponectin knockout mice had fat depot weights that were comparable to wild-type mice, which indicate a novel sex-specific role of adiponectin in determining adiposity.

ACKNOWLEDGEMENTS

I am grateful to a lot of people who contributed in various ways for the successful completion of this thesis work. It was quite an experience for me as an international student. Beyond earning an academic degree, it was a journey that strengthened me and taught me many things in my professional as well as personal life.

First of all, I am extremely indebted to my 'Super'visor, Dr. Jeffrey Wigle for giving me an opportunity to work with him and for his relentless support, momentum and tremendous patience all throughout my stay in his lab and completion of my thesis. Thank you so much for all the encouragements, freedom and the knowledge you have imparted, which has helped me to start think, work and explore independently.

Secondly, I would like to thank my committee member, Dr. Peter Zahradka for all his support, time (I must say he always found time for me even in the middle of his busy works) and consideration as his own student during my time here. He was always a great relief during committee meetings with your wits and at the same time motivating me to think critically about my research. I am very much grateful to Dr. Carla Taylor for being so supportive and for her constant guidance during my animal studies. Her sweet smile and pleasant face has always made my day a better one and her patience to sit with me and listen to my questions have helped me tackle the various aspects of statistics, an area I was weak at.

I would also like to thank Dr. Mark Nachtigal, for being in my advisory committee and prompting me to think critically- deeper and broader about my project. Additionally, I thank him for being an excellent Cell Biology teacher. I further extend my sincere appreciation to Dr. Leigh Murphy for taking time out of her busy schedule in the main campus to chair my committee meetings and for providing us with invaluable inputs which later helped to streamline my thesis project.

I acknowledge the financial supports from Canadian Diabetes Association, Research Manitoba, St. Boniface Hospital Foundation, University of Manitoba and Sir Ratan Tata Trust, which enabled me to

pursue my research studies as well as various Travel Awards from University of Manitoba and Institute of Cardiovascular Science to present my work at national and international conferences.

I would like to take this opportunity to thank all the staff at the R.O. Burrell animal facility at SBRC for their assistance and cooperation during my animal studies; administrative staffs especially Susan Zettler, Cathy Webber, Tuntun Sarkar, Shweta, Mary Brown and Tracy for easing the administrative paper works; all Wigle-Zahradka-Taylor lab members for their assistance, expertise, support and fun time I had with them. Especially David Cheung, Jaime Clark (for her help during long hours of termination), Leslee Tworek (always ready with clarifications and technical help of any sort), Youjia Du (my personal cheerleader Elsa! ☺), Fahmida Jahan, Lena Hong, our bubbly summer student Qing Dai, Garret DeGagne (indeed Sunshine!), former students Dr. Azadeh Yeganeh and Dr. Josette Northcott (I simply wish if I could have been in lab before she left). Thanks Josette for helping me selflessly at many occasions.

Thank you so much Dr. Xavier Lieben Louis and Cinda Chechi for helping me settle down in the city since my arrival in Winnipeg and for being there as an extended family where I could always run to whenever I felt homesick.

I extend my gratitude to many other SBRC staffs, friends and families who I have met in Canada including David and Candy, Deepthi, Dr. Bagchis, Dr. Sijo Joseph and family for their friendships and emotional support which made my journey here a pleasant one.

Finally and most importantly, I would like to express my deep gratitude to my parents, P. M. Muraleedharan and K. Omana; and my sister Truptha Muraleedharan for their blessings, selfless love, confidence and the freedom they gave me to chase my dreams. Special thanks to my mother for being my ultimate support and strength now and always. Thank you so much Amma! ☺

Dedication

This thesis is dedicated to my loving family in India.

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LIST OF ABBREVIATIONS

ACC	Acetyl CoA Carboxylase
ACO	Acyl CoA Oxidase
Acrp30	Adipocyte Complement-Related Protein (30 kDa)/Adiponectin
ACS	Acyl-CoA Synthase
AdipoR1/2	Adiponectin Receptor 1/2
AMP	Adenosine Mono Phosphate
AMPKα	AMP Activated Protein Kinase Alpha
aP2	Adipocyte Fatty Acid Binding Protein P2
apM1	Adipose Most Abundant Gene Transcript 1
APN KO	Adiponectin Knockout
APPL1	Adaptor protein containing pleckstrin homology domain, phosphotyrosine-binding domain and leucine zipper motif 1
APS	Ammonium persulfate
ATP	Adenosine Triphosphate
AUC	Area Under Curve
BAT	Brown Adipose Tissue
BC	Body Composition
BCA	Bicinchoninic Acid
BMI	Body Mass Index
BP	Blood Pressure
BSA	Bovine Serum Albumin
Bwt	Body Weight
CAD	Coronary Artery Disease
CPT1	Carnitine Palmitoyl Transferase 1
CRP	C-reactive protein
CVD	Cardiovascular Disease
db/db	Leptin Receptor Deficient mouse model
DDT	Dichloro Diphenyl Trichloroethane
DIO	Diet-Induced Obesity
DMRT	Duncan's Multiple Range Test
DNL	*De Novo* Lipogenesis
EMT	Epithelial-to-Mesenchymal Transition
eNOS	Endothelial Nitric Oxide Synthase
ERK1/2	Extracellular Signal Regulated Kinase 1/2
ERα	Estrogen Receptor Alpha
FABP3	Fatty Acid Binding Protein 3
FAS	Fatty Acid Synthase
FAT/CD36	Fatty Acid Translocase/Cluster of Differentiation 36
FFA	Free Fatty Acids
G6Pase	Glucose-6-Phosphatase
gAcrp30	Globular Adiponectin
GBP28	Gelatin Binding Protein

Geno	Genotype
GLUT4	Glucose Transporter 4
GPCR	G Protein-Coupled Receptor
GPI	Glycosyl Phosphatidyl Inositol
GSK-3	Glycogen Synthase Kinase 3
H&E	Hematoxylin and Eosin
HAEC	Human Aortic Endothelial Cells
HDL	High Density Lipoprotein
HFD	High Fat Diet
HIF1α	Hypoxia-Inducible Factor 1 Alpha
HMW	High Molecular Weight
HNF1α	Hepatic Nuclear Factor 1 Alpha
HNF4α	Hepatic Nuclear Factor 4 Alpha
HRP	Horseradish Peroxidase
ICAM-1	Intercellular Adhesion Molecule 1
IGF-1	Insulin-like Growth Factor 1
IRS	Insulin Receptor Substrates
JNK	c-Jun N-terminal Kinase
KO	Knockout
LDL	Low Density Lipoprotein
LFD	Low Fat Diet
LMW	Low Molecular Weight
LPL	Lipoprotein Lipase
min	Minutes
MKP-1	Mitogen-activated Protein Kinase Phosphatase-1
mM	Millimoles
MMW	Medium Molecular Weight
MSC	Mesenchymal stem cells
mTOR	mammalian Target of Rapamycin
N.D.	Not Determined
N.S.	Not Significant
NADH	Nicotinamide Adenine Dinucleotide
NAFLD	Non-Alcoholic Fatty Liver Disease
NF-κB	Nuclear Factor Kappa B
ob/ob	Leptin Deficient mouse model
OCT	Optimal Cutting Temperature
OGTT	Oral Glucose Tolerance Test
p38 MAPK	p38 Mitogen-Activated Protein Kinase
PAD	Peripheral Arterial Disease
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PDGF	Platelet-Derived Growth Factor
PEPCK	Phospho enol Pyruvate Carboxykinase
PFA	Paraformaldehyde
PGC1α	Peroxisome Proliferator-activated Receptor γ Coactivator 1 Alpha

PI3K	Phosphatidylinositol 3-Kinase
PIP_2	PI 3, 4-Bisphosphate
PIP_3	PI 3, 4, 5-Triphosphate
PKB	Protein Kinase B
PPARα	Peroxisome Proliferator-Activated Receptor Alpha
PPARγ	Peroxisome Proliferator-Activated Receptor Gamma
PPRE	PPAR-Responsive Element
PVDF	Polyvinylidene Fluoride
PWV	Pulse Wave Velocity
QMR	Quantitative Nuclear Magnetic Resonance
RAR	Retinoic Acid Receptor
RXR	Retinoic X Receptor
S	Serine
SAPK	Stress-Activated Protein Kinase
SCAT	Subcutaneous Adipose Tissue
SEM	Standard Error of the Mean
SNP	Single Nucleotide Polymorphisms
SR-A	Class A Scavenger Receptor
TBST	Tris Buffered Saline Tween 20
TCA	Tri Carboxylic Acid
T-cad	Truncated Cadherin
TEMED	Tetramethylethylenediamine
TFs	Tissue Factors
TGFβ	Transforming Growth Factor beta
TGs	Triglycerides
TIMP-1	Tissue Inhibitor of Metalloproteinase-1
Tris	Tris(hydroxymthyl)aminomethane
TZD	Thiazolidine Diones
UCP 1/2	Uncoupling Protein 1/2
Vaspin	Visceral Adipose Tissue derived Serpin
VAT	Visceral Adipose Tissue
VCAM-1	Vascular Cell Adhesion Molecule-1
VEGF	Vascular Endothelial Growth Factor
VLDL	Very Low Density Lipoprotein
VSMC	Vascular Smooth Muscle Cell
WAT	White Adipose Tissue
WT	Wild-type

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1	Figure 2	NRC Research Press: Northcott et al. (2012) Canadian Journal of Physiology and Pharmacology
2	Figure 4	Endocrine Society: Kadowaki and Yamauchi (2005) Endocrine Reviews
3	Figure 5	Nature Publishing Group: Okada-Iwabu et al. (2013) Nature

INTRODUCTION

Obesity is rapidly increasing with 30% of the world's population being either obese or overweight in 2014. This substantial recent rise in global obesity presents a major public health epidemic in both developed and developing countries (Ng et al. 2014). The United States has the highest proportion of the world's obese people (13%) while China and India together have 15%, with the rates being adjusted for differences in population size and ages. However, while men had higher rates of obesity in developed countries, women had higher rates of obesity in developing countries. Also in developed countries, the peak obesity rates are moving to younger ages (Ng et al. 2014).

This high prevalence of obesity is alarming as it is a major risk factor for the development of disorders such as hypertension, Type 2 diabetes, renal failure, cardiovascular diseases, certain forms of cancer and osteoarthritis (Kaplan 1989; Masaki and Yoshimatsu 2008). In 2010, obesity and being overweight were estimated to have caused 3.4 million deaths, most of which were from cardiovascular diseases (Lim et al. 2012). In most cases, obesity results from the excess consumption of food relative to energy expenditure and thus increased adiposity or body fat mass. Body mass index (BMI) is the most widely used proxy of adiposity and it is defined as the weight-to-height ratio (kg/m^2). Individuals with a BMI greater than or equal to 25 are considered overweight and those having a BMI greater than or equal to 30 are considered obese.

Although the correlation of BMI with body fat percentage is positive, it is only a surrogate measure of adiposity because it is based on weight rather than fat mass and thus fails to reflect the changes in body composition that occur with age, sex and ethnic differences. For instance, older adults tend to have more body fat than younger ones for equivalent BMI and also

the loss of height through ageing may result in increased BMI without increased weight in elderly. Similarly, women have a greater body fat percentage than men with an equivalent BMI. Thus it has been recommended to use waist-to-height, waist-to-hip ratio or body fat composition as better predictors of adiposity than BMI (Chan et al. 2003). Furthermore, studies show that even a moderate increase in BMI makes South Asians more prone to insulin resistance and related diseases. Thus it has been suggested that lower cut-off values for BMI should be adopted in Asia as compared to Western countries (de Wilde et al. 2013).

A recent study involving a cohort of a healthy English population (n=2993) showed significant differences in BMI between different sex and age groups using body fat percentage as the covariate and thus reveals the necessity to redefine age- and sex-specific overweight and obese BMI cut-off points to more fairly reflect the critical adiposity levels throughout a population (Nevill and Metsios 2015). However, BMI remains the current gold standard to establish obesity due to the relative ease of its determination and its clear association with increased mortality from cardiovascular disease.

Altogether, obesity exerts a tremendous burden on the world economy and health care system. Over the past three decades no nation has seen a decrease in this accelerated rate of obesity which suggests intense study will be required to tackle the pandemic and minimize the risk of new cases of diabetes, heart disease and other obesity-related complications.

As mentioned above, energy homeostasis requires a balance between fat storage and energy utilization. This balance is maintained by the regulation of fat cells, which is an endocrine unit as well. However, excess adiposity results in an inflammatory secretome, particularly from central visceral fat depots and enhances insulin resistance, excessive lipotoxicity and hypertension that escalate atherosclerosis including coronary artery disease. These effects are

found to be opposed by adipocyte hormones such as adiponectin and visfatin that help maintain normal energy utilization (Berg et al. 2002).

1. Adipose Tissue

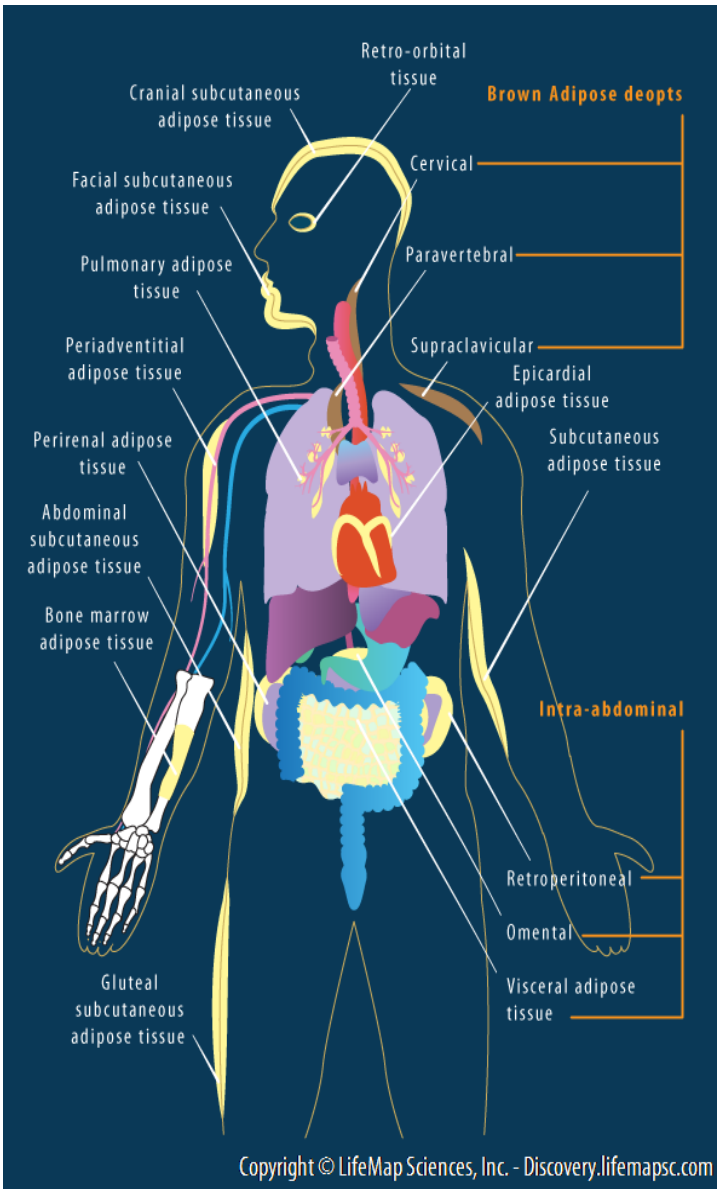
Adipose tissue is a highly specialised organ which stores excess energy in the form of triglycerides (TGs) and thus serves as a major fuel reservoir in vertebrates. Adipose tissue also functions as a metabolic sink and protects other tissues from lipotoxicity. It is composed mainly of adipocytes along with connective tissue matrix, vascular and neural tissues and other non-fat cells such as inflammatory cells (macrophages), pre-adipocytes and fibroblasts. Obesity results when there is an imbalance between energy intake and energy expenditure leading to energy surplus. As a result, adipocytes expand due to increased lipid synthesis and storage. The pathological conditions accompanying obesity result from this excess fat accumulation, which adversely alters adipose tissue homeostasis. Apart from its classic function of storing fat, white adipose tissue (WAT) has been found to be an endocrine organ and regulates insulin sensitivity and other physiological processes (Havel 2002).

Another important adipose tissue type is the brown adipose tissue (BAT), which dissipates energy as heat by uncoupling the electron transport chain in the mitochondria via a protein called uncoupling protein 1 (UCP 1) (Ricquier and Bouillaud 2000). BAT serves as a thermogenic source both in infants as well as in adults living in colder regions (Nedergaard et al. 2007).

1.1 Major white adipose tissue depots in the body

The major WAT depot is the visceral adipose tissue (VAT) located within the abdominal cavity between internal organs and hence also known as intra-abdominal fat. In rodents, this depot includes several different fat pads such as the mesenteric, peri-renal, gonadal known as epididymal in males and parametrical in females; and epicardial adipose tissue. Omental fat is the predominant VAT in humans seen attached to the stomach and spleen as shown in Figure 1. While mesenteric WAT is located along the intestine, peri-renal WAT is located around the kidneys and epicardial WAT is found around the heart.

Subcutaneous adipose tissue (SCAT) is found underneath the skin and is another major WAT depot. Inguinal adipose tissue is the predominant SCAT depot in rodents and is situated anterior to the upper region of the hind limbs (Figure1) (Gupta and Gupta 2015).



http://discovery.lifemapsc.com/library/images/human-fat-distribution

Figure 1: Fat tissue depots in the human body. The various white adipose tissue (WAT) depots such as omental WAT, peri-renal WAT, mesenteric WAT, epicardial WAT, subcutaneous WAT *etc* are shown in yellow and various brown adipose tissue (BAT) depots are shown in brown colour.

1.2 Adipose tissue depot distribution and its pathophysiological implications

The distribution of WAT rather than simply total body fat is critical in predicting cardiometabolic health risk. Individuals with higher abdominal or VAT than SCAT carry a greater risk of developing comorbidities associated with obesity. The close proximity of VAT to liver facilitates the drainage of visceral blood containing various secretory molecules such as cytokines and adipokines and free fatty acids to the liver through the portal vein and thus has a more pronounced effect on hepatic function than SCAT (Nielsen et al. 2004).

Additionally, smaller adipocytes, the normal physiological buffer or sink which actively absorb free fatty acids and TGs, are more abundant in SCAT than in VAT (Misra and Vikram 2003). Visceral white adipose tissue in turn contains more larger adipocytes, which tend to be more insulin resistant, hyperlipolytic and resistant to the anti-lipolytic effect of insulin (Abate et al. 1995; Frayn 2000). Insulin resistance results in the reduced uptake of glucose and lipids into the adipocytes. Hence individuals with abdominal obesity have lower rates of glucose disposal and lipid β-oxidation. Consequently insulin resistance of adipocytes could be a major factor linking visceral adiposity to cardiovascular risk (Kadowaki et al. 2003).

Another important factor is the mesothelial layer that lines the human and rodent VAT but is absent around the SCAT (Chau et al. 2014; Darimont et al. 2008). Adipose mesothelial cells secrete interleukin 8 (IL-8) *in vitro* upon treatment with inflammatory cytokines such as IL-1β and TNFα (Topley et al. 1993). This secretion correlates with the association of elevated circulating IL-8 with BMI, waist circumference, insulin resistance and development of atherosclerosis and cardiovascular disease (Kim et al., 2006). Fibrotic remodeling of adipose tissue is a pronounced feature in obese subjects with metabolic syndrome. Mesothelial cells are shown to readily undergo epithelial-to-mesenchymal transition (EMT) in a transforming growth

factor β (TGFβ) dependent manner in lung, liver and kidney fibrosis and this process is implicated in adipose tissue remodeling as well (Gupta and Gupta 2015).

Furthermore, population based studies have reported that subjects with excess VAT depots had higher blood cholesterol and TG levels, lower high density lipoprotein (HDL) cholesterol and an increased risk of developing hepatic insulin resistance, and eventually progressing to Type 2 diabetes (Ibrahim 2010)**.** Thus abdominal obesity as measured by waist circumference is a predictor for mortality independent of BMI. This correlation also explains, as mentioned earlier, the preference of using waist-to-hip ratio to the commonly used weight-to-height ratio (BMI) to predict the health risks of obesity.

1.3 Adipose Tissue Expansion

Cellularity of VAT and SCAT is another predictor for the metabolic complications accompanying obesity. In obesity, adipose tissue can expand by accumulating excess fat in pre-existing adipocytes leading to hypertrophy (Joe et al. 2009; Wang et al. 2013b). Cellular hypertrophy is characteristic of adipose tissue from obese subjects with metabolic disorders. Because adipocytes rapidly distend while storing excess fat, their growth can surpass neovascularization via angiogenesis and thus the blood supply becomes limiting. This condition leads to hypoxia, fibrosis, adipose endocrine dysfunction, inflammation and cell death. As a result, excess circulating lipids begin to accumulate in peripheral non-adipose organs such as liver, skeletal muscle, pancreas, kidney and heart leading to lipotoxicity (Gustafson et al. 2009). There is also evidence that adipose can only accumulate a certain amount of fat regardless of circumstances leading to obesity; once that threshold is reached, the fat overflows into the non-adipose tissues (Perez-Diaz et al. 2014). Excess fat in the form of diacylglycerols and ceramides,

can ultimately lead to insulin resistance in these tissues by impairing insulin signaling pathways (Larsen and Tennagels 2014; Perry et al. 2014) as well as reducing insulin secretion from pancreatic β cells (Noushmehr et al. 2005).

Increased fat mass in obesity can also be due to an increased availability of adipocytes for lipid loading due to adipocyte hyperplasia. Adipocyte hyperplasia is characteristic of WAT from metabolically healthy obese individuals and is correlated with low levels of inflammation and fibrosis (Klöting et al. 2010; Sun et al. 2014). Thus the distribution of excess fat among numerous, smaller adipocytes preserves the function and insulin sensitivity of the adipose tissue. Moreover, it preserves the balance between the secretion of anti-inflammatory and anti-diabetic adipokines to the pro-inflammatory ones (Gupta and Gupta 2015). However, the factors that regulate the balance between hypertrophy and hyperplasia in an expanding fat pad remain to be determined.

1.4 Adipogenesis

Adipose tissue is a highly dynamic organ and approximately 10% of the body's adipocytes are regenerated each year (Spalding et al 2008). New adipocytes arise from pre-adipocytes, which are commonly isolated *in vitro* from the stromal vascular fraction. Pre-adipocytes originate from mesenchymal stem cells, which could also differentiate into osteoblasts and chondrocytes (Pittenger et al. 1999). Adipogenesis is a tightly regulated cell differentiation process by which mature adipocytes are generated. The key features of differentiated adipocytes are morphological change, growth arrest and the high level of expression of lipogenic genes and adiponectin.

Peroxisome proliferator-activated receptor γ (PPARγ) is the most important transcription factor in the regulation of adipocyte differentiation or adipogenesis *in vivo* as well as *in vitro*. It belongs to the Class II nuclear hormone receptor family which also includes retinoic acid receptors (RAR) and thyroid hormone receptors (Aranda and Pascual 2001). Fatty acids and their derivatives such as prostaglandins act as its natural ligands. The ligand bound PPARγ heterodimerizes with the 9-*cis* retinoic acid receptor or retinoic X receptor (RXR), which acts as a co-activator (Mukherjee et al. 1997). PPARγ is responsible for activating a number of genes involved in fatty acid and TG synthesis and uptake, such as adipocyte fatty acid binding protein P2 (aP2) (Tontonoz et al. 1994), and acyl-CoA synthase (ACS) (Schoonjans et al. 1995) and lipoprotein lipase (LPL) (Schoonjans et al. 1996). Phosphorylation of PPARγ at serine 273 upregulates the expression of adiponectin and adipsin contributing to the insulin sensitization along with its adipogenic properties (Choi et al 2010). Adipose-specific PPARγ knockout mice showed lipoatrophy (both BAT and WAT depots) and consequently have a severe metabolic phenotype characterized by elevated blood glucose and insulin levels, fatty liver, pancreatic hypertrophy, insulin resistance, and high bone mass but with loss of bone marrow fat (Wang et al. 2013a).

Drugs such as fibrates and thiazolidinediones (TZD), and xenobiotics such as pesticides, fungicides, phthalates and bisphenol A (BPA) also act as PPARγ ligands. Recently, Skinner et al. (2013) observed that the incidence of obesity was increased in the third generation offspring of female rats exposed to environmental chemicals such as BPA, phthalates and pesticides like dichlorodiphenyltrichloroethane (DDT). They attributed this effect to the 'epigenetic transgenerational inheritance of obesity' and the adipogenic action of obesogens via disruption of PPARγ signalling (Grün and Blumberg 2006; Grün et al. 2006; Manikkam et al. 2013). Whether

this observation in rodents could be extrapolated to humans and linked to the recent alarming rise in the global obesity rate is intriguing and warrants further investigation.

In addition, adipogenesis is affected by insulin. Pre-adipocytes express more insulin-like growth factor-1 (IGF-1) receptor than insulin receptors and consequently insulin acts mainly by binding to the IGF-1 receptor during the early stages of differentiation. However, post-differentiation, the expression of insulin receptor becomes predominant (Smith et al 1988). The IGF-1 receptor becomes auto-phosphorylated upon binding to insulin, which in turn activates the insulin receptor substrates (IRS). Downregulation of downstream signaling kinases such as phosphatidylinositol 3-kinase (PI3K) and Akt/protein kinase B (PKB) has been shown to inhibit adipogenesis (Garofalo et al 2003). Mammalian target of rapamycin (mTOR) is another signaling cascade downstream of insulin which can inhibit PPARγ transactivation and block adipogenesis (Kim & Chen, 2004). As a result, two different downstream insulin signaling cascades act in parallel to regulate adipogenesis. Furthermore, Akt was shown to phosphorylate the anti-adipogenic members of nuclear proteins such as the forkhead family members FOXO1 and FOXO2, which results in their inactivation. This represents an alternative pathway for the regulation of adipogenesis downstream of insulin (Fan et al. 2009; Gerin et al. 2009). Furthermore, inhibition of Akt and mTOR by rapamycin affected adipocyte differentiation and obesity in C57BL/6 mice (Chang et al. 2009).

2. Sex Dimorphism

Although women have a higher percentage of whole body fat mass, they generally have a more favourable cardiovascular risk profile and a lower amount of VAT than men. However, the cardioprotective effect seen in women is eliminated when corrected for the sex difference in

VAT levels (Tankó et al, 2003a). Studies show that peripheral adiposity or a larger hip circumference in women has an independent anti-atherogenic effect (Lissner et al, 2001; Tanko et al, 2003b). This could be explained by the observation that although large adipocytes are observed in both abdominal and femoral SCAT in both sexes, smaller adipocytes are more abundant in women, particularly in the femoral depot (Tchoukalova et al 2010), which besides being more sensitive to insulin, contributes to the increased secretion of anti-inflammatory and anti-atherogenic adipokines. Comparison of the morphology and adipogenesis of abdominal SCAT and VAT depots in pre- and post-menopausal women showed hyperplasia only in the SCAT while hypertrophy was present in both depots (Drolet et al. 2008). In obesity, as the adipose tissue enlarges, the number of adipocytes increases in women but not in men (Tchoukalova et al. 2008). On the other hand, the proportion of hypertrophic adipocytes is higher in men than women.

Importantly, sex hormones play a crucial role in adipose metabolism and adipogenesis. Estrogen enhances preadipocyte proliferation (Roncari and Van 1978) through the MAPK-dependent and c-fos signaling pathways (Lacasa et al 1997). In contrast, testosterone treatment promotes myogenic commitment of mesenchymal stem cells while blocking the adipogenic lineage (Singh et al 2003), which explains the sex difference observed in body composition (lean muscle mass versus fat mass). While the mitogenic effect of estrogen is observed in both abdominal SCAT and VAT, women are more sensitive than men (Anderson et al 2001). Notably, the transcription of cytochrome P450 aromatase, the enzyme responsible for the synthesis of estrogen from androgens, is expressed more in the femoral than in the abdominal SCAT depot (Bulun and Simpson, 1994; Yang et al, 2006). Additionally, the hypoxia-inducible factor 1 alpha (HIF1α) activated pro-fibrotic pathway is negatively regulated by estrogen receptor alpha (ERα)

signaling, which explains some of the protective actions of estrogen on adipose tissue function in obesity (Kim et al 2014). However, further studies are required to understand in greater detail how sex hormones modulate adipogenesis and the expression and function of various adipokines.

3. Convergence of carbohydrate and lipid metabolism in the body

Glucose from dietary carbohydrates is converted to pyruvate in hepatocytes via glycolysis. Acetyl CoA, which is generated from pyruvate, enters the tricarboxylic acid (TCA) or Kreb's cycle for energy production in the form of adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NADH). Excess acetyl CoA leaves the mitochondria as citrate and enters the lipogenic pathway in the cytosol. It is first converted to malonyl CoA by acetyl CoA carboxylase (ACC), which is further converted to fatty acids such as palmitate by fatty acid synthase (FAS) enzyme. Acetyl CoA is additionally used for cholesterol synthesis by a multi-step pathway.

Fatty acids produced by *de novo* lipogenesis (DNL) in the liver, along with dietary fatty acids from circulation, are used to generate triglycerides (TGs) by the sequential esterification of the glycerol backbone in the liver. Hepatic TGs are secreted as very low density lipoprotein (VLDL) into the circulation and are acted upon by lipoprotein lipase (LPL). The free fatty acids (FFA) thus released are taken up by adipose tissue where they are re-esterified for storage in lipid droplets as TGs. These pathways are tightly regulated to maintain a metabolic homeostasis according to the nutritional and hormonal conditions. DNL takes place in most cells including adipocytes (Wang et al. 2015).

3.1. Insulin Action

Insulin secretion from pancreatic beta cells increases as a response to postprandial hyperglycemia. Insulin effectively reduces blood glucose levels by increasing translocation of glucose transporter 4 (GLUT4) molecules to the plasma membrane of cells of peripheral organs such as skeletal muscle, adipose tissue and liver. This increases the facilitated diffusion of glucose into these cells**.** Once inside the cell, glucose becomes phosphorylated and therefore cannot be transported out by GLUT4. Glucose is stored as glycogen in liver and muscle and as TGs in adipose tissue.

Under conditions of surplus energy in the form of dietary lipids, WAT enlarges and becomes insulin resistant resulting in the downregulation of glucose uptake and DNL leading to hyperglycemia. A study corroborating this model has shown that the increased expression of lipogenic enzymes in adipocytes is associated with enhanced insulin sensitivity in humans independent of obesity (Roberts et al 2009). In addition, resistance to the anti-lipolytic effect of insulin increases lipolysis in adipocytes leading to increased release of FFA into the circulation, resulting in hyperlipidemia. In contrast, insulin sensitive hepatocytes continue to take up blood glucose and undergo DNL, resulting in increased TG production that leads to hepatic insulin resistance and eventually to non-alcoholic fatty liver disease (NAFLD) (Hudgins et al. 2011).

Insulin exerts its effects through the insulin receptor tyrosine kinase (IR) which upon ligand binding dimerizes and is autophosphorylated at tyrosine 960 (Van Obberghen and Kowalski 1982). This phosphorylation event facilitates the stable association and phosphorylation of docking proteins called insulin receptor substrate 1 and 2 (IRS-1 and IRS-2). IRS phosphorylates PI3K whose products, PI 3,4-bisphosphate (PIP_2) and PI 3,4,5-triphosphate (PIP_3), remain in the cytosolic leaflet of the plasma membrane and act as activators of the serine-

threonine kinases PDK1 and Akt. PDK1 phosphorylates Akt, which is directly involved in GLUT4 translocation to the plasma membrane and thus increased glucose uptake in insulin sensitive cells (Karp 2006). Activated Akt further induces glycogen synthesis through inhibition of glycogen synthase kinase 3 (GSK-3). Insulin signaling induces fatty acid and cholesterol synthesis in parallel via the regulation of SREBP transcription factors (Shao and Espenshade, 2012; Wong and Sul, 2010). In obesity, as a result of inflammation, increased levels of TNFα and IL-1 phosphorylate and activate the c-Jun N-terminal Kinases (JNKs) / Stress-activated kinases (SAPKs) signaling pathway. This results in the phosphorylation of IRS-1 at Serine 307 leading to the blockade of downstream insulin signaling and thus insulin resistance in obesity (Aguirre et al. 2000).

4. Adipokines

Apart from its classic function of dietary energy storage in the form of TGs, adipose tissue also secretes a number of potent peptide hormones and cytokines collectively called adipokines. These adipokines exert effects on energy metabolism in other tissues as well as on appetite through its action on neuroendocrine pathways and on the brain itself (Guilherme et al. 2008) in addition to influencing immunity, blood pressure and angiogenesis (Ibrahim 2010). Adipokines include adiponectin, omentin, leptin, resistin, adipsin, chemerin, vaspin, visfatin, TNFα and interleukins. Different adipose tissue depots (for instance, VAT and SCAT) secrete different proportions of these adipokines. Furthermore, the amount of fat being stored in each depot determines the amount of various adipokines secreted (Northcott et al. 2012). While some adipokines such as adiponectin and omentin are anti-inflammatory others such as leptin, resistin and visfatin possess pro-inflammatory properties (Figure 2).

Adiponectin, the most abundant adipokine secreted by adipocytes, exhibits both anti-inflammatory and insulin sensitizing properties along with anti-atherogenic properties. It is more highly expressed in VAT than in SCAT (Motoshima et al. 2002) and there are numerous studies highlighting the negative correlation of serum adiponectin levels with BMI/obesity. Adiponectin is described in more detail below.

Leptin was the first adipokine to be discovered and primarily functions as an appetite suppressor and a regulator of body weight gain (Zhang et al. 1994). Thus circulating levels of leptin are directly proportional to BMI and the degree of adiposity. SCAT is the major source of leptin (Wajchenberg 2000). However, in obesity elevated leptin levels fail to regulate weight gain due to a condition called leptin resistance. Elevated leptin levels may accelerate atherosclerosis by increasing monocyte recruitment in mice (Gruen et al 2007) and by increasing secretion of other pro-atherogenic cytokines like TNFα, IL-6 and vascular endothelial growth factor (VEGF) in humans which induces inflammation and/or angiogenesis (Beltowski 2006; Guzik et al. 2003). Leptin can also cause platelet aggregation and arterial thrombosis which can result in a stroke or myocardial infarction (Konstantinides et al. 2001).

In contrast, omentin is an anti-inflammatory adipokine which is secreted by VAT but not by SCAT (Yang et al. 2006a). Omentin is found to promote vasorelaxation and angiogenesis, and prevent pro-inflammatory signalling in endothelial cells, and thus has effects on coronary atherosclerosis and obesity-related disease such as hypertension. Omentin is also expressed by human epicardial adipocytes (Fain et al. 2008) and it has been reported that subjects with obesity and Type 2 diabetes have decreased omentin levels (De Souza Batista et al. 2007; Tan et al. 2008; Zhong et al. 2011).

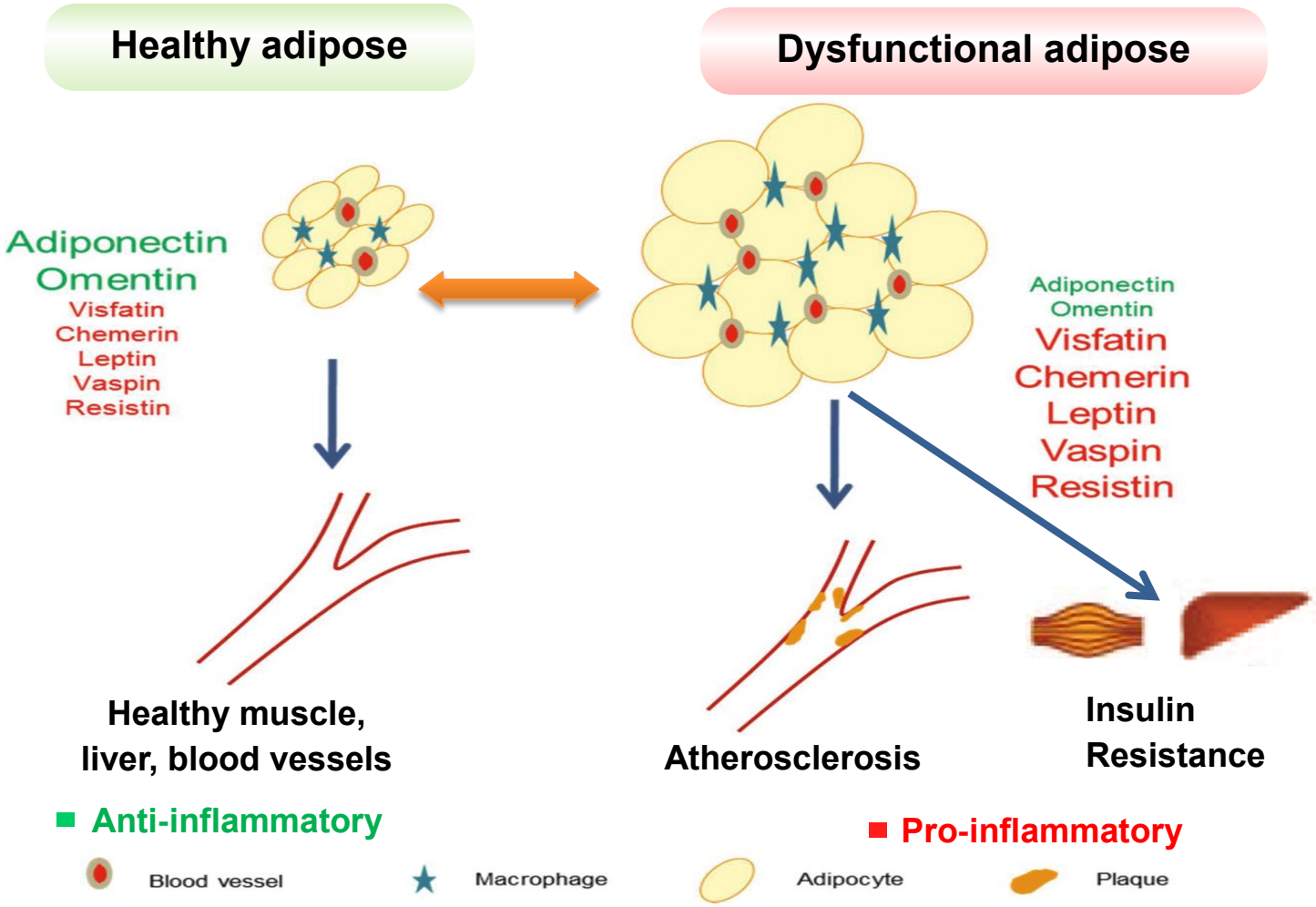
Resistin or ‘resistance to insulin’ is a pro-inflammatory adipokine with anti-insulin and pro-atherogenic properties. While in rodents it is primarily secreted by adipocytes (Rajala et al. 2004; Steppan et al. 2001), in humans, macrophages appears to be the major cell source of resistin (Fain et al. 2003; Patel et al. 2003). Studies show that resistin reduces glucose uptake by decreasing insulin-stimulated GLUT4 translocation in cardiomyocytes (Graveleau et al. 2005) possibly by stimulating the JNK-IRS1 pathway (Kang et al. 2011). Moreover, resistin increases the expression of intercellular adhesion molecule 1 (ICAM-1) and vascular adhesion molecule 1 (VCAM-1) and thus enhances monocyte adhesion to endothelial cells (Skilton et al. 2005; Verma et al. 2003). Several population based studies have shown a positive correlation between resistin levels and obesity associated diabetes (Gerber et al. 2005; Steppan et al. 2001). Elevated serum resistin is an indicator of metabolic syndrome (Malo et al. 2011) and is positively associated with inflammatory markers, TGs, LDL-cholesterol in humans (Ohmori et al. 2005; Reilly et al. 2005).

Visfatin is another adipokine reported to be elevated in obese individuals with visceral fat accumulation (Araki et al. 2008). It has been shown to promote endothelial dysfunction. Vaspin (visceral adipose tissue derived serpin) is an adipokine shown to ameliorate glucose intolerance and insulin resistance in high-fat high-sucrose fed obese mice (Hida et al. 2005). Vaspin expression decreases in subcutaneous fat depot as the visceral fat increases in obesity (Lee et al. 2011). However, while higher plasma vaspin is associated with metabolic syndrome in men, its association with coronary atherosclerosis is observed only in women (Choi et al. 2011).

In addition to these adipokines, IL-6 and TNFα are the two important pro-inflammatory cytokines secreted by resident and infiltrated adipose macrophages. Since VAT is more infiltrated with inflammatory cells than SCAT, it is a more predominant source of these cytokines. Consequently, both cytokines are increased in abdominal obesity and are pro-diabetic,

impairing insulin sensitivity and insulin signaling. Furthermore, TNFα triggers vascular inflammation by activating the nuclear factor-κ B (NF-κB) (Ibrahim 2010).

Therefore, it is evident that in obesity as fat accumulates and adipocytes enlarge, the secretion of anti-inflammatory adipokines such as adiponectin and omentin decreases, and pro-inflammatory adipokines such as leptin, resistin and visfatin increases leading to disorders such as insulin resistance and associated cardio-metabolic diseases as demonstrated in Figure 2.



Adapted with permission from NRC Research Press: Northcott et al. (2012) Can. J. Physiol. Pharmacol.

Figure 2: Impaired adipocyte endocrine properties in obesity. Healthy adipocytes secrete more anti-inflammatory adipokines such as adiponectin and omentin while in obesity as fat accumulates and adipocytes enlarge, they secrete more pro-inflammatory adipokines such as leptin, visfatin, resistin and this leads to pathologies like insulin resistance and atherosclerosis.

5. Adiponectin

Adiponectin is an adipocyte-derived peptide hormone discovered by four research groups independently and was named as adipocyte complement-related protein (30 kDa) (Acrp30) (Scherer et al. 1995), AdipoQ (Hu et al. 1996), apM1-adipose most abundant gene transcript 1 (K. Maeda et al. 1996) and GBP28 – gelatin binding protein (Nakano et al. 1996). In humans, adiponectin is encoded by the *ADIPOQ* gene of size 17 kb on chromosome locus 3q27 (Takahashi et al. 2000), which has been identified as a Type 2 diabetes susceptibility locus (Kissebah et al. 2000; Vionnet et al. 2000). The gene is composed of 3 exons with the start codon in exon 2 and the stop codon in exon 3 (Saito et al. 1999; Takahashi et al. 2000) and it encodes a 244 amino acid peptide.

Single nucleotide polymorphisms (SNP) in the *ADIPOQ* gene have been identified in population based studies as being associated with Type 2 diabetes: (1) Individuals with both of the alleles containing SNP 276 (located downstream of the translational start site and consisting of guanine instead of thymidine (G/G phenotype) have a doubled risk of developing diabetes). This SNP is observed in 40% of Japanese subjects. (2) The I164T missense mutation in the globular domain is associated with lower serum adiponectin levels independent of BMI. (3) A haplotype seen in German and North American individuals containing SNPs 276 and SNP 45 in exon 2 which is associated with obesity and insulin resistance. (4) In white French subjects, SNPs 11377 and 11391 in the promoter region are linked to hypoadiponectinemia and Type 2 diabetes (Kadowaki et al. 2006).

Adiponectin is a very abundant serum protein and is present in the serum at a concentration range from 2-17 µg/ml, with women having a significantly higher level than men

in both healthy and obese conditions (Arita et al. 1999). It constitutes about 0.01% of the total plasma proteins (Iglesias et al. 2006).

5.1 Regulation of adiponectin gene expression

The adiponectin gene is expressed predominantly in WAT by mature adipocytes; however, expression in BAT (T37i brown adipocyte cell line) (Iacobellis et al. 2013; Viengchareun et al. 2002), bone-forming cells (Berner et al. 2004) and cardiomyocytes (Piñeiro et al. 2005) has been reported. Its expression is regulated by a number of hormonal and environmental factors. Adiponectin expression is attenuated by obesity, TNFα and glucocorticoids. Its expression is increased by weight loss, insulin-like growth factor 1 (IGF-1) and insulin (Fasshauer et al. 2002; Halleux et al. 2001; Maeda et al. 2001). While insulin stimulate adiponectin gene expression upon short term exposure of the 3T3-L1 adipocyte cells (Scherer et al. 1995), another study showed a decrease in adiponectin gene expression upon longer exposure to insulin (Fasshauer et al. 2002). Oxidative stress resulting from hypoxic conditions during adipose expansion and inadequate blood supply in obesity was also found to downregulate the expression of adiponectin (Furukawa et al. 2004). Furthermore, Iwaki et al. (2003) found a PPAR-responsive element (PPRE) in the human adiponectin gene promoter region. Adiponectin expression was shown to increase with upregulation of PPARγ expression. Additionally, PPARγ agonists such as TZD which is used for insulin sensitizing in diabetic patients increase serum adiponectin levels. Therefore, PPARγ is considered a significant transcriptional regulator of adiponectin gene expression.

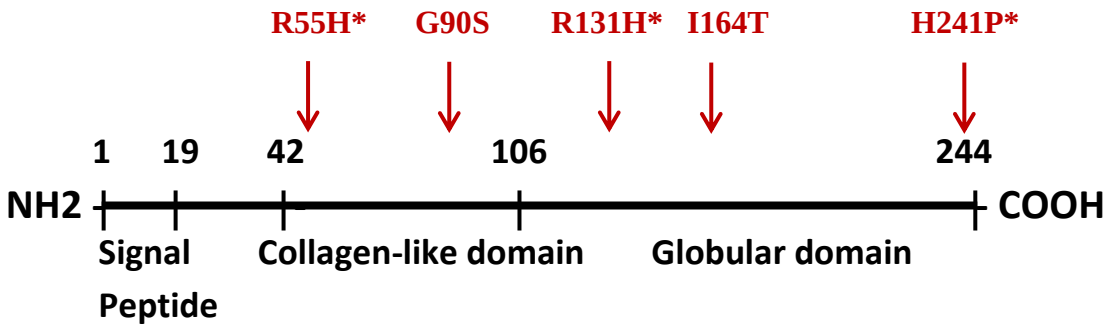
Sex dimorphism in the levels of circulating adiponectin suggests a role of sex hormones such as estrogen and testosterone in the regulation of plasma adiponectin levels (Combs et al.

2003; Nishizawa et al. 2002; Xu et al. 2005) and may also partially account for the fact that females are more insulin sensitive than men. Thus, although the exact mechanism behind regulation of adiponectin needs further studies, it is affected by multiple factors such as lifestyle, aging and sex.

5.2 Protein structure of adiponectin

The primary sequence of adiponectin consists of a signal peptide at the amino terminus, a short variable region followed by a conserved collagenous domain homologous to collagen VIII and X, and a carboxyl terminal half containing a globular domain, which shows sequence similarity to the complement factor C1q (Figure3). Thus adiponectin belongs to the C1q globular domain protein family (Maeda et al. 1996; Shapiro and Scherer 1998). Adiponectin can form a number of multimers such as low molecular weight (LMW) trimers, medium molecular weight (MMW) hexamers and high molecular weight (HMW) 12-18 oligomers in the circulation (Onay-Besikci et al. 2004; Waki et al. 2003). Interestingly, adiponectin shows a striking structural homology to TNFα without significant sequence similarity (Berg et al. 2002; Shapiro and Scherer 1998).

Studies observe a lower abundance of HMW oligomers in the circulation of men relative to women (Waki et al. 2003) suggesting sex dimorphism in both total and multimeric circulating adiponectin levels. The remarkable abundance of this regulatory hormone leads to the hypothesis that it could be secreted in an inactive form. In support of this, Fruebis et al. (2001) showed the presence of a truncated form of adiponectin containing only the globular domain in human blood plasma. While *in vitro* studies suggested trypsin and leucocyte elastase as the protease responsible for this proteolytic cleavage (Waki et al. 2005).



Adapted from Jungtrakoon et al. 2011 PLoS One

Figure 3: Schematic representation of the protein structure of adiponectin. Adiponectin has an N-terminal signal peptide followed by a collagen like domain homologous to collagen VIII and X and a C-terminal globular domain homologous to complement C1q factor. Full-length adiponectin undergoes proteolytic cleavage to form globular adiponectin. Some of the amino acid variants identified in the adiponectin protein sequence are shown by arrows with those seen in Type 2 diabetes patients indicated with an asterisk.

5.3 Adiponectin Receptors

The globular and monomeric full-length forms of adiponectin exert their beneficial effects on energy homeostasis through two main receptors known as adiponectin receptor 1 and 2 (AdipoR1 and R2). Both are cell membrane proteins with the seven transmembrane domain structure of G protein-coupled receptor (GPCR) and share 66.7% sequence homology. However, adiponectin receptors have their amino terminus on the cytoplasmic side of the plasma membrane and an extracellular carboxyl terminus (Figure 4), which is opposite to the orientation observed for typical GPCR proteins (Yamauchi et al. 2003). The membrane spanning regions of these proteins are found to be conserved with marked homology of human AdipoR1 to gene products in *Saccharomyces cerevisiae*, *Caenorhabditis elegans* and *Drosophila melanogaster* of 29%, 56% and 60% identity, respectively. AdipoR1 is ubiquitously expressed in endothelial cells, cardiomyocytes and adipose tissue, but it is most abundantly expressed in skeletal muscle and has high affinity for globular adiponectin. AdipoR2 has similar affinity for globular and full-length adiponectin and is abundantly present in hepatocytes.

T-cadherin is a putative third receptor of adiponectin which binds preferentially to oligomeric adiponectin. It is a cadherin family member that is attached to the plasma membrane by a glycosyl phosphatidyl inositol (GPI) moiety (Ranscht and Dours-Zimmermann 1991). It is different from other cadherin molecules as it lacks a cytoplasmic domain and hence is called truncated cadherin (T-cad) and is not involved in cell-cell adhesion (Takeuchi et al. 2002; Zhou et al. 2002). Since it lacks a cytoplasmic domain, it is assumed not to take part in signaling, acting rather as an adiponectin-binding protein or a co-receptor with a yet to-be identified receptor. However, T-cad is considered biologically significant as it is highly conserved throughout evolution in vertebrates. It is highly expressed in vascular endothelial cells and

smooth muscle cells (Takeuchi et al. 2007) and is found to play a major role in the angiogenic and anti-atherogenic properties of adiponectin. Furthermore, T-cad is found to mediate the protective effect of adiponectin on endothelial cells against oxidative stress-induced apoptosis in endothelial cells, which enhances tumour-associated angiogenesis (Joshi et al. 2005). This is in agreement with high expression of T-cad in intra-tumoral vascular endothelial cells (Adachi et al. 2006; Wyder et al. 2000).

Expression of these adiponectin receptors on adipose tissue suggests an autocrine or paracrine action of adiponectin on adipose metabolism. Similarly, expression in pancreatic β cells alludes to a role of adiponectin in insulin secretion (Kharroubi et al. 2003). However, the expression of both AdipoR1 and R2 has been found to be decreased in skeletal muscle and adipose tissue of the insulin resistant *ob/ob* mouse model and this may explain the lower sensitivity to adiponectin and impaired glucose tolerance in obesity (Tsuchida et al. 2004). Consequently, signaling downstream of the receptors was impaired in these obese models. Moreover, skeletal muscle from Type 2 diabetic patients has been observed to express adiponectin R1 and R2 at a lower level than healthy subjects (Civitarese et al. 2004). Thus in obesity, not only is a decrease in plasma adiponectin levels but also a downregulation of adiponectin receptors on peripheral target cells that leads to insulin resistance, which in turn aggravates hyperinsulinemia (Tsuchida et al. 2004).

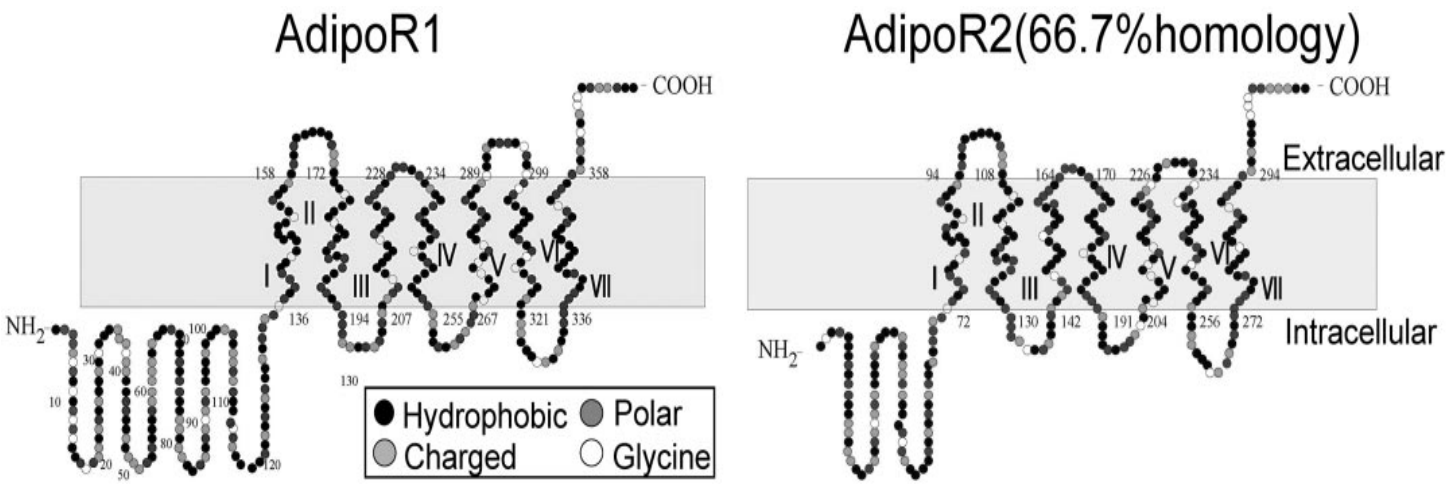


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Figure 4: Structure of adiponectin receptors. Adiponectin receptor 1 and 2 (AdipoR1 and R2) have seven transmembrane domains with an intracellular amino-terminus and extracellular carboxyl terminus. While AdipoR1 is abundant in skeletal muscle and preferentially binds to globular adiponectin, AdipoR2 is abundant in liver and has similar affinity for both globular and full-length adiponectin.

5.4 Insulin sensitizing effect of Adiponectin

Adiponectin exerts its insulin sensitizing effect by acting on various peripheral organs/tissues of which liver and skeletal muscle are the primary sites. Mao et al. (2006) showed that the cytoplasmic N-terminus of adiponectin receptors interacted with adaptor protein containing pleckstrin homology domain, phosphotyrosine-binding domain and leucine zipper motif 1 (APPL1) which is stimulated upon adiponectin binding to the receptors. APPL1 in turn activates AMP Activated Protein Kinase (AMPK) which mediates most of the beneficial roles associated with adiponectin and is summarized in Figures 5 and 6.

Adiponectin modulates insulin sensitivity by mainly targeting liver and skeletal muscle. Liver abundantly expresses AdipoR2 and studies have shown that treatment with only full-length adiponectin and not globular adiponectin lead to the phosphorylation and inhibition of ACC via AMPK activation, thereby reducing fatty acid synthesis, resulting in increased fatty acid oxidation in liver (Yamauchi et al. 2002). In addition, AMPK activation reduces the expression of hepatic gluconeogenic enzymes such as phosphoenol pyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) (Lochhead et al. 2000), thus decreasing plasma glucose levels. However, in liver, processes like glycogen synthesis, glucose uptake and glycolysis are not influenced by adiponectin (Berg et al. 2001; Combs et al. 2001). Furthermore, Liu et al. (2012) have shown that adiponectin knockout mice had lower mRNA levels for key enzymes involved in several glucose and lipid metabolic pathways including glycolysis, TCA cycle, TG synthesis and cholesterol synthesis in liver, with a simultaneous decreased binding of hepatic nuclear factor 4 alpha (HNF4α) to the promoter of these hepatic genes in comparison to the wild-type mice. This suggests that adiponectin may regulate HNF4α and thereby the hepatic metabolic gene expression.

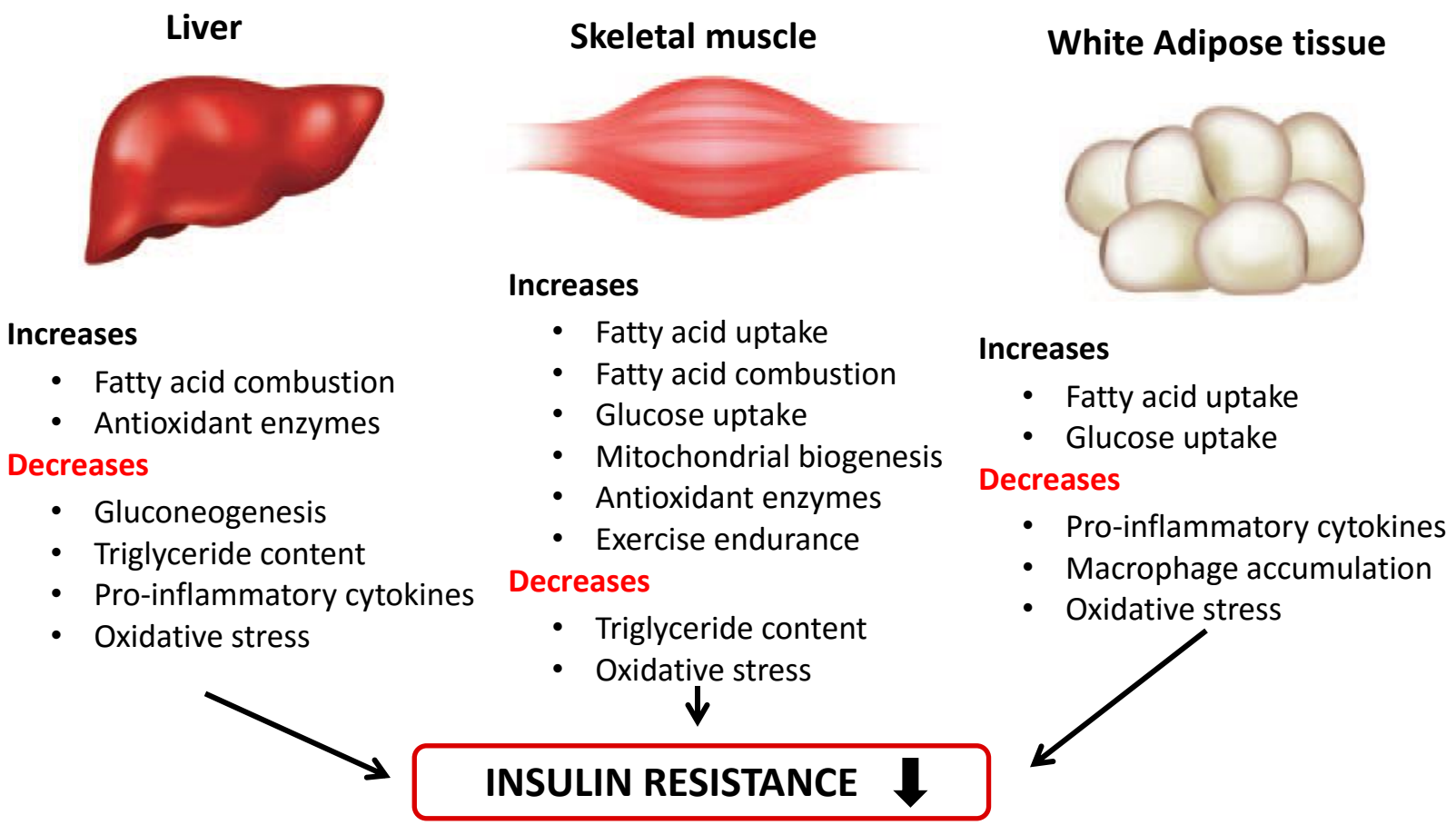

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Figure 5: Mechanism of adiponectin action on its target tissues. Adiponectin exerts its insulin sensitizing properties by acting mainly on the liver, skeletal muscle and white adipose tissue by modulating gluconeogenesis, fatty acid metabolism, macrophage accumulation, pro-inflammatory cytokine secretion and oxidative stress.

In skeletal muscle, similar to hepatocytes, adiponectin stimulates fatty acid oxidation via phosphorylation of AMPK and ACC2. Both full-length (fAcrp30) and globular adiponectin (gAcrp30) decreased β-oxidation with the globular form having a more pronounced effect consistent with its higher affinity for AdipoR1, which is highly expressed on myocytes. Adiponectin treatment showed increased glucose uptake via AMPK activation but independent of the PI3K pathway (Yamauchi et al. 2002). Ceddia et al. (2005) showed that gAcrp30 treatment increased translocation of GLUT4 to the myocyte membrane and thus resulted in increased glucose uptake. In addition, adiponectin increased the expression of fatty acid translocase (FAT/CD36) and thus fatty acid uptake into the cells. Moreover, adiponectin receptors in muscle cells activated PPARα gene expression and activity. PPARα is a major transcriptional regulator of peroxisomal and mitochondrial β-oxidation, which works via AMPK and p38 mitogen-activated protein kinase (p38 MAPK) activation. PPARα increases the expression of several target genes involved in fatty acid metabolism such as acyl CoA oxidase (ACO), the rate-limiting enzyme of the β-oxidation pathway in peroxisomes; carnitine palmitoyl transferase 1 (CPT1) and fatty acid binding protein 3 (FABP3), which are responsible for the fatty acid transport into mitochondria, where fatty acid oxidation occurs (Yoon et al. 2006). Furthermore, Qiao et al. (2012) has shown *in vivo* and *in vitro*, in skeletal muscle and in C2C12 myotubes, respectively, that adiponectin suppressed mitogen-activated protein kinase phosphatase-1 (MKP-1) expression and thereby enhanced p38MAPK phosphorylation which in turn increased mitochondrial biogenesis via enhanced peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α) gene expression and activity.

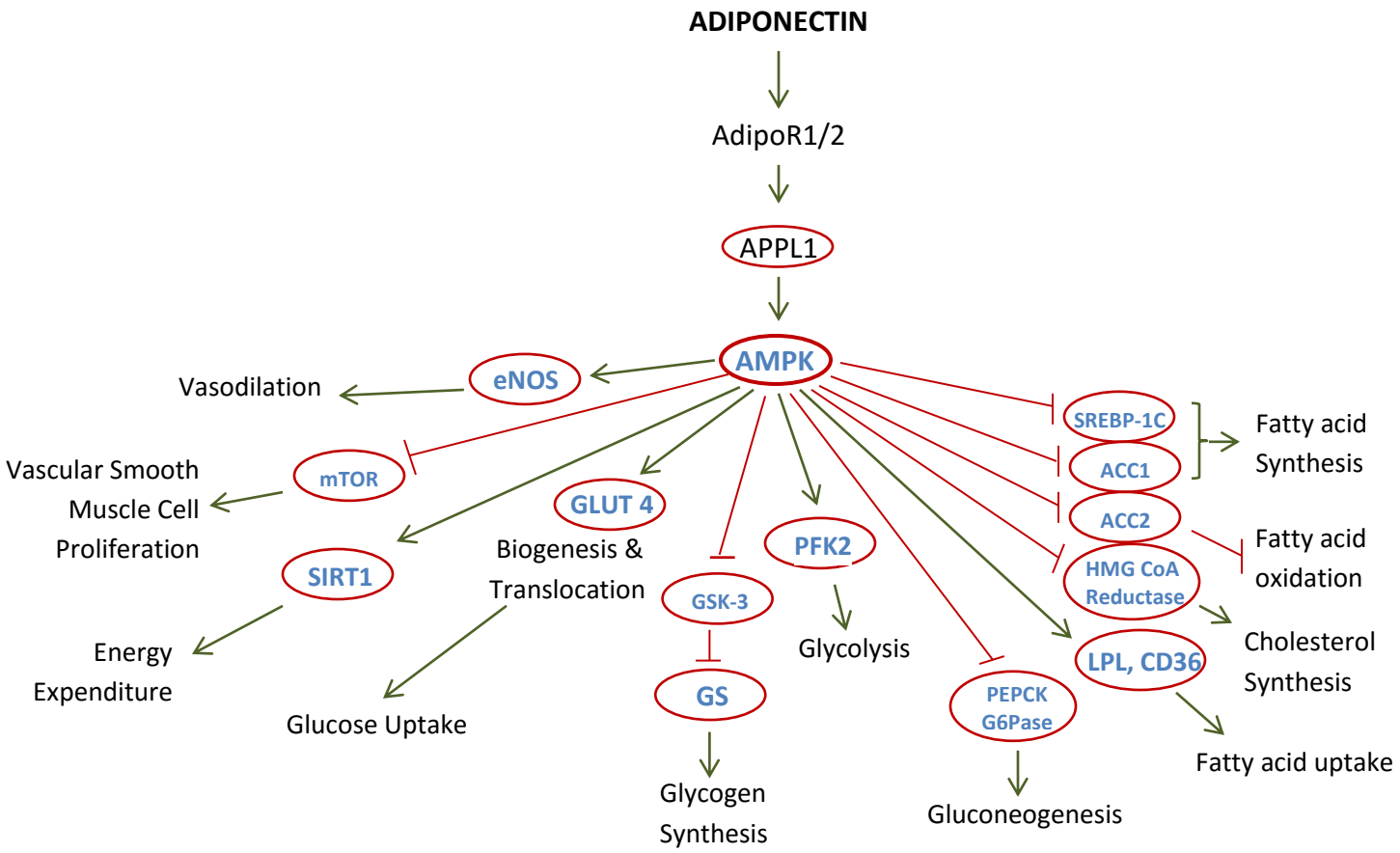


Figure 6: Adiponectin signaling is dependent on AMP activated protein kinase (AMPK). Adiponectin binds to its receptors on the cell membrane and activates AMPK signaling in the cell and promotes its anti-diabetic and anti-atherosclerotic actions. ACC: Acetyl coenzyme A carboxylase, AdipoR: Adiponectin receptors, APPL1: Adaptor protein containing pleckstrin homology domain, phosphotyrosine-binding domain and leucine zipper motif 1, CD36/FAT: Fatty acid translocase, eNOS: endothelial Nitric oxide synthase, GS: Glycogen synthase, GSK-3: Glycogen synthase kinase 3, HMG CoA: 3-Hydroxy-3-methylglutaryl coenzyme A, LPL: Lipoprotein lipase, mTOR: mammalian target of rapamycin, PEPCK: Phosphoenol pyruvate carboxy kinase, PFK2: Phosphofructo kinase 2, SIRT1: Sirtuin 1 and SREBP-1C: Sterol regulatory element-binding protein 1C.

6. Cardiovascular diseases and Adiponectin

Cardiovascular disease (CVD) is the leading cause of death and disability in the world (WHO Factsheet, 2012). Risk factors for CVD are an unhealthy diet, obesity, raised blood pressure (hypertension), physical inactivity, diabetes, tobacco use and raised lipids. Among these factors, hypertension and atherosclerosis are the most common disorders affecting the blood vessels that accelerate the development of arterial diseases and cardiac hypertrophy, which eventually can lead to myocardial infarction and stroke.

The basic structure of blood vessels consists of three main layers *viz.* tunica interna (intima), tunica media and tunica externa (adventia). The intima is the innermost layer consisting of endothelial cells which has a role in modulating the permeability and contractility of the blood vessel via the secretion of locally active nitric oxide. Additionally, the endothelial cell enhances blood flow by decreasing surface friction. The medial layer mainly consists of smooth muscle cells and elastic fibers, which mediate vasoconstriction and vasodilation to regulate blood flow and blood pressure. It is the most variable layer and varies in structure and function in different types of blood vessels. The media is separated from the adventia by the external elastic lamina. The advential layer consists mainly of elastic and collagen fibers and helps to anchor the vessel to the surrounding tissues (Tortora and Derrickson 2011).

6.1 Atherosclerosis

Atherosclerosis is the progressive thickening of the walls of the blood vessels characterized by cholesterol-rich plaques called atheromas. The plasma levels of lipoproteins that transport TGs and cholesterol in the body play a major role in the progression of

atherosclerosis. When the levels of very low density lipoprotein (VLDL) and low density lipoprotein (LDL) that transport cholesterol from the liver to the body cells are elevated as in obese subjects, these particles accumulate in the inner layer of large vessels such as arteries and undergo oxidation. This accumulation of oxidized lipids causes tissue damage and triggers inflammation. As a result of the immune response, endothelial cells and the smooth muscle cells secrete chemokines that attract monocytes to the site, and they differentiate into macrophages. These macrophages then ingest the oxidized LDLs and become foam cells. Inflammatory cells such as T-lymphocytes along with foam cells and macrophages infiltrate the inner lining of the vessel and form the fatty streak that progresses to the characteristic atherosclerotic plaque. Cytokines secreted by macrophages cause the proliferation and migration of smooth muscle cells to the top of the plaque forming a cap over it and narrowing the vessel lumen (Coleman et al. 2006). Blood flow is hindered when this cap is ruptured, along with clot formation due to the secretion of tissue factors (TFs) by the foam cells. If these events occur in an artery supplying the heart, it results in coronary artery disease (CAD) and ultimately myocardial infarction, and if occurs in those supplying other body parts, peripheral arterial disease (PAD) and cerebrovascular diseases results.

6.2 Anti-atherogenic effects of adiponectin

In obesity, elevated levels of VLDL and LDL and increased systemic inflammation combined with reduced levels of high density lipoprotein (HDL) contribute to the development of atherosclerotic vessel disease. In addition, the altered secretome of enlarged adipocytes also play a pivotal role in various vascular diseases.

Epidemiological studies in men have observed a 2-fold increase in CAD prevalence in patients with hypoadiponectinemia (<4.0 μg/ml) after adjusting for other factors such as hypertension, BMI, dyslipidemia, diabetes and smoking (Kumada et al. 2003). Another study showed a similar association where plasma adiponectin levels in diabetic patients with CAD was significantly lower than those of diabetic patients without CAD (4.0±0.4 versus 6.6±0.4 μg/ml, $P<0.001$ in men and 6.3±0.8 versus 7.6±0.7 μg/ml in women) (Hotta et al. 2000). Additional research has revealed details of the various anti-atherogenic actions of adiponectin.

Adiponectin treatment inhibits NF-κB activation, thereby effectively reducing TNFα-stimulated VCAM-1, ICAM-1 and IL-8 expression in human aortic endothelial cells (HAEC) and thus the attachment of monocytes to TNFα-activated endothelial cells (Kobashi et al. 2005; Ouchi et al. 1999). Adiponectin was also found to inhibit the transformation of macrophages to foam cells by reducing the cholesterol content in human macrophages by suppressing the class A scavenger receptor (SR-A) expression (Ouchi et al. 2001). Furthermore, adiponectin increases the expression of tissue inhibitor of metalloproteinase-1 (TIMP-1) in macrophages via stimulation of the anti-inflammatory cytokine IL-10 (Kumada et al. 2004). However, while gAcrp30 abrogated platelet-derived growth factor (PDGF) stimulated vascular smooth muscle cell (VSMC) proliferation, full-length Acrp30 stimulated the p44/42 MAPK or extracellular signal-regulated kinase 1/2 (ERK1/2) and the Akt pathway with concomitant DNA and protein synthesis in VSMCs (Fuerst et al. 2012). Additionally, adiponectin is a major regulator of endothelial nitric oxide synthase (eNOS), which is a key determinant of endothelial function and angiogenesis. Adiponectin increases eNOS activity by its phosphorylation via the AMPK pathway (Chen et al. 2003; Ouchi et al. 2004). It also

enhances eNOS expression in endothelial cells by the amelioration of oxidized LDL-suppressed eNOS activity (Motoshima et al. 2004; Xi et al. 2005). In addition to its protective effect against hypertension by vasodilation, endothelial nitric oxide (NO) suppresses vascular inflammatory responses (Moncada 1991).

Consistent with the *in vitro* studies, both gAcrp30 and fAcrp30 attenuated atherosclerotic lesions and suppressed the expression of SR-A, TNF and VCAM-1 in apolipoprotein E-deficient atherosclerotic mouse models (Okamoto et al. 2002; Yamauchi et al. 2003). Moreover, adiponectin knockout models on an atherogenic diet exhibited hypertension and reduced eNOS expression and activity in aorta. Furthermore, blockage of eNOS activity by L-NAME inhibited the attenuation of high blood pressure by adiponectin replenishment (Ohashi et al. 2006; Ouchi et al. 2003).

Adiponectin decreases the oxidative stress induced by hyperglycemia in endothelial cells and thus protects against development of atherosclerosis in diabetes (Ouedraogo et al. 2006; Stocker and Keaney 2004). Moreover, mammary arteries which abundantly express T-cadherin are resistant to atherosclerosis and coronary arterial endothelial cells which express less T-cadherin are more susceptible to atherosclerotic change (Qin et al. 2003).

Taken together, adiponectin exerts a regulatory effect on vascular tone by increasing the bioavailability of NO by elevating eNOS and modulating vascular remodeling through inhibition of vascular smooth muscle cell proliferation and migration. Thus, a lack of adiponectin could contribute to the development of atherosclerosis and subsequent arterial diseases.

6.3 Arterial Stiffness

Arterial stiffness is recognized as being an independent predictor of incipient cardiovascular diseases associated with obesity and metabolic syndrome (Cavalcante et al. 2011). Large arteries such as the aorta and coronary arteries are progressively injured over the course of ageing and undergo cumulative reparative inflammatory responses, elastin fiber degradation and collagen accumulation (Laurent et al. 2005) favoring wall calcification and remodeling (Yu and Blumenthal 1965) and eventually leading to stiffness (Ahmadi et al. 2011). However, this process is extensively accelerated by the presence of obesity and its comorbidities.

The proximal aorta, which serves not only as a conducting system but also as a crucial buffering reservoir is more elastic to support the systolic impulse and accommodate large stroke volume. Likewise, the thoracic aorta is more elastic than the distal vessels, the latter vessels having a more elevated collagen to elastin fiber ratio (Lee and Kamm 1994). Therefore, the attenuated aortic distensibility due to adverse structural and functional alterations in their vessel wall could be an early detectable biomarker of cardiovascular diseases such as hypertension, atherosclerosis, CAD and cardiac hypertrophy.

Insulin resistance, diabetes and hypertension, comorbidities that are highly prevalent in obese subjects, are associated with arterial stiffness in population based studies, but there are conflicting findings in healthy volunteers (Cruickshank et al. 2002; Mitchell et al. 2010). Furthermore, hypertension has been implicated as both a cause and a consequence of vessel stiffening. Inflammatory molecules such as TNF, IL-6 and C-reactive protein were all found linked to aortic stiffness (Mahmud and Feely 2005). In addition, deficiency of adiponectin in obesity, which potentiates insulin resistance and hypertension and contributes to injury-induced

neointimal formation (Kubota et al. 2002), could also be contributing to the pathophysiology of arterial stiffness and necessitates further investigation in animal models.

Pulse wave velocity (PWV) is the current gold standard technique to non-invasively quantify aortic stiffness. The basic principle of PWV analysis is the association between the speed at which a pulse wave propagates along a vascular segment and the elasticity of the arterial wall. The stiffer (*i.e.* less elastic) the vessel wall is, the higher the pulse wave velocity. Thus it requires two variables – the distance between two points in the vessel and the time required by the pulse wave to pass that distance. The measurement of carotid-femoral PWV is under consideration as a diagnostic tool as it is simple, robust and reproducible (Calabia et al. 2011). However, various methods are employed to detect the pulse wave such as pressure transducers, arterial tonometry and ultrasound. Mechanical methods using transducers have the disadvantages of requiring specific devices, prolonged learning period and anatomical limitations of some patients, whereas the ultrasound method is faster, has a shorter learning curve and more versatility without anatomical limitations (Calabia et al. 2011). A unit increase of PWV is predicted to raise the risk of CVD by 10% (Vlachopoulos et al. 2010). Nevertheless, due to lack of age- and sex-specific reference cutoff values for PWV, the use of arterial stiffness as a CVD biomarker in clinical practice is still rudimentary. However, a recent study has established reference and normal values for PWV based on a large European population after standardizing results for different methods of PWV measurement (Boutouyrie and Vermeersch 2010).

7. Animal models of Obesity

Human obesity results from a combination of complex and chronic pathophysiological changes and therefore requires the development of clinically relevant animal models to

investigate it systematically. Such a model will enable us to design and test effective preventive methods or therapeutic drugs to tackle the pandemic. Most anti-obesity drugs have been withdrawn from the market due to their side-effects (Kang and Park 2012). Therefore the choice of appropriate animal models is crucial for the pre-clinical assessment of drugs, to identify both positive as well as negative effects on physiology and pathophysiology.

Some of the animal models currently being used to study the underlying molecular mechanisms of human obesity are murine and rat genetic loss-of-function models, transgenic gain-of-function models, polygenic models and models exposed to environmental factors (Wang and Liao 2012).. Genetic models are helpful to tease out the role of a particular molecule or a signaling pathway. Monogenic models such as leptin deficient (*ob/ob*) or leptin receptor deficient (*db/db*) models of obesity due to hyperphagia may be useful for developing a distinct and rapid adiposity phenotype. Genetic disease models such as apolipoprotein E or LDL receptor knockout mouse show impaired clearing of plasma cholesterol and develop atherosclerosis in a shorter time while the wild-type mice are resistant to developing vascular disease. This also enables the study of the effect of pharmaceuticals at various stages of disease progression (Nilsson et al. 2012).

In contrast, the diet-induced obesity (DIO) mouse model serves as a well-established tool in obesity research as it mimics the current scenario of a fat-rich western diet, which is a major contributor to the present obesity pandemic. The C57BL/6 mouse strain is widely used in DIO models as it gains weight and becomes obese when fed a high fat diet rich in lard compared to a chow diet and develops comorbidities such as hyperlipidemia, insulin resistance and hyperglycemia as in human obesity (Winzell and Ahre 2004). Additionally different formulations of diet similar to human consumption could be used to study the effect of different

dietary components on adiposity. However, the smaller tissue or vascular vessel size of mouse models pose difficulty in working with the samples but their genetic similarity with humans and ease of genetic manipulation makes them the current disease model of choice.

7.1. Adiponectin Knockout mouse models

The global adiponectin knockout (APN KO) mouse model developed by Nawrocki et al. (2006) has a portion of the 5' upstream region of the *ADIPOQ* gene, exon 1, 2 and the coding region of exon 3 replaced by the neomycin resistance gene. This mouse model develops severe insulin resistance on a normal diet as well as when challenged with a high fat diet but is glucose intolerant only on high fat diet. Additionally, the PPARγ agonist TZD ameliorated glucose intolerance only partially in *ob/ob* mice lacking adiponectin indicating that the insulin sensitizing effect of TZDs is mediated by adiponectin via activation of the AMPK pathway. Similarly, Kubota et al. (2002) generated a APN KO model with exon 2 and 3 deleted that exhibited severe insulin resistance under basal conditions. However, the APN KO model generated by Maeda et al. (2002) with only exon 2 deleted developed insulin resistance only when challenged with a high fat/high sucrose diet. Another model from the Ma et al. (2002) group with an exon 2 deletion, had an unexpected increase in fatty acid oxidation in skeletal muscle but no effect on insulin sensitivity. In the current study, we have used the Nawrocki et al (2002) APN KO mouse model due to its distinct metabolic phenotype of insulin resistance on normal as well as high fat diet and the ease of PCR-based genotyping, which provides individual PCR amplification products for the WT allele (246 base pairs) and the disrupted (KO) allele (482 base pairs). Additionally, this APN KO mouse model has exhibited a sex difference in cardiac remodeling in response to the alterations in eNOS and adiponectin expression during DIO (Durand et al. 2012).

RATIONALE

Adiponectin exerts a number of beneficial metabolic effects such as insulin-sensitizing, anti-inflammatory, anti-lipotoxic and anti-atherogenic actions on hepatocytes, endothelial cells, skeletal, cardiac and smooth muscle cells as evident from rodent studies and further substantiation by clinical observations. However, there is a sex dimorphism in the serum levels of adiponectin with females having significantly higher levels than males (Hung et al. 2008) and estrogen has been reported to modulate expression of adiponectin and its receptors (Tan et al. 2006). Interestingly, serum adiponectin levels were found to be decreased in female ERα knockout mice (Bryzgalova et al. 2006) and were also associated with polymorphisms in the human ERα gene (Yoshihara et al. 2009). Although estrogen plays a cardioprotective role in pre-menopausal females and protects against hepatic lipid accumulation and insulin resistance (Deschamps et al. 2011; Moolman 2006; Shen and Shi 2015), no study has yet looked into the integrated metabolic actions of adiponectin and estrogen and thus how sex differences may impact adiponectin action.

Importantly, the adiponectin knockout mouse model developed by Nawrocki et al (2006), used in the present study, has exhibited a sex difference in cardiac remodeling in response to the alterations in eNOS and adiponectin during DIO. Upon high fat diet feeding, cardiac function was significantly reduced in male mice deficient in either eNOS or adiponectin or both eNOS and adiponectin. On the other hand, females only showed reduced function when both eNOS and adiponectin were absent. Furthermore, cardiac function was conserved at the expense of significantly increased left ventricular mass with the overexpression of adiponectin in male eNOS KO mice. In contrast, overexpression of adiponectin protected female eNOS KO mice from both decreased function and increased left ventricular hypertrophy (Durand et al. 2012).

Adiponectin is the most abundant adipokine secreted by adipose tissue. However, in obesity, adipose tissue expansion paradoxically results in reduced adiponectin secretion. Whether this reduction in adiponectin levels has a feedback effect on adipose tissue expansion and/or adipogenesis requires further investigation. Moreover, PPARγ which is a key player in adipogenesis and upregulator of adiponectin expression is inhibited by estrogen via ERβ (Foryst-Ludwig et al. 2008). In cultured bone marrow mesenchymal stem cells (MSCs), estrogen suppresses the adipocyte lineage and VAT adipocyte hypertrophy through the combined actions of membrane and nuclear ERα (Pedram et al. 2016).

Arterial stiffness which is increasingly being recognized as an independent predictor of inchoate vascular disease is associated with obesity and metabolic syndrome (Cavalcante et al. 2011). In population based studies, insulin resistance and hypertension, two comorbidities highly prevalent in obese subjects, were associated with arterial stiffness (Mulè et al. 2006; Payne et al. 2010; Wohlfahrt et al. 2014). Furthermore, hypertension has been considered as both cause and consequence of vascular stiffness (Mitchell 2014). Thus a deficiency of adiponectin, which potentiates insulin resistance and hypertension, could be contributing to the pathophysiology of arterial stiffness and warrants further investigation in animal models.

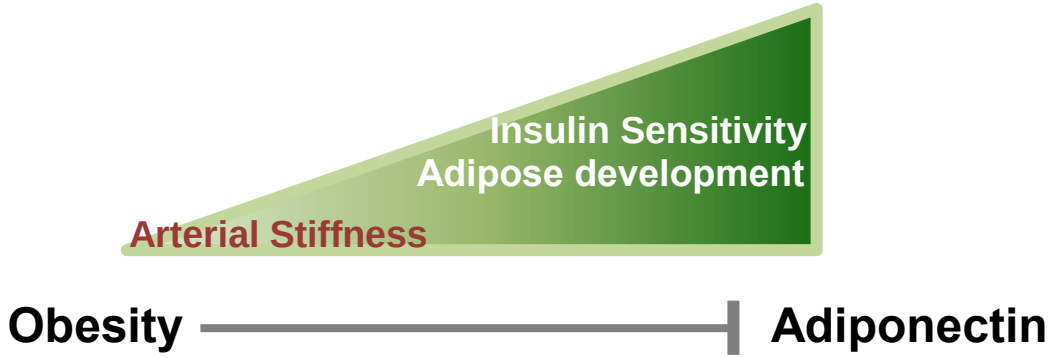
Additionally, adiponectin exerts a regulatory effect on vascular tone by increasing the bioavailability of NO (Cheng et al. 2007). It modulates vascular remodeling by inhibiting vascular smooth muscle cell proliferation and migration and thereby regulates the development of atherosclerosis (Fuerst et al. 2012). Likewise, estrogen is shown to have a vasoprotective effect by its induction of eNOS expression and activity in human umbilical vein endothelial cell (HUVEC) and brain endothelial cells (Nevzati et al. 2015) via activation of AMPK through a ERβ/Ca^{2+}/Calmodulin-dependent protein kinase kinase β (CaMKKβ) pathway (Yang and Wang

2015). This mechanism could partially account for the increased incidence of cardiovascular diseases in post-menopausal women who have decreased ovarian estrogen production. Thus, even though a sex difference is well established in the pathophysiology of obesity and metabolic disorders, the underlying molecular mechanism is not yet fully understood. As such, it is imperative to research the effect of sex dimorphism on the metabolic and vascular functions of adiponectin, a potent hormone secreted by adipose tissue.

HYPOTHESIS

We hypothesize that the

1. Lack of adiponectin negatively affects adipose tissue development, insulin sensitivity and vascular tissue function in diet-induced obesity.
2. Metabolic and vascular defects observed in the absence of adiponectin are sex-dependent.



OBJECTIVES

In the proposed study, our objectives are to:

1. Characterize the metabolic and vascular effects of loss of adiponectin function in a model of diet-induced obesity, and
2. Determine the effect of sex on the metabolic and vascular phenotypes in adiponectin knockout mice fed a high fat diet.

MATERIALS AND METHODS

1. Adiponectin knockout mice colony establishment

Three (one male and two female) whole body adiponectin knockout (APN KO) mice on the C57BL/6 background were obtained from Dr. Philipp Scherer, University of Texas, Southwestern Medical Centre. The two female knockout mice died prior to successful breeding. Therefore the remaining male APN KO mouse was bred with wild-type C57BL/6 mice to obtain heterozygous offspring. The heterozygous mice were crossed to generate homozygous knockout mice, which were used to establish our APN KO colony. The animal care protocol was approved by the University of Manitoba Animal Care Committee and followed the guidelines of the Canadian Council on Animal Care.

1.1. Genomic DNA Isolation

Genomic DNA was isolated by incubating tails snips overnight in 500 µl of tail lysis buffer (1 M Tris-HCl pH 8.0, 0.5 M EDTA pH 8.0, 5 M NaCl, 10% SDS) along with 2.5 µl of Proteinase K enzyme (Sigma Cat.# P2308; Stock solution 25 mg/ml in double distilled water) at 55°C. After digestion, the tubes were vortexed briefly and then centrifuged at 21,000 g for 5 minutes. The supernatant was transferred into a fresh 1.5 ml microfuge tube and mixed with 1 ml of 95% ethanol, followed by centrifugation at 21,000 g for 10 minutes. The supernatant was discarded and the pellet was washed with 70% ethanol and re-centrifuged at 21,000 g for 5 minutes. After discarding the supernatant, the pellet was air dried for between 30-40 minutes and dissolved in 50 µl of sterile double distilled water and stored at -20°C.

1.2. Genotyping Adiponectin knockout mice

Genotyping PCR was done using AccuStart II GelTrack PCR SuperMix (2×) (Quanta Biosciences Cat.# 95135-500) with 0.5 µl of 10 µM primers (Table 1) and 1 µl of the isolated

genomic DNA in a final volume of 50 µl. The PCR conditions were according to Nawrocki et al (2006) and are shown in Table 2. The PCR reaction products were run on a 2% agarose gel containing the Sybr Safe DNA gel stain (Invitrogen Cat.# S33102) along with an O'GeneRuler Low Range DNA Ladder (Thermo Scientific Cat.# SM1203), and the presence of a 482 bp amplicon (disrupted adiponectin allele) and/or a 260 bp amplicon (wild-type allele) were detected using Gel Doc (BioRad, USA) (Nawrocki et al. 2006).

2. Animal Study Design

2.1. Study I

In order to characterize the metabolic and vascular effects of loss of adiponectin function in obesity, male 8-week old adiponectin knockout *(*APN KO) and C57BL/6 wild-type (WT) control mice were fed a high fat diet (HFD, 60% energy from fat) for 12 weeks to induce obesity and insulin resistance (n=10/group). APN KO and C57BL/6 were fed a low fat diet (LFD, 10% energy from fat) and used as lean controls (n=10/group). Eight week old male APN KO and WT mice (n=10/group) before starting on diets were used as baseline controls. A total of 62 mice (30 WT and 30 APN KO) were used. The LFD and HFD diets were the same as Nawrocki et al (2006) and obtained from Research Diets (New Brunswick, NJ, USA); their compositions are given in Table 3. The mice were kept in individual cages at 21±1°C with a fixed 12 hour light 12 hour dark cycle and fed *ad libitum* the respective diets. Weekly body weights and other parameters were recorded as indicated in Figure 7. Oral glucose tolerance test (OGTT) was done at week 8 and 12 to determine insulin resistance development. The animal care protocol was approved by the University of Manitoba Animal Care Committee and followed the guidelines of the Canadian Council on Animal Care.

Table 1: Genotyping PCR Primers

Name	**Sequence**
ACRP-forward	GGACCCCTGAACTTGCTTCAC
ACRP-reverse	CACCCACAGTAATTCCATGGG
Neo-reverse	GAATGGGCTGACCGCTTCCTCGTG

Table 2: Genotyping PCR Conditions

Steps	Cycles	Temperature (°C)	Duration
i. Denaturation	1	95	15 minutes
ii. Amplification a. Denaturation b. Annealing c. Extension	35	 94 64 72	 30 seconds 30 seconds 1 minute
iii. Termination	1	4	∞

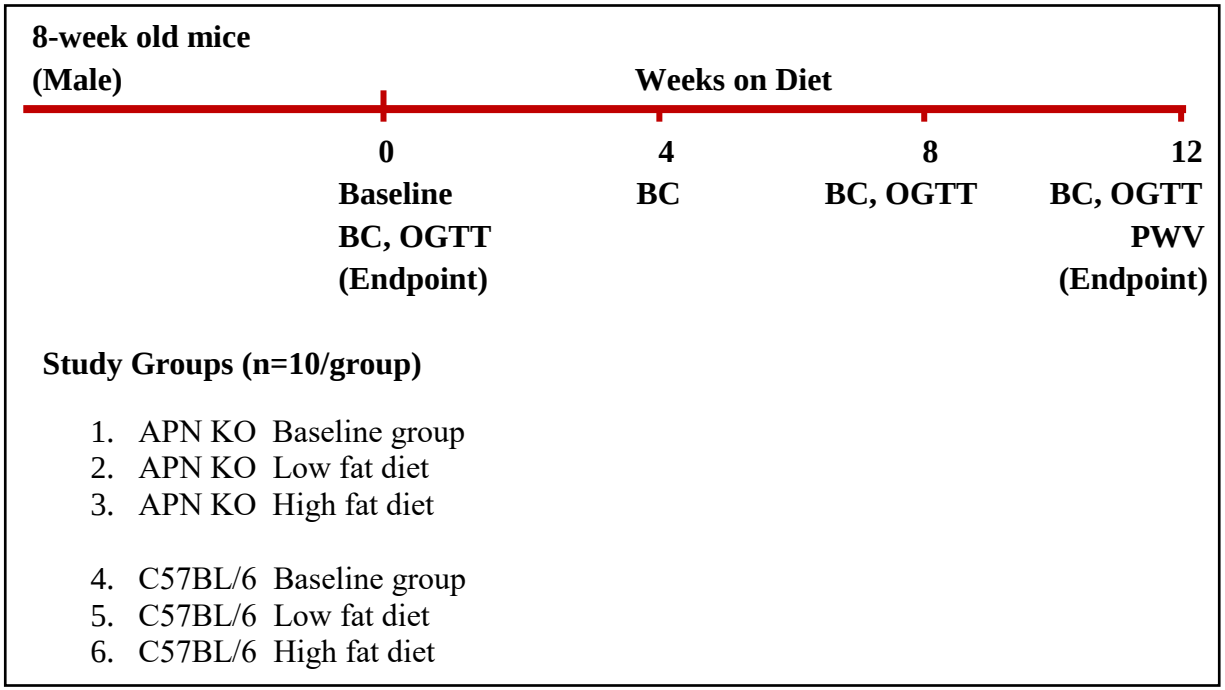


Figure 7: Study I Design. Eight week old male adiponectin knockout (APN KO) and wild-type C57BL/6 mice were fed high fat diet (60% energy from fat) or low fat diet (10% energy from fat) n=10/group for 12 weeks. **BC**-Body Composition was measured every 4 weeks at baseline, 4, 8 and 12 weeks. **OGTT**-Oral Glucose Tolerance Test was carried out at baseline, 8 and 12 weeks. **PWV**-Pulse Wave Velocity was measured at week 12 before the endpoint.

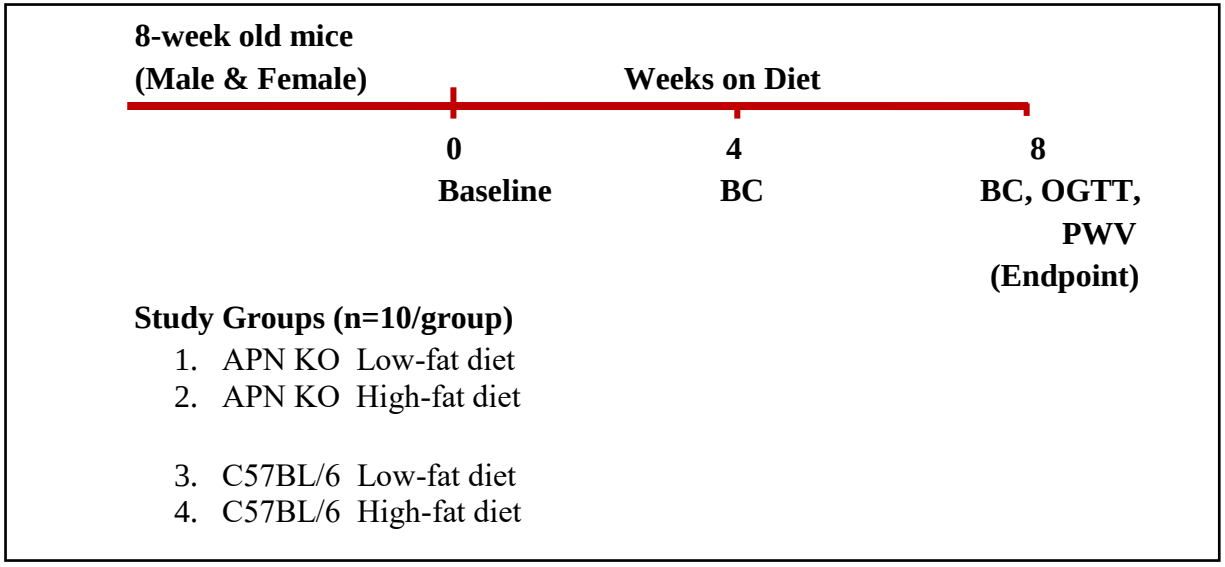


Figure 8: Study II Design. Eight week old male and female adiponectin knockout (APN KO) and wild-type C57BL/6 mice were fed high fat diet (60% energy from fat) or low fat diet (10% energy from fat) for 8 weeks, n=10/group. **BC**-Body Composition was measured every 4 weeks at baseline, 4 and 8 weeks. **OGTT**-Oral Glucose Tolerance Test was carried out at week 0 and week 8. **PWV**-Pulse Wave Velocity was measured at week 8 before the endpoint.

2.2. Study II

In order to study the effect of sex in this model of obesity, 8-week old male and female APN KO and C57BL/6 WT mice were fed a HFD for 8 weeks. APN KO and WT mice fed LFD for 8 weeks served as lean controls (n=10/group). A total of 80 mice (40 males and 40 females) were used. Weekly body weights were measured along with other parameters as indicated in Figure 8. The 8 week study duration was based on the results obtained in Study I.

3. Body composition

Body composition (BC) (total fat mass, total lean mass, total water and total free water) was measured at weeks 0, 4, 8 and 12 on diet, using the EchoMRI-700™ whole body quantitative nuclear magnetic resonance (QMR) instrument (Echo Medical Systems, Houston TX, USA) by placing the animals in restraining tubes appropriate to their size. % Fat mass and % lean mass were calculated using the corresponding body weight.

4. Oral Glucose Tolerance Test (OGTT)

Animals were fasted for 6 hours and tail vein blood glucose was measured using an Alpha TRAK blood glucose monitoring system (calibrated for rodents) and Alpha TRAK2 blood glucose test strips (Abbott Laboratories, USA) at times 0 (pre glucose load), 30, 60, 90 and 120 minutes after a glucose bolus (2.5 mg/g body weight using a 50% [w/v] glucose solution) given orally (Study I) or by gavage (Study II). Area under the curve (AUC) was calculated using the trapezoidal method (Purves 1992). The base calculation is as follows, where C = blood glucose concentration:

$$AUC_{times0\text{-}1} = [(C_0 + C_1)/2] \times \text{time interval}_{0\text{-}1}$$

AUC was repeated for each time interval and then the sum of all the time intervals was taken to estimate the insulin-dependent movement of glucose from the blood into the tissues.

5. Arterial Stiffness

At weeks 8 and 12 on diet in Study II and Study I, respectively, the arterial stiffness was determined by measuring PWV or peak flow velocity. PWV was measured non-invasively on the femoral artery by a pulsed-wave Doppler ultrasound system (Indus Instruments, Texas, USA) and carried out by the R. O. Burrell Lab staff at the Albrechtsen Research Centre at St. Boniface Hospital. Each mouse was anesthetized with Isoflurane and then placed in the supine position with its limbs taped to the non-invasive electrodes on the MouseMonitor S which captures processes, filters the ECG signals and outputs the filtered ECG waveform to the signal processing system. The peak flow velocity signals were captured by positioning the tip of the pulsed Doppler tubing mounted probe near the femoral artery. A 20 MHz probe was used for mice. All of the pulsed Doppler transducers were plugged into the appropriate 20 MHz module housed in the MouseDoppler Multichannel Mainframe to generate the pulsed Doppler signal. The MouseDoppler™ then processed the signals using a fast Fourier transform algorithm and displayed the results as a real-time grayscale Doppler ultrasound spectrogram which was analyzed by the Doppler spectrogram analysis software. Three consecutive spectrograms were taken for each animal and the data presented are the average of these three readings.

6. Tissue collection

At the end of the study, the baseline control animals and the ones on diet for 8 and 12 weeks were euthanized by decapitation. Trunk blood was collected into a 2 ml microfuge tube

containing Sodium Heparin (PPC, Canada Cat.# C504710), mixed by inverting 6-7 times, put on ice and later centrifuged at 1000 g for 15 minutes at 4°C. The plasma layer was collected and stored at -80°C for future analysis. Various fat depots such as epididymal (males), parametrical (females), peri-renal, inguinal, mesenteric and epicardial white adipose tissues (WAT) were harvested, weighed and flash frozen in liquid nitrogen and stored at -80°C. Additionally, other tissues such as liver, BAT, kidney, pancreas, heart, aorta and skeletal muscle were collected, weighed and stored for future. A small piece of liver tissue was embedded in optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA, Cat.# 4583) and epididymal adipose tissue was embedded in Cryo-Gel (Leica Microsystems, Electron Microscopy Sciences, Hatfield, PA, Cat.# 62806-01); both were frozen immediately in a dry ice-ethanol bath and stored at -80°C until used for histological analysis.

7. Hepatic lipid droplet assessment

Eight μm thick sections of frozen liver in OCT compound were cut using a cryotome (Microm HM550) set at -15°C. The sections were then fixed in 4% paraformaldehyde (PFA) for 7 minutes and washed in 1× phosphate buffered saline (PBS) for 5 minutes. The fixed sections were then stained using hematoxylin and eosin (H & E) or Oil Red O staining.

A Mayer's hematoxylin working solution was made by dissolving 50 g aluminium potassium sulfate (alum), 1 g hematoxylin (Fischer Scientific Cat.# 345-25), 0.2 g sodium iodate and 20 ml glacial acetic acid in 1 L of double distilled water. Once fully dissolved, the solution was brought to boil, cooled and filtered before use. Eosin solution was prepared by dissolving 5 g eosin Y (Fischer Scientific Cat.# E-511) in 500 ml double distilled water.

The fixed sections were then washed in double distilled water for 15 minutes followed by incubation for 4 minutes in hematoxylin solution. The slides were again rinsed in double distilled water and washed in cold tap water for 5 minutes. The differentiation step was done by a quick dip in acidified ethanol (1 ml concentrated hydrochloric acid and 400 ml 70 % ethanol) followed by a quick rinse in cold tap water and double distilled water. Excess water was blotted before counter-staining with eosin Y dye for 3 minutes. The counter-stained sections were then dehydrated by incubation in 95% ethanol for 5 minutes and then twice in absolute ethanol for 5 minutes each, followed by a clearing step in xylene for 15 minutes. The sections were then immediately mounted carefully without air bubbles using VectaMount^{TM} mounting medium (Vector Laboratories Cat.# H-5000) and dried overnight in a fumehood.

The fixed sections were also stained with Oil Red O, a fat soluble dye, to visualize hepatic lipid droplets. An Oil Red O stock solution was made by combining 0.7 g of Oil Red O (Sigma-Aldrich, St. Louis, MO cat # O0625) with 200 ml of 60% isopropanol. The mixture was stirred overnight, filtered through a 0.2 μm filter and stored at 4°C. An Oil Red O working solution was made by combining 120 ml of stock solution with 80 mL of double distilled water. The prepared sections were placed in 60% isopropanol for 5 minutes and then placed in the Oil Red O working solution for 30 minutes. The slides were then rinsed twice in 60% isopropanol, followed by a wash in double distilled water. The slides were then counter-stained with haematoxylin for 1 minute, rinsed again in double distilled water and placed in tap water for 1 minute. After a final rinse in double distilled water, the slides were mounted using aqua mounting media and dried at room temperature in the fumehood.

The stained sections were visualized with a Zeiss Axioskop 2 plus microscope (Zeiss, Thornwood, NY) and images were captured with a Zeiss Axiocam digital camera using Axio

Vision 4.6 (Zeiss, Thornwood, NY). Unstained vacuoles were counted as lipid droplets for H&E stained sections; lipids were stained orange red in Oil Red O stained sections. A visual comparison was made between study groups based on 6 different sections from each animal in the groups. A total of 4 animals per group were studied and the observers were blinded to the identity of slides being examined. The scoring system gave '-' to lipid droplet free sections and '+' for droplet containing sections. The number of '+' is directly proportional to the number of lipid droplets observed.

8. Statistical Analysis

In Study I, two-way ANOVA, with genotype and diet as main effects, was used for endpoint data while repeated measures two-way ANOVA was used for time course data such as weekly body weight, body composition and glucose concentrations during OGTT (SAS Version 9.2, SAS institute, Cary, NC). Following ANOVA, means testing with Duncan's multiple range test (DMRT) was performed. In Study II, three-way ANOVA with sex, genotype and diet as main effects was used for endpoint data while repeated measures three-way ANOVA was used for time course data such as weekly body weight, body composition and glucose concentrations during OGTT. If the data were not normal or homogeneous, it was analyzed after log transformation, or nonparametric testing was used. Nonparametric testing used the Kruskal-Wallis test, followed by least significant difference post hoc testing with Tukey correction for multiple comparisons. Outliers (± 2.5 standard deviations from the mean) were removed from the data set before analysis. Data are expressed as means ± standard error of the mean (SEM). A P-value ≤ 0.05 was considered to be significant and trend towards significance was noted at

$P \leq 0.10$. Statistically significant differences among groups at a given time point are indicated by different lower case letters for time course data.

Table 3: Low Fat Diet and High Fat Diet Formulations*

Diet Name	**Low Fat Diet (LFD)**		**High Fat Diet (HFD)**	
Product #	D14051101		D12492	
%	gram	**kcal**	gram	**kcal**
Protein	29	**30**	26	**20**
Carbohydrate	58	**60**	26	**20**
Fat	4	**10**	35	**60**
Total		**100**		**100**
kcal/gam	3.8		5.2	
Ingredients	**gram**	**kcal**	**gram**	**kcal**
Casein	300	1200	200	800
L-Cystine	4.5	18	3	12
Corn Starch	404.7	1619	0	0
Maltodextrin	125	500	125	500
Sucrose	68.8	275	68.8	275
Cellulose	50	0	50	0
Soybean Oil	25	225	25	225
Lard	20	180	245	2205
Mineral Mix	10	0	10	0
DiCalcium Phosphate	13	0	13	0
Calcium carbonate	5.5	0	5.5	0
Potassium Citrate	16.5	0	16.5	0
Vitamin Mix	10	40	10	40
Choline Bitartrate	2	0	2	0
FD&C Yellow Dye #5	0.05	0	0	0
FD&C Blue Dye #1	0	0	0.05	0
Total	**1055.05**	**4057**	**773.85**	**4057**

*OpenSource Diets, Research Diets, New Brunswick, NJ, USA.

RESULTS

1. Genotyping and establishment of adiponectin knockout mouse colony

A whole body adiponectin knockout male mouse on the C57BL/6 background obtained from Dr. Philip Scherer, University of Texas, was bred with wild-type C57BL/6 mouse and heterozygous animals were obtained. The crossing of adiponectin knockout animals obtained from heterozygous breeding pairs was carried out to establish the colony in the R.O. Burrell lab at St. Boniface Hospital Albrechtsen Research Centre. The genomic DNA isolation from tail snips was standardized and genotyping PCR for the mouse models were done to further obtain animals for the study. PCR-based genotyping provided 246 base pair sized amplicon for wild-type allele and 482 base pair sized amplicon for disrupted adiponectin knockout allele (Figure 9).

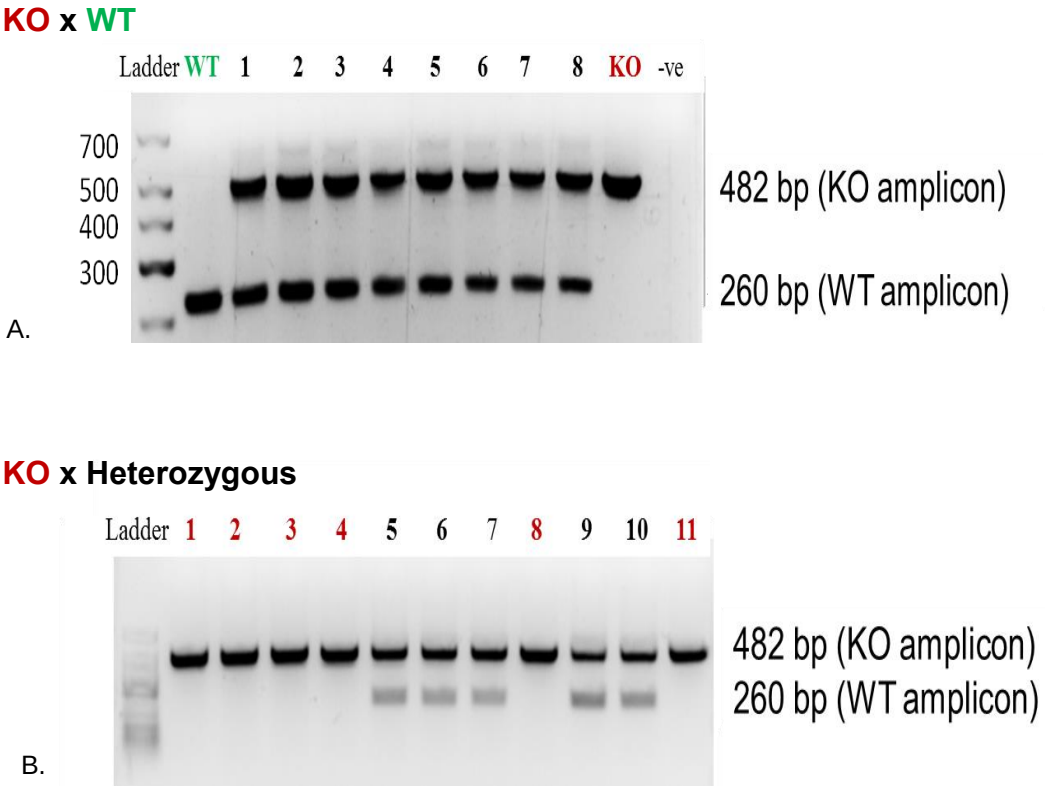


Figure 9: PCR-based genotyping of the adiponectin knockout mouse model. F1 generation of (A) Adiponectin Knockout × Wild-type C57BL/6 mouse with lane 1-8 showing Heterozygous genotype with both 260bp and 482bp bands and (B) Adiponectin Knockout × Heterozygous mouse with lanes 1-4, 8 and 11 showing the knockout genotype and the other lanes showing the Heterozygous genotype. **KO:** Adiponectin Knockout mice, **WT:** Wild-type C57BL/6 mice.

2. Metabolic and vascular effects of loss of adiponectin function in a male model of diet induced obesity

2.1. Body weight

Eight week old male adiponectin knockout (APN KO) and wild-type C57BL/6 (WT) mice were either fed a high fat diet (60% energy from fat) for 12 weeks to induce obesity and insulin resistance (n=10/group) or were fed a low fat diet (10% energy from fat), for 12 weeks to be used as lean controls (n=10/group). On the high fat diet, APN KO and WT mice showed comparable gain in body weight over the first 8 weeks, however, from week 9 onwards APN KO mice gained significantly more body weight than did the WT mice (Figure 10). Thus after 12 weeks on the high fat diet, APN KO mice weighed significantly more than the WT mice (45.1±1.3 g vs 40.1±1.1 g, P=0.0008) but there were no differences in the final body weights between genotypes when on low fat diet (Figure 11).

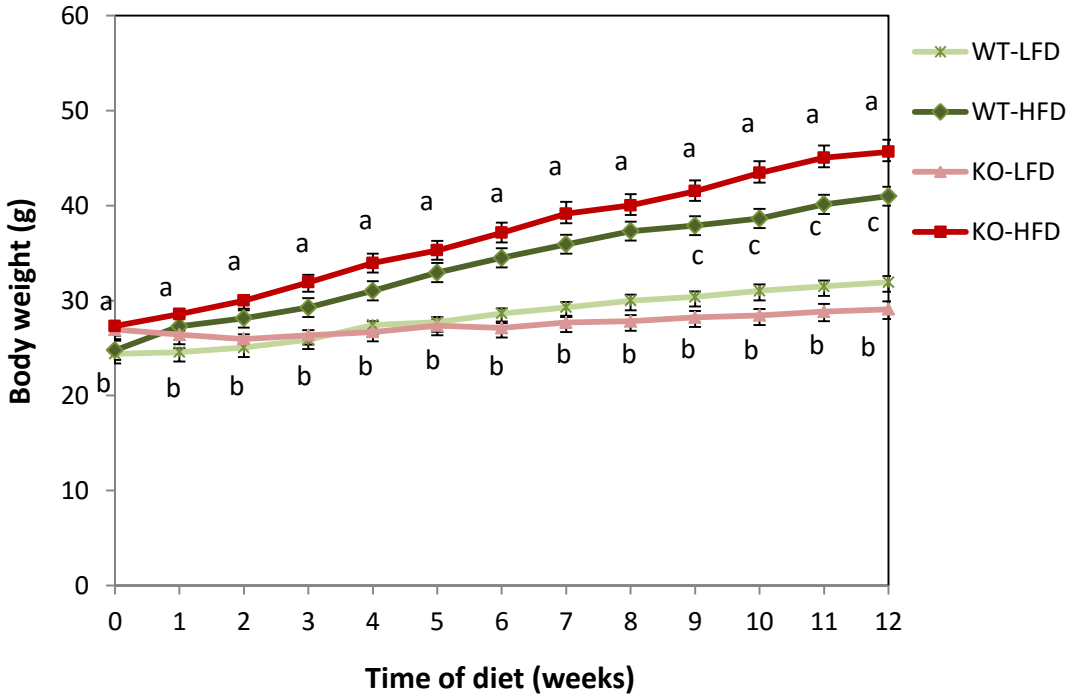


Figure 10: Weekly body weights of mice fed either a low or a high fat diet. Eight week old male wild-type C57BL/6 or adiponectin knockout mice were fed either a low fat diet or high fat diet for 12 weeks. After week 8, KO mice gained significantly more weight than WT mice on the high fat diet. Data are expressed as means ± SEM (n=10/group). Repeated measures ANOVA was used and statistically significant differences ($P<0.05$) among groups at a given time point of diet are indicated by different lower case letters. **LFD**: Low fat diet, **HFD**: High fat diet, **KO**: Adiponectin Knockout mice, **WT**: Wild-type C57BL/6 mice.

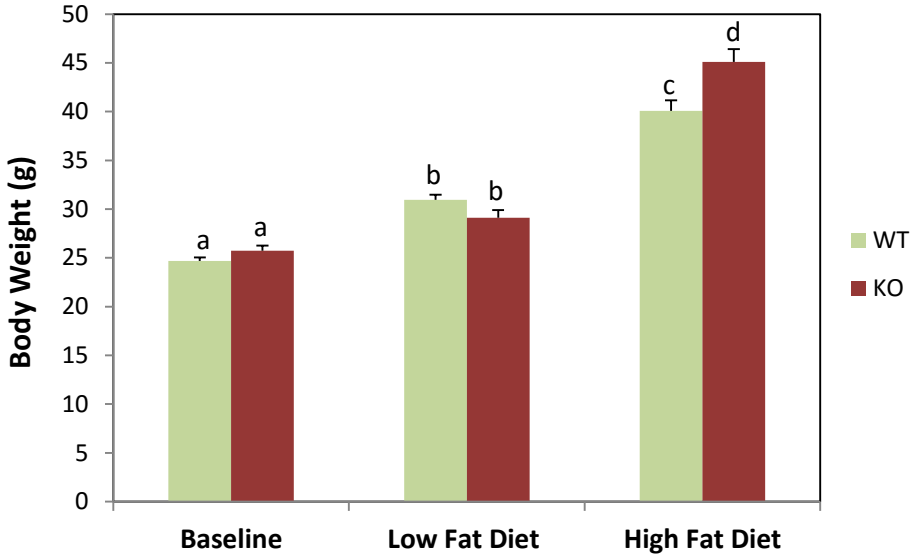


Figure 11: Initial and final body weights. Mice were weighed at baseline (before starting on diet) and after 12 weeks on either a low or a high fat diet. Data are expressed as means ± SEM (n=10/group). Statistically significant differences among groups ($P<0.05$) are indicated by different lower case letters. There were significant main effects for diet ($P<0.0001$) and genotype×diet ($P=0.001$). **KO**: Adiponectin Knockout mice, **WT**: Wild-type C57BL/6 mice.

2.2. Adiposity

2.2.1. White Adipose Tissue Depot Weights

Although APN KO mice weighed significantly more than the WT mice after 12 weeks on high fat diet, their epididymal fat pad weight was significantly less (5.98±0.42 g/100 g bwt vs 7.24±0.35 g/100 g bwt, P=0.035) (Figure 12A). In contrast, the other visceral fat pads such as peri-renal and mesenteric fat pads was not different from WT mice when fed HFD (Figure 12 B & C). Furthermore, both in the baseline and LFD fed groups, there were significant reductions in individual fat pads (epididymal, peri-renal and mesenteric) (Figure 12) and their sum as visceral fat(3.57±0.43 g/100 g bwt vs 7.37±0.32 g/100 g bwt, P<0.0001) and subcutaneous (inguinal) fat pad weights (1.44±0.14 g/100 g bwt vs 2.52±0.12 g/100 g bwt, P<0.0001) in the APN KO mice relative to the WT mice (Figure 13).

2.2.2. Other Tissue/Organ Weights

Additionally, a significant difference in liver weight normalized to 100 g body weight was observed between WT and APN KO in the low fat diet groups (4.25±0.20 g/100 g bwt vs 5.09±0.10 g/100 g bwt, P=0.0013) and high fat diet groups (3.16±0.16 g/100 g bwt vs 4.03±0.17 g/100 g bwt, P=0.0007) (Table 5). Furthermore, APN KO had significantly reduced skeletal muscle and heart weights both normalized to 100 g body weight in the high fat diet group as compared to the WT group. No significant difference was observed between genotypes in either diet groups for normalized weights of epicardial fat tissue, brown adipose tissue, pancreas and kidney (Table 5).

With regard to original weights of tissues before normalizing to body weight, brown adipose tissue, liver, pancreas and lung fluid from APN KO group weighed significantly

more than the WT group when on the high fat diet as compared to the WT group. However, in both WT and APN KO high fat diet groups, the weights of the epicardial white adipose tissue on a increased significantly relative to low fat diet group (Table 4).

2.2.3. Body composition

The above findings of reduced fat depot weights were corroborated by whole body composition analysis using EchoMRI™ which showed a reduction in % fat mass in APN KO in comparison with the WT mice when fed a low fat diet for 12 weeks (11.5±1.5% vs 24.7±1.2%, $P<0.0001$) with a concomitant increase in % lean mass (88.65±2.4% vs 71.83±1.2%, $P<0.0001$). However, there were no differences in overall % fat mass and % lean mass between genotypes when fed a high fat diet (Figure 14).

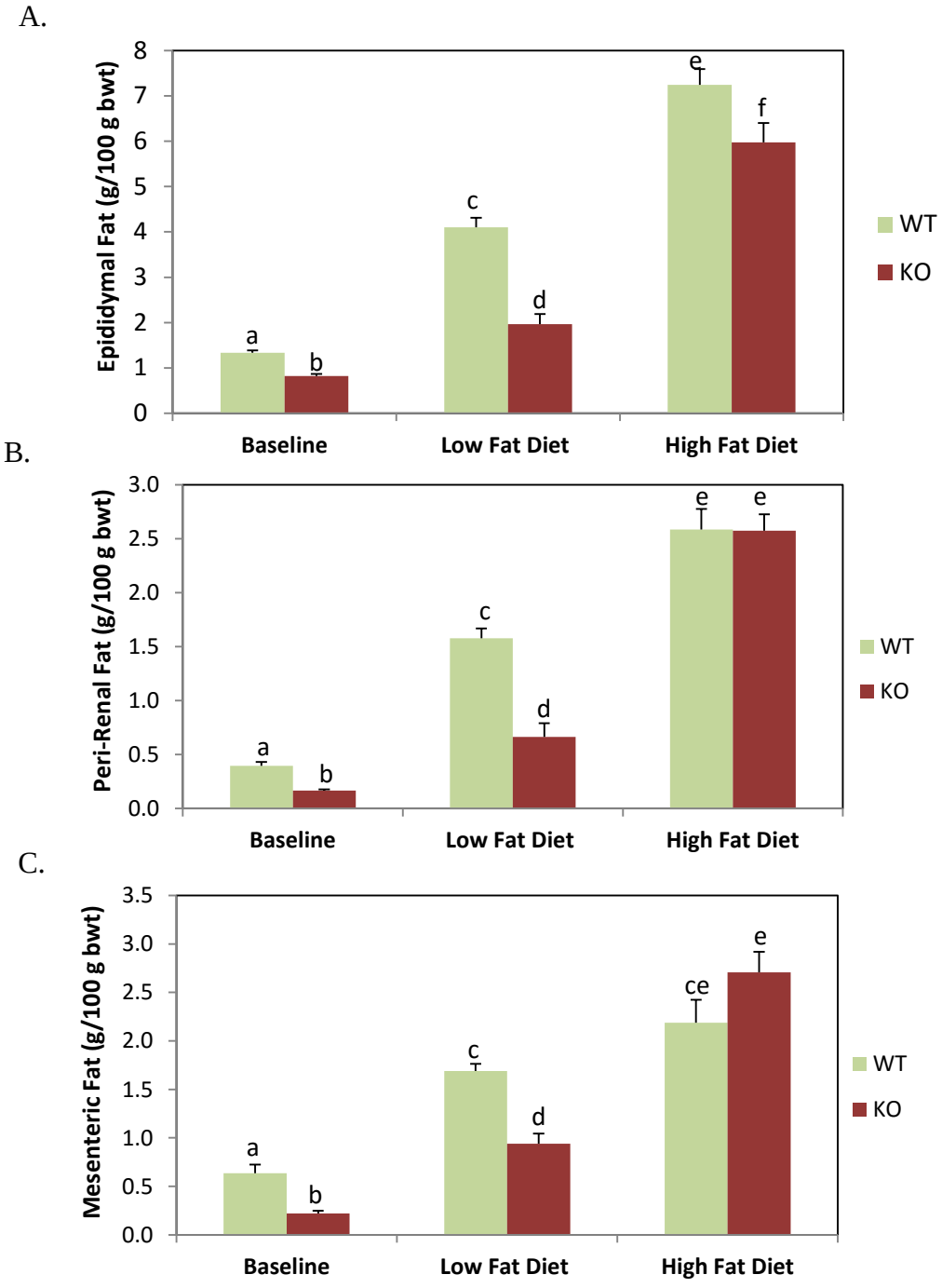
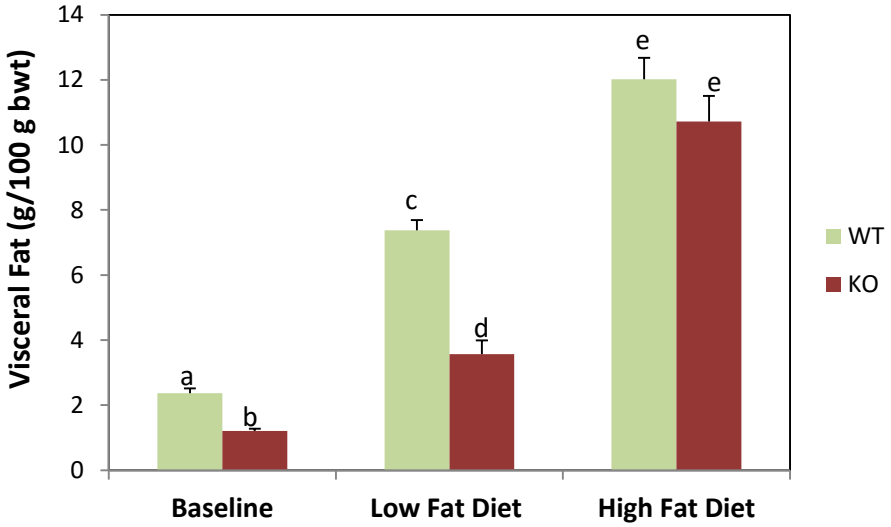


Figure 12: Comparison of weights of white adipose tissue depots. (A) Epididymal (B) Peri-renal and (C) Mesenteric fat pad weights at baseline before starting on diet and after 12 weeks on high or low fat diet. Data are expressed as means ± SEM (n=10/group). Statistically significant differences among groups ($P<0.05$) are indicated by different lower case letters. There were significant main effects for genotype, diet and genotype×diet. **KO**: Adiponectin Knockout mice, **WT**: Wild-type C57BL/6 mice.

A.



B.

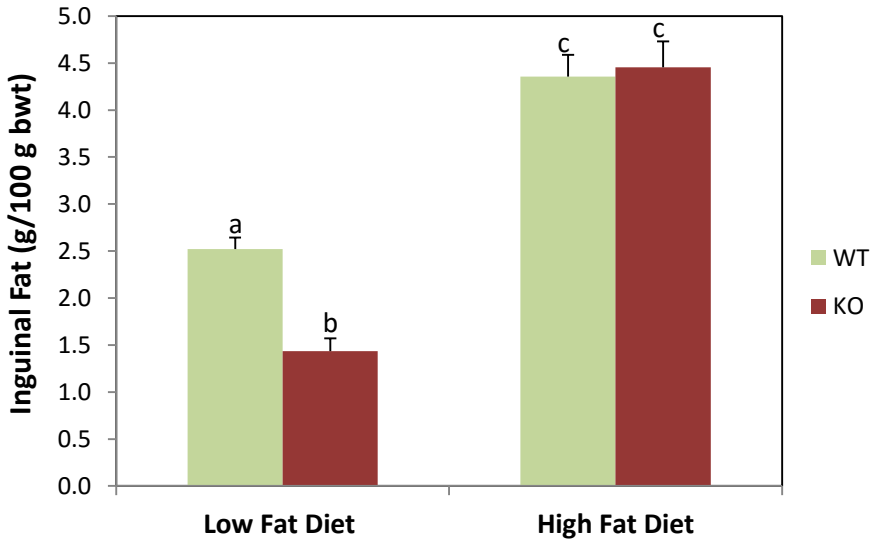


Figure 13: Comparison of visceral and subcutaneous (inguinal) adipose tissue weights. Fat pads were harvested and weighed at baseline before starting on diet and after 12 weeks on high or low fat diet where the visceral fat tissue weight is the total weight of epididymal, peri-renal and mesenteric fat pads. Data are expressed as means ± SEM (n=10/group). Statistically significant differences among groups ($P<0.05$) are indicated by different lower case letters. There were significant main effects for genotype ($P<0.0001$), diet ($P<0.0001$) and genotype×diet ($P=0.0001$) for visceral fat while subcutaneous fat had significant main effects for diet ($P<0.0001$) and genotype×diet ($P=0.006$) only. **KO**: Adiponectin Knockout mice, **WT**: Wild-type C57BL/6 mice.

Table 4: Tissue/Organ weights[#]

Tissues (g)	**Baseline**		**Low Fat Diet**		**High Fat Diet**		**Geno**	**Diet**	**Geno x Diet**
	WT	**KO**	**WT**	**KO**	**WT**	**KO**			
Epicardial WAT	N.D.	N.D.	0.027 ± 0.003^{a}	0.021 ± 0.003^{a}	0.047 ± 0.004^{b}	0.054 ± 0.005^{b}	N.S.	<0.0001	N.S.
BAT	0.17 ± 0.01^{a}	0.12 ± 0.01^{a}	0.25 ± 0.02^{b}	0.21 ± 0.01^{b}	0.25 ± 0.04^{bc}	0.30 ± 0.02^{c}	N.S.	<0.0001	0.03
Liver	1.37 ± 0.06^{a}	1.52 ± 0.05^{a}	1.31 ± 0.06^{a}	$1.48\pm.03^{a}$	1.27 ± 0.09^{a}	1.83 ± 0.12^{b}	<0.0001	N.S.	0.02
Pancreas	0.33 ± 0.02^{a}	0.35 ± 0.01^{a}	0.30 ± 0.02^{a}	0.32 ± 0.02^{a}	0.29 ± 0.01^{a}	0.38 ± 0.02^{b}	0.0038	N.S.	N.S.
Skeletal muscle	0.66 ± 0.03^{a}	0.70 ± 0.02^{a}	0.93 ± 0.03^{b}	0.82 ± 0.03^{c}	0.97 ± 0.06^{bd}	0.95 ± 0.05^{d}	N.S.	<0.0001	N.S.
Kidney	0.35 ± 0.006^{a}	0.32 ± 0.02^{a}	0.37 ± 0.01^{ab}	0.38 ± 0.01^{b}	0.37 ± 0.02^{ab}	0.40 ± 0.01^{b}	N.S.	0.0005	N.S.
Heart	0.14 ± 0.007^{a}	0.14 ± 0.007^{a}	0.14 ± 0.007^{a}	0.14 ± 0.006^{a}	0.16 ± 0.005^{b}	0.15 ± 0.004^{ab}	N.S.	0.04	N.S.
Lung fluid	0.14 ± 0.008^{a}	0.14 ± 0.01^{a}	0.18 ± 0.02^{a}	$0.15\pm.005^{a}$	0.17 ± 0.02^{ab}	0.19 ± 0.01^{b}	N.S.	0.004	N.S.

[#]Data are expressed as means ± SEM (n=8-10/group). Two-way ANOVA with genotype and diet as main effects was used and within a row, statistically significant differences among groups ($P<0.05$) are indicated by different lower case letters as superscript.

BAT: Brown Adipose Tissue, **Geno**: Genotype, **KO**: Adiponectin Knockout mice, **N.D.**: Not determined, **N.S.**: Not significant, **WAT**: White Adipose Tissue and **WT**: Wild-type C57BL/6 mice.

Table 5: Normalized Tissue/Organ weights[#]

Tissues (g/100 g bwt)	**Baseline**		**Low Fat Diet**		**High Fat Diet**		**Geno**	**Diet**	**Geno* Diet**
	WT	**KO**	**WT**	**KO**	**WT**	**KO**			
Epicardial WAT	N.D.	N.D.	0.088 ± 0.01^{a}	0.069 ± 0.01^{a}	0.117 ± 0.01^{b}	0.120 ± 0.01^{b}	N.S.	<0.0001	N.S.
BAT	0.69 ± 0.04^{a}	0.48 ± 0.03^{c}	0.82 ± 0.05^{b}	0.72 ± 0.03^{b}	0.69 ± 0.07^{a}	0.74 ± 0.04^{ab}	0.03	0.0004	0.02
Liver	5.53 ± 0.21^{a}	5.92 ± 0.17^{a}	4.25 ± 0.20^{b}	5.09 ± 0.10^{c}	3.16 ± 0.16^{d}	4.03 ± 0.17^{e}	<0.0001	<0.0001	N.S.
Pancreas	1.32 ± 0.08^{a}	1.37 ± 0.03^{a}	0.93 ± 0.07^{b}	1.08 ± 0.05^{b}	0.78 ± 0.06^{bd}	0.85 ± 0.05^{d}	N.S.	<0.0001	N.S.
Skeletal muscle	2.66 ± 0.09^{a}	2.74 ± 0.06^{ab}	2.93 ± 0.07^{b}	2.81 ± 0.11^{b}	2.58 ± 0.11^{a}	2.23 ± 0.08^{c}	N.S.	<0.0001	N.S.
Kidney	1.40 ± 0.03^{a}	1.20 ± 0.07^{b}	1.21 ± 0.04^{b}	1.30 ± 0.03^{b}	0.92 ± 0.05^{c}	0.90 ± 0.02^{c}	N.S.	<0.0001	0.005
Heart	0.56 ± 0.02^{a}	0.56 ± 0.03^{a}	0.47 ± 0.03^{b}	0.48 ± 0.02^{b}	0.39 ± 0.01^{c}	0.34 ± 0.01^{d}	N.S.	<0.0001	N.S.

[#]Data are expressed as means ± SEM (n=8-10/group). Two-way ANOVA with genotype and diet as main effects was used and within a row, statistically significant differences among groups ($P<0.05$) are indicated by different lower case letters as superscript.

BAT: Brown Adipose Tissue, **Geno**: Genotype, **KO**: Adiponectin Knockout mice, **N.D.**: Not determined, **N.S.**: Not significant, **WAT**: White Adipose Tissue and **WT**: Wild-type C57BL/6 mice.

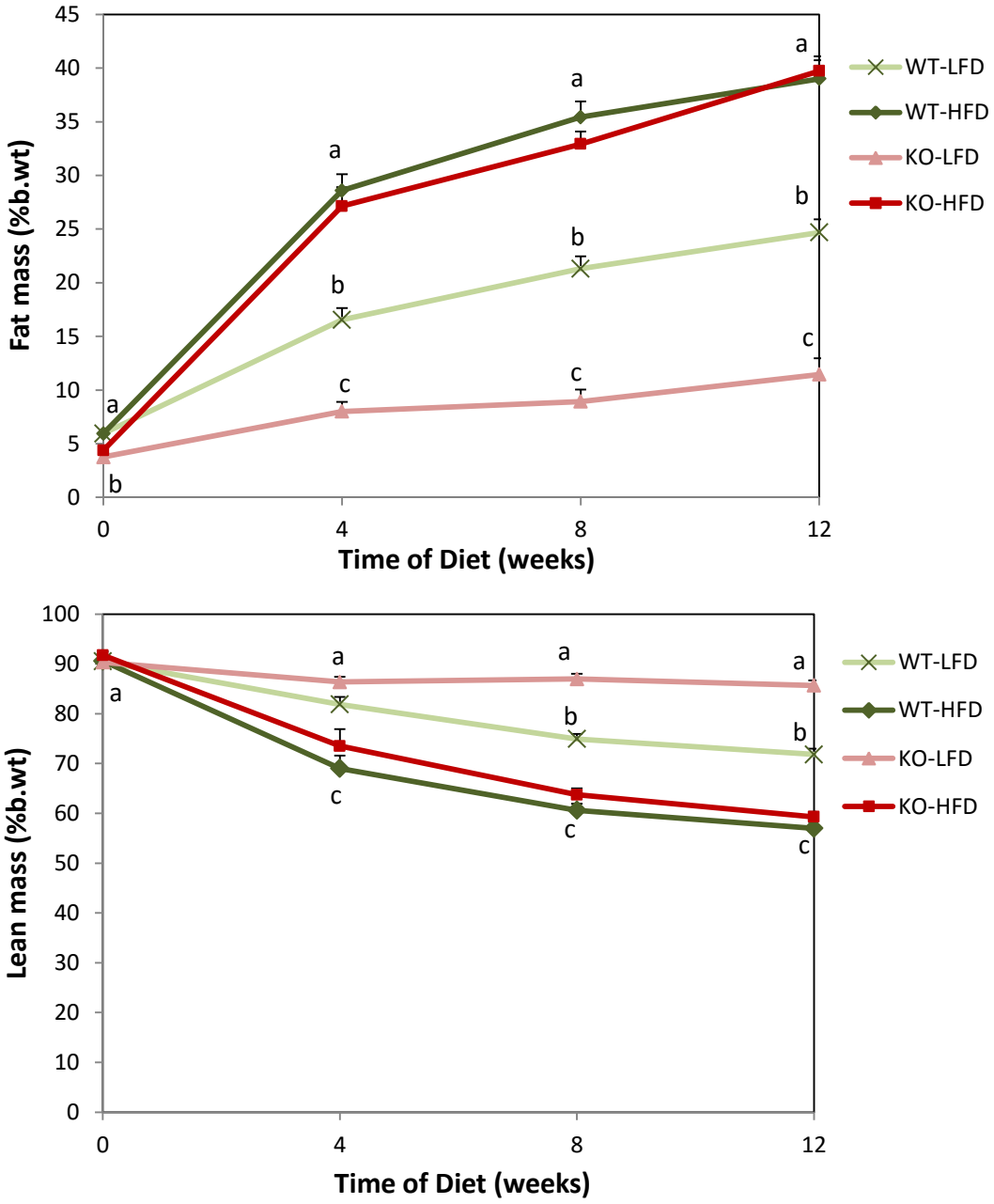


Figure 14: Comparison of % fat mass and % lean mass between adiponectin knockout and wild-type mice. Total fat and lean mass of mice fed low fat diet or high fat diet was obtained by whole body composition analysis with an EchoMRI™. Data are expressed as means ± SEM (n=4-10/group). Repeated measures ANOVA was used and statistically significant differences (P<0.05) among groups at a given time point of diet are indicated by different lower case letters. Significant main effects for fat mass were geno (P=0.016), diet (P<0.0001), geno×diet (P=0.0012) and time×geno×diet (P=0.0004) and for lean mass was geno (P<0.0001). **LFD:** Low Fat Diet, **HFD:** High Fat Diet, **KO**: Adiponectin Knockout mice, **WT**: Wild-type C57BL/6 mice.

2.3. Insulin Resistance

In the baseline group, both APN KO mice and WT mice had comparable glucose tolerance except at 30 minutes after the glucose bolus, APN KO had significantly higher blood glucose concentration than the WT group as shown in Figure 15. Even though, both genotypes gained a significant amount of body weight over a 12 week period on high fat diet, only the APN KO mice developed insulin resistance as measured by oral glucose tolerance test at week 8 and 12 (Figure 16A & 17A). Furthermore, APN KO mice on both the high and the low fat diets for 12 weeks developed insulin resistance (Area under curve (AUC) mM/L × min = 437±70 and 438±57) as compared to the WT mice fed low or high fat diets (AUC mM/L × min = 251±27 and 245±43) (Figure 17B). At week 8 on diet, APN KO mice on low fat diet group showed a trend towards developing insulin resistance with respect to high fat diet fed groups as evident from AUC (Figure 16B).

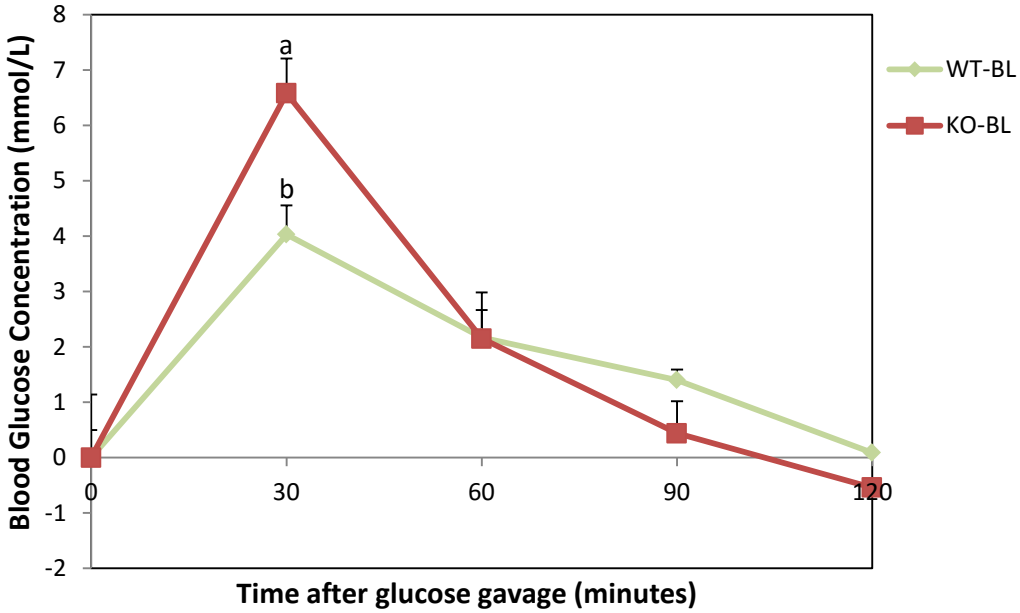
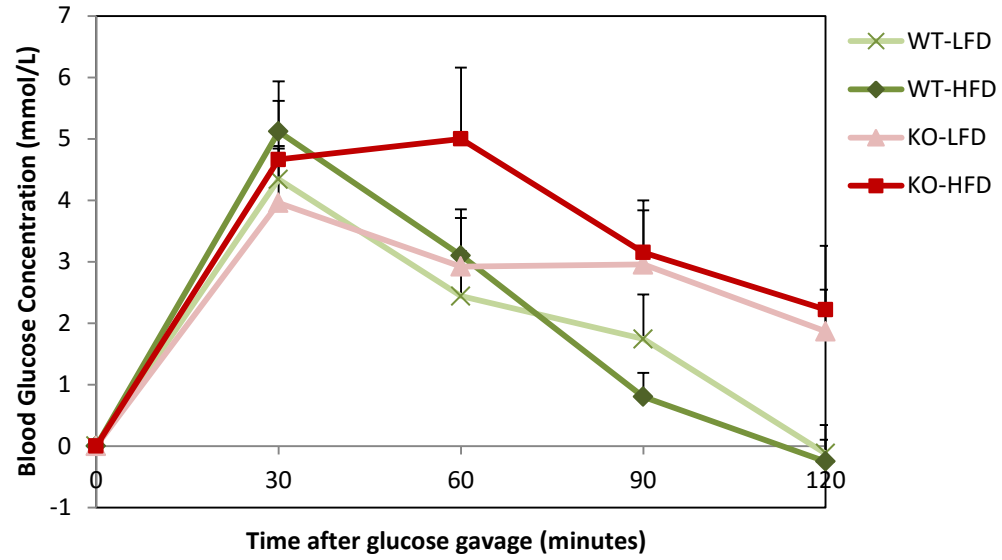


Figure 15: Comparison of glucose tolerance between adiponectin knockout and wild-type mice at the baseline. Oral glucose tolerance test was performed at baseline, i.e. before starting on low or high fat diets. Animals were fasted for 6 h and blood glucose was measured at times 0 (pre glucose load), 30, 60, 90 and 120 minutes after glucose dosage (2.5 mg/g body weight). The glucose tolerance curve was plotted. Data are expressed as mean ± SEM (n=10/group). Repeated measures ANOVA was used and statistically significant differences ($P<0.05$) among groups at a given time point are indicated by different lower case letters. **KO**: Adiponectin Knockout mice, **WT**: Wild-type C57BL/6 mice.

A.



B.

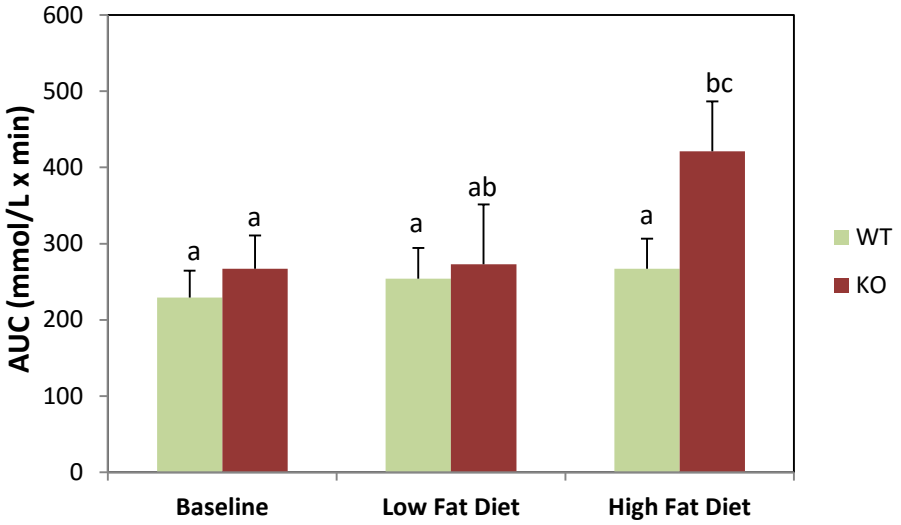
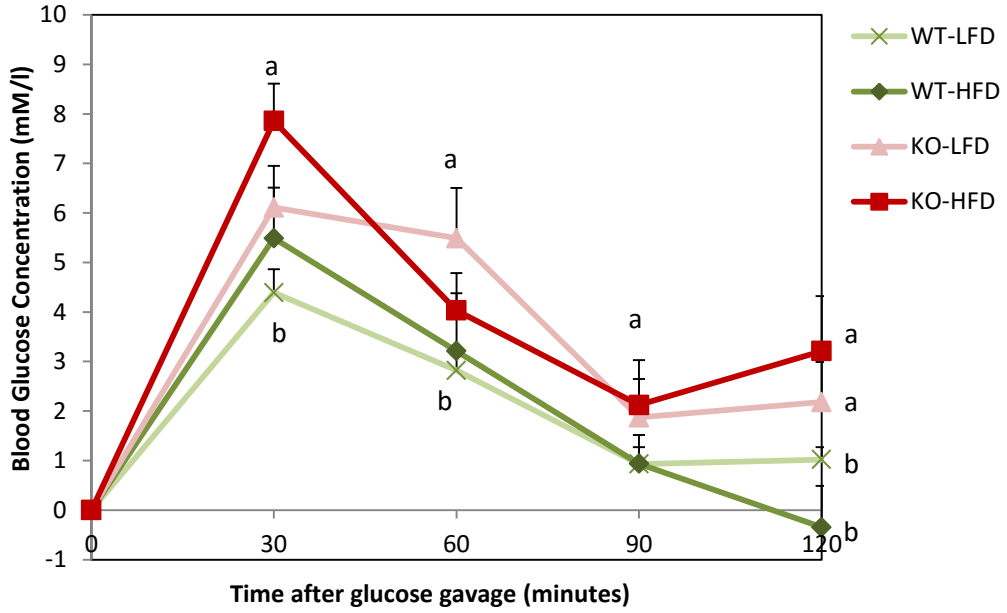


Figure 16: Comparison of glucose tolerance between adiponectin knockout and wild-type mice at week 8 on diet. Oral glucose tolerance test was performed at baseline and after 8 weeks on low or high fat diets. Animals were fasted for 6 h and blood glucose was measured at times 0 (pre glucose load), 30, 60, 90 and 120 minutes after glucose dosage (2.5 mg/g body weight). The glucose tolerance curve was plotted (A) and area under curve (AUC) (B) was calculated. Data are expressed as mean ± SEM (n=7-10/group). Repeated measures ANOVA was used for glucose tolerance curve and statistically significant differences ($P<0.05$) among groups at a given time point are indicated by different lower case letters. Two-way ANOVA was used for AUC. There were no significant main effects. **KO**: Adiponectin Knockout mice, **WT**: Wild-type C57BL/6 mice.

A.



B.

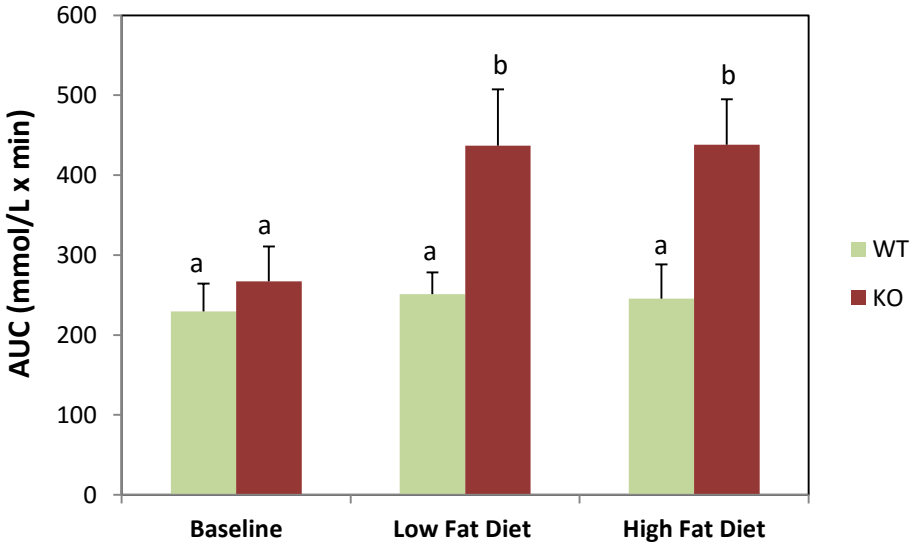


Figure 17: Comparison of glucose tolerance between adiponectin knockout and wild-type mice at week 12 on diet. Oral glucose tolerance test was performed at baseline and after 12 weeks on low or high fat diets. Animals were fasted for 6 h and blood glucose was measured at times 0 (pre glucose load), 30, 60, 90 and 120 minutes after glucose dosage (2.5 mg/g body weight). The glucose tolerance curve was plotted (A) and area under curve (AUC) (B) was calculated. Data are expressed as mean ± SEM (n=7-10/group). Repeated measures ANOVA was used for glucose tolerance curve and statistically significant differences ($P<0.05$) among groups at a given time point are indicated by different lower case letters. Two-way ANOVA was used for AUC. There were significant main effects for genotype ($P=0.001$). **KO**: Adiponectin Knockout mice, **WT**: Wild-type C57BL/6 mice.

2.3.1. Histological analysis of hepatic lipid content

As the liver has a major role in glucose disposal after feeding, we examined hematoxylin and eosin stained liver sections from APN KO and WT mice on low and high fat diets for morphological changes (n=4/group). Despite no significant difference in liver weight at 12 weeks on diet, analysis of liver sections showed that numerous lipid droplets appeared in both the APN KO and wild-type mice on high fat diet for 12 weeks as unstained empty vacuoles compared to healthy liver tissues from mice fed low fat diet for the same time period, which were devoid of these vacuoles.(Figure 18). Additionally, the scores from two blinded observers showed similar results for the lipid droplet content of the liver sections as shown in Table 6.

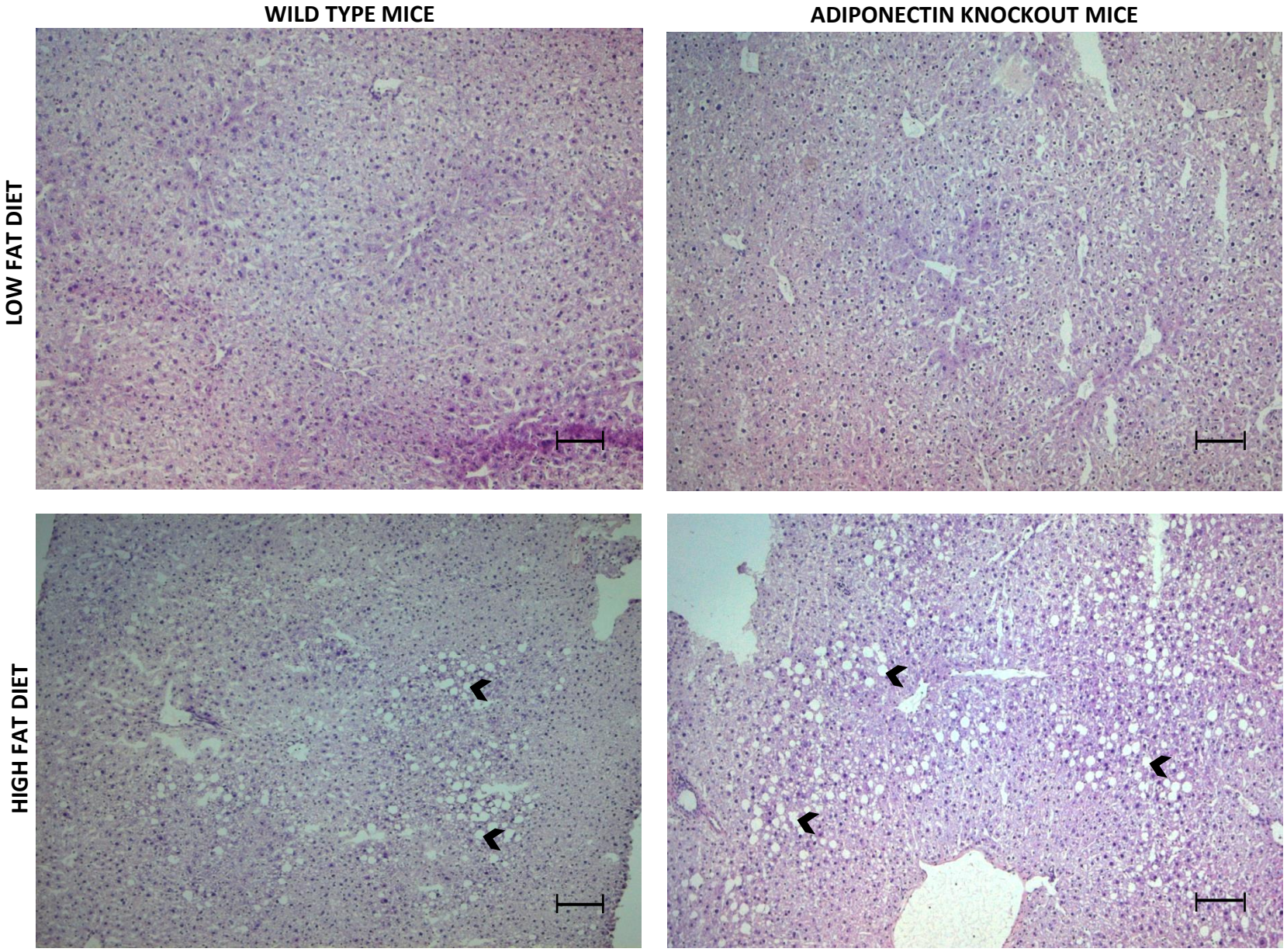

WILD TYPE MICE
ADIPONECTIN KNOCKOUT MICE
LOW FAT DIET
HIGH FAT DIET

Figure 18: Histological analysis for lipid droplets in liver from adiponectin knockout and wild type mice on diet for 12 weeks. Eight μm thick sections of frozen liver in OCT compound were prepared using a cryotome and fixed using 4% paraformaldehyde. Six sections per animal (n=4/group)were stained using hematoxylin and eosin and images were taken by bright field microscopy. A representative micrograph from each group is shown with 10Xmagnification. Arrow heads indicate unstained lipid droplets in the liver sections. Scale bar represents 100 μm.

Table 6: Blinded scores of the lipid droplet content of the hepatic sections from male animals on diet for 12 weeks

MALES (12 weeks on diet)	Animal ID #	Blinded observer 1	Blinded observer 2
WT-LFD	#327	+	++
	#338	-	-
	#346	+++	+++
	#348	-	+
WT-HFD	#333	++	++
	#340	+++	+++
	#341	++++	++++
	#349	+	+
KO-LFD	#129	-	+
	#130	-	-
	#131	-	-
	#123	-	+
KO-HFD	#124	+++	+++
	#132	-	+
	#133	+++	+++
	#107	++++	++++

2.4. Arterial Stiffness

Arterial stiffness was determined by Doppler pulse wave velocity analysis of the femoral artery. Pulse wave velocity was increased in the APN KO mice fed a high fat diet relative to APN KO mice fed a low fat diet for 12 weeks (12.56±0.78 cm/s vs 9.47±0.95 cm/s, P=0.0035; n=7-10/group). Pulse wave velocity was not different between WT mice on either the low or high fat diets (10.63±0.73 cm/s and 10.86±0.50 cm/s), thus revealing that only mice deficient in adiponectin developed arterial stiffness in response to a high fat diet (Figure 19).

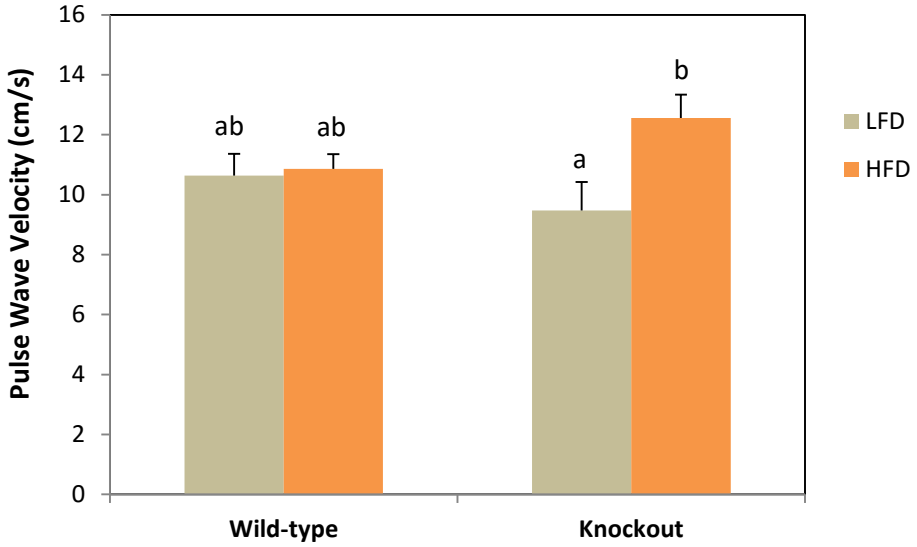


Figure 19: Arterial stiffness as determined by pulse wave velocity. Pulse wave velocity was measured non-invasively by pulsed-wave Doppler ultrasound on the femoral artery of mice fed either a low or high fat diet for 12 weeks. Data are expressed as means ± SEM (n=7-10/group). Statistically significant differences among groups ($P<0.05$) are indicated by different lower case letters. There were significant main effects for diet and genotype × diet. **LFD**: Low Fat Diet, **HFD**: High Fat Diet.

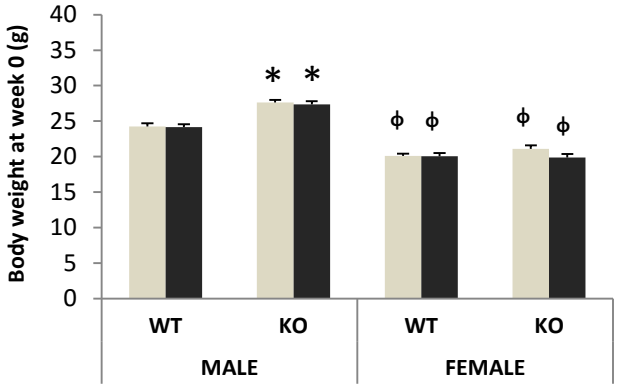
3. Metabolic effects are sex-dependent in a diet-induced obese adiponectin knockout mouse model

3.1. Body weight

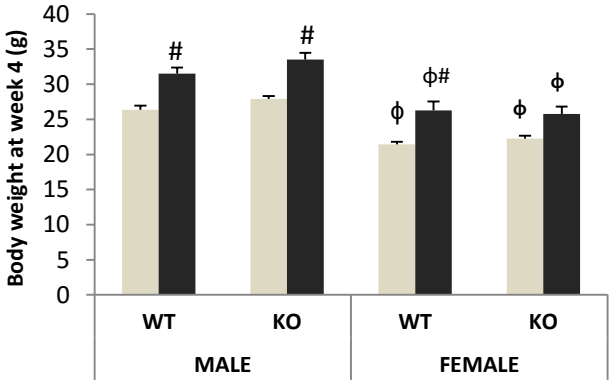
Male and female 8-week old adiponectin knockout (APN KO) and wild-type C57BL/6 (WT) mice were fed a high fat diet (60% energy from fat) for 8 weeks (n=10/group) to induce obesity and insulin resistance or were fed a low fat diet (10% energy from fat) for 12 weeks (n=10/group) to be used as lean controls. At the start of the study, a significant genotype difference in males was observed in the body weight of males with APN KO mice weighing more than the WT control mice. Additionally females weighed less than the males for both genotypes (Figure 20a A). Upon high fat diet feeding for 8 weeks, all mice showed significant increases in body weight as compared to their low fat diet counterparts (Figure 20a C). Importantly, the initial difference observed between genotypes in males was not observed in either diet group (Figure 20a C). However, females of both genotypes weighed significantly less than males on low fat diet after 8 weeks. For those on high fat diet, the female APN KO weighed less than their male counterparts while female WT on HFD was similar to males (Figure 21).

Furthermore, when the body weight was plotted as a function of time, sex, diet and genotype, there was a significant interaction between time and sex ($P=0.03$), genotype ($P=0.03$) and diet ($P<0.0001$) (Figure 20b).

A.



B.



C.

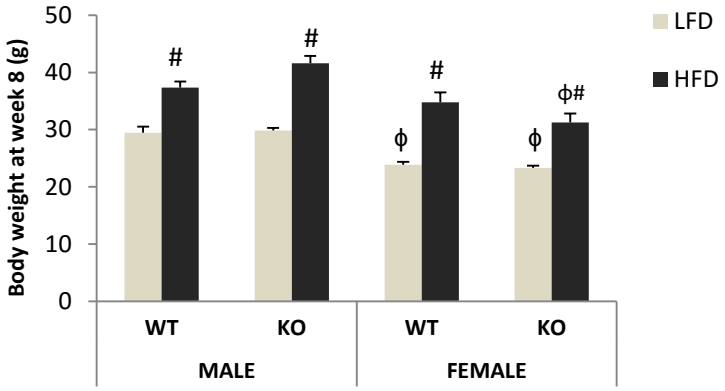
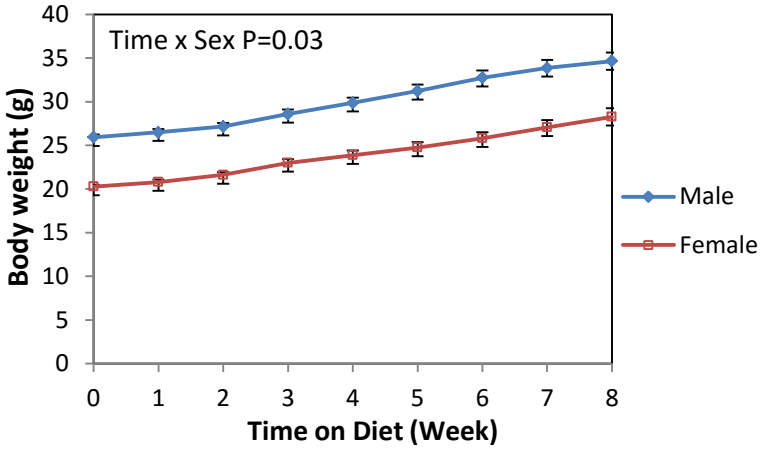


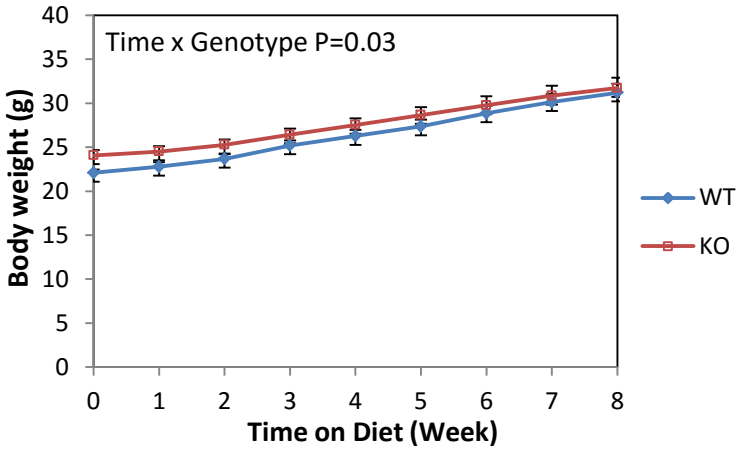
Figure 20a: Comparison of weekly body weights of mice fed either a low or a high fat diet. Eight-week old male and female wild-type C57BL/6 or adiponectin knockout mice were fed either a low fat diet or high fat diet for 8 weeks. Data are expressed as means ± SEM (n=9-10/group). Repeated measures 3-way ANOVA was used to determine significant main effects, and lsmeans with Tukey-Kramer correction for multiple comparisons was used for means testing between specific groups; $P<0.05$ was considered statistically significant. There were significant main effects for time ($P<0.0001$), time ×sex ($P=0.03$), time×genotype ($P=0.03$), time×diet

($P<0.0001$) and time × sex × genotype × diet ($P=0.01$). Within each week, ϕ indicates differences ($P<0.05$) between sexes within a diet and genotype; # indicates differences ($P<0.05$) between diets within a sex and genotype and * indicates differences ($P<0.05$) between genotypes within a sex and diet. **LFD**: Low fat diet, **HFD**: High fat diet, **KO**: Adiponectin Knockout mice, **WT**: Wild-type C57BL/6 mice.

A.



B.



C.

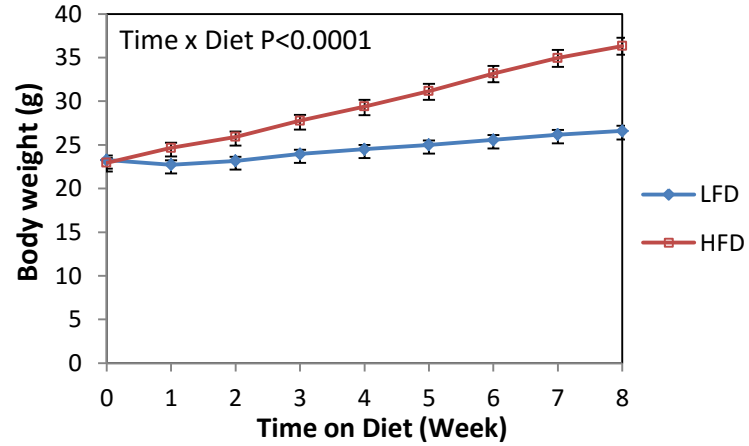


Figure 20b: Analysis of body weight as a function of time, sex, genotype and diet. Eight-week old male and female wild-type C57BL/6 (**WT**) or adiponectin knockout (**KO**) mice were fed either a low fat diet (**LFD**) or high fat diet (**HFD**) for 8 weeks. Body weights are shown as a function of time and sex (A), genotype (B) and diet (C). Data are expressed as means ± SEM (n=37-41/group). Data were analyzed by repeated measures 3-way ANOVA and $P<0.05$ was considered statistically significant.

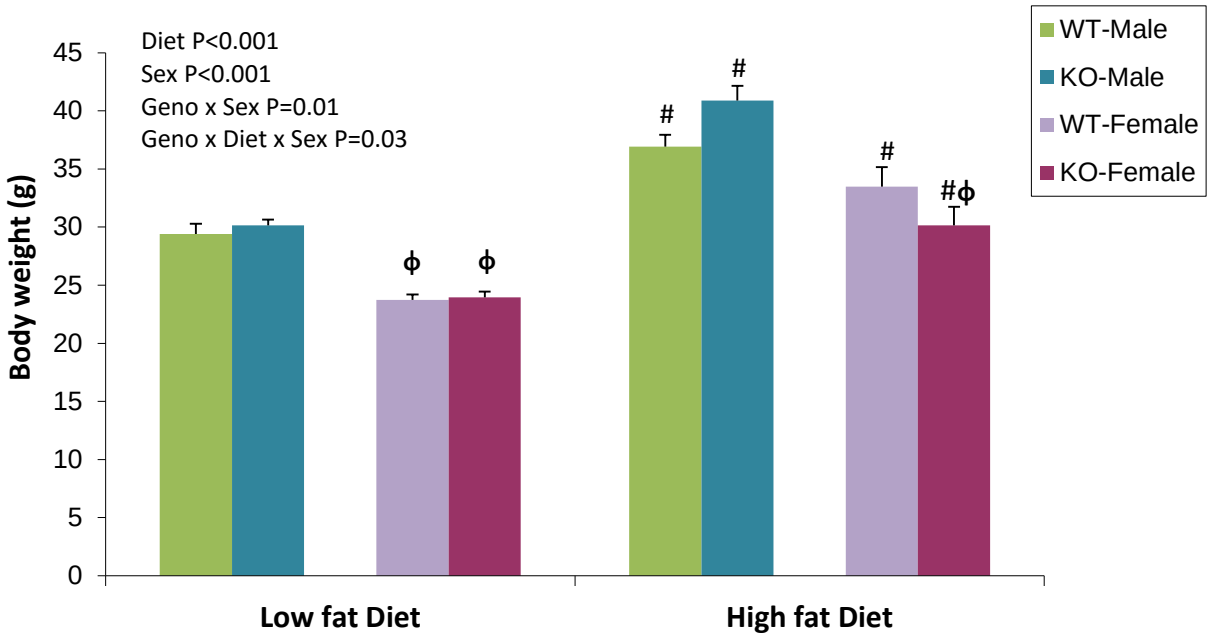


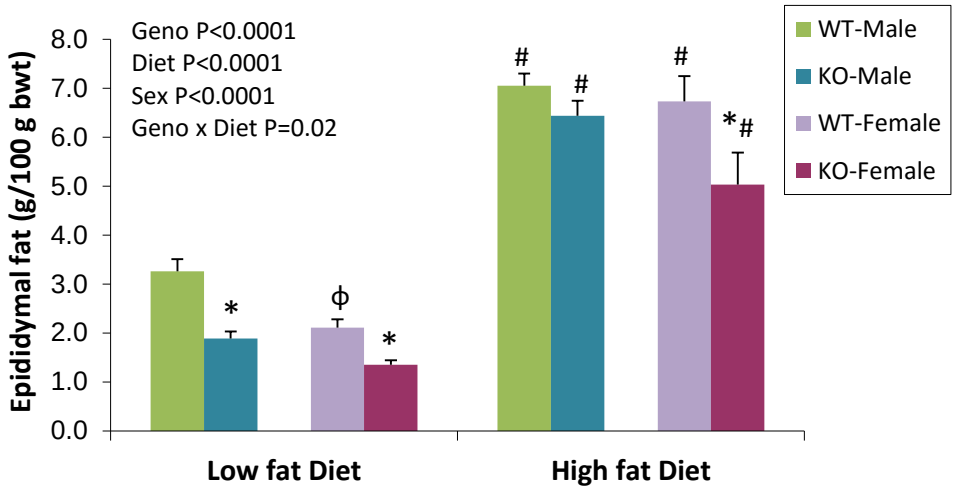
Figure 21 Comparison of body weights after 8 weeks on diet. Male and female wild-type C57BL/6 or adiponectin knockout mice fed either a low fat diet or high fat diet was weighed after 8 weeks on diet. Data are expressed as means ± SEM (n=10/group). Three-way ANOVA was used with sex, genotype and diet as main effects, and lsmeans with Tukey-Kramer correction for multiple comparisons was used for means testing between specific groups. $P<0.05$ was considered statistically significant. ϕ indicates differences ($P<0.05$) between sexes within a diet and genotype; # indicates differences ($P<0.05$) between diets within a sex and genotype and * indicates differences ($P<0.05$) between genotypes within a sex and diet. **KO**: Adiponectin Knockout mice, **WT**: Wild-type C57BL/6 mice.

3.2. Adiposity

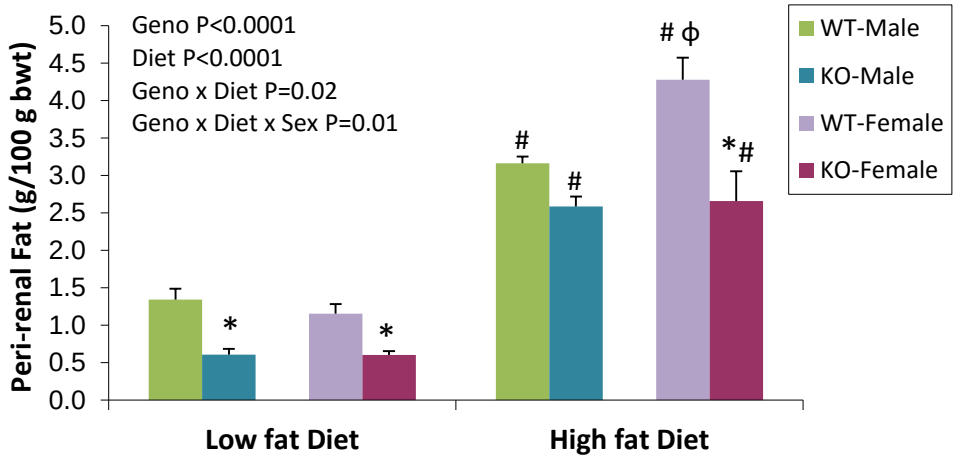
3.2.1. White Adipose Tissue Depot Weights

Although APN KO mice had similar body weights as compared to the WT mice after 8 weeks on low fat diet, the the epididymal-parametrial, peri-renal and mesenteric fat depots in both male as well as female, weighed significantly less in APN KO mice versus WT mice. However on the high fat diet, male APN KO mice gained comparable epididymal, peri-renal and mesenteric fat mass as WT mice, whereas female APN KO did not and hence had significantly less parametrial, peri-renal and mesenteric fat mass than the female WT mice (Figure 22A). A significant genotype effect was observed in all three depots of female mice on control diet as well as high fat diet (Figure 22). A sex difference was observed in epididymal-parametrial and mesenteric fad depot mass with female WT mice having less than the male WT mice on low fat diet, but this difference was not observed in the high fat diet animals and APN KO mice on control diet (Figure 22A & C). WT females on HFD had more peri-renal fat than WT males on HFD (Figure 22B).

A.



B.



C.

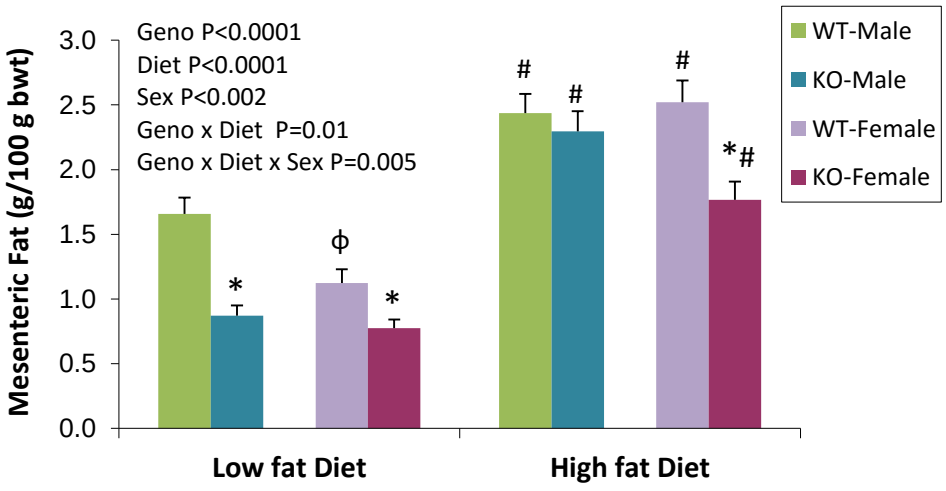


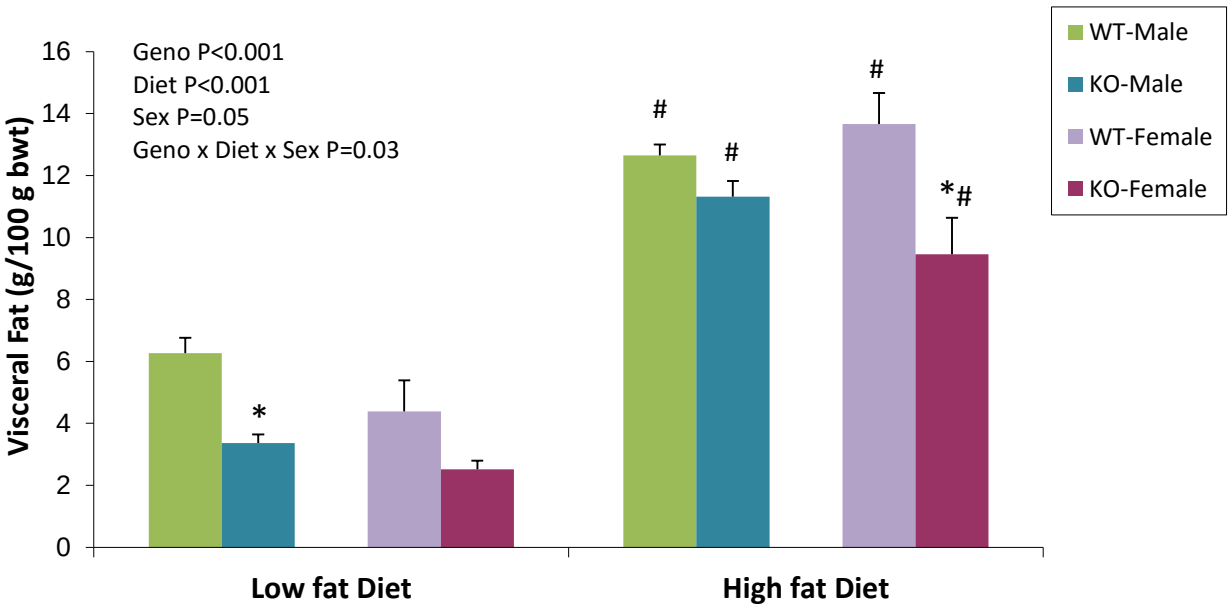
Figure 22: Comparison of white adipose tissue depot weights. (A) Epididymal-parametrial, (B) peri-renal and (C) mesenteric fat pad weights relative to body weight after 8 weeks on high or low fat diet. Data are expressed as means ± SEM (n=10/group). Three-way ANOVA was used

with sex, genotype and diet as main effects, and lsmeans with Tukey-Kramer correction for multiple comparisons was used for means testing between specific groups. $P<0.05$ was considered statistically significant. ϕ indicates differences ($P<0.05$) between sexes within a diet and genotype; # indicates differences ($P<0.05$) between diets within a sex and genotype and * indicates differences ($P<0.05$) between genotypes within a sex and diet. **bwt**: body weight, **KO**: Adiponectin Knockout mice, **WT**: Wild-type C57BL/6 mice.

After 8 weeks on low fat diet, both the visceral (the sum total of epididymal, peri-renal and mesenteric) fat pads and subcutaneous (inguinal) fat pads in the male APN KO mice weighed less than those of the male WT mice. In contrast, females did not show any genotype difference in these depots on a low fat diet. On high fat feeding for 8 weeks, female APN KO mice had significantly less visceral fat mass as compared to their WT counterparts whereas males did not show any genotype difference. On the other hand, with high fat feeding both males and female APN KO mice had significantly lower inguinal fat mass, a subcutaneous depot than their WT counterparts (Figure 23B). However, there was no sex difference between groups in these fat depots, but overall there was a main sex effect (P=0.05) in visceral fat depot (Figure 23A).

For mice on low and high fat diets, it was interesting to observe a significantly larger epicardial fat depot in both genotypes in females (Figure 24). Similar to other fat pads, male APN KO had significantly less epicardial fat mass than male WT mice on a low fat diet, but females did not show any genotype difference in this depot on the same diet. However on high fat diet, the weight of the epicardial fat depot of male APN KO mice was comparable to male WT mice. On the contrary, a diet effect was observed only in female WT mice (Figure 24).

A.



B.

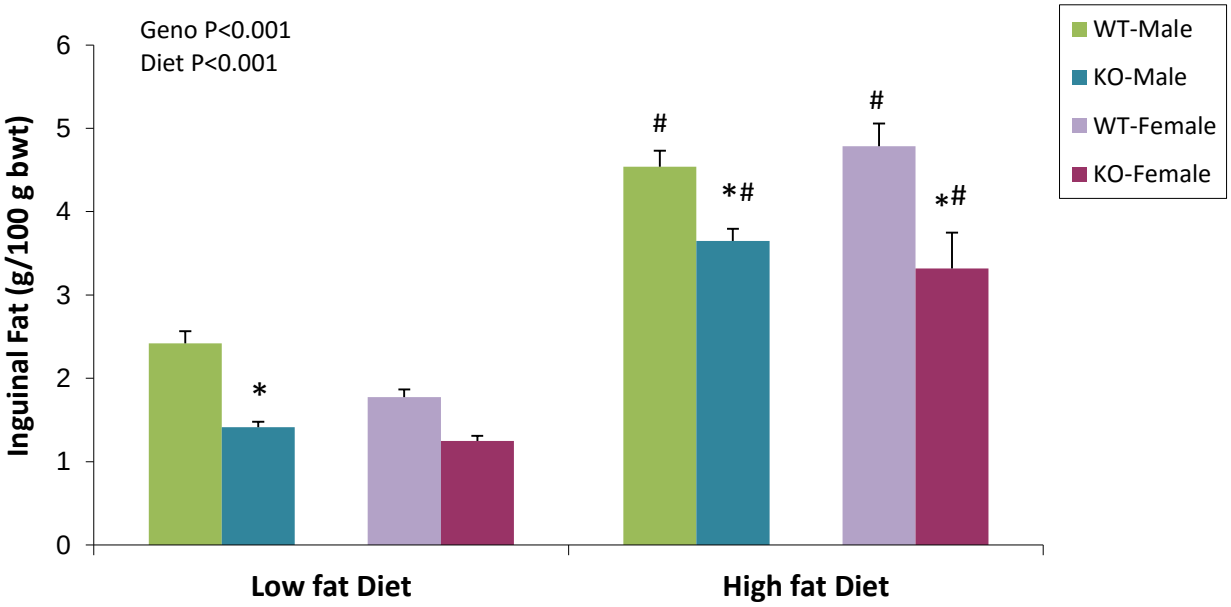


Figure 23: Comparison of visceral and subcutaneous (inguinal) adipose tissue depot weights. (A) Visceral fat (sum of epididymal--parametrial, peri-renal and mesenteric fat pads) and (B) inguinal fat pad weights relative to body weight after 8 weeks on low or high fat diet. Data are expressed as means ± SEM (n=10/group). Three-way ANOVA was used with sex, genotype and diet as main effects. P<0.05 was considered statistically significant. ϕ indicates differences (P<0.05) between sexes within a diet and genotype; # indicates differences (P<0.05) between diets within a sex and genotype and * indicates differences (P<0.05) between genotypes within a sex and diet. **bwt**: body weight, **KO**: Adiponectin Knockout mice, **WT**: Wild-type C57BL/6 mice.

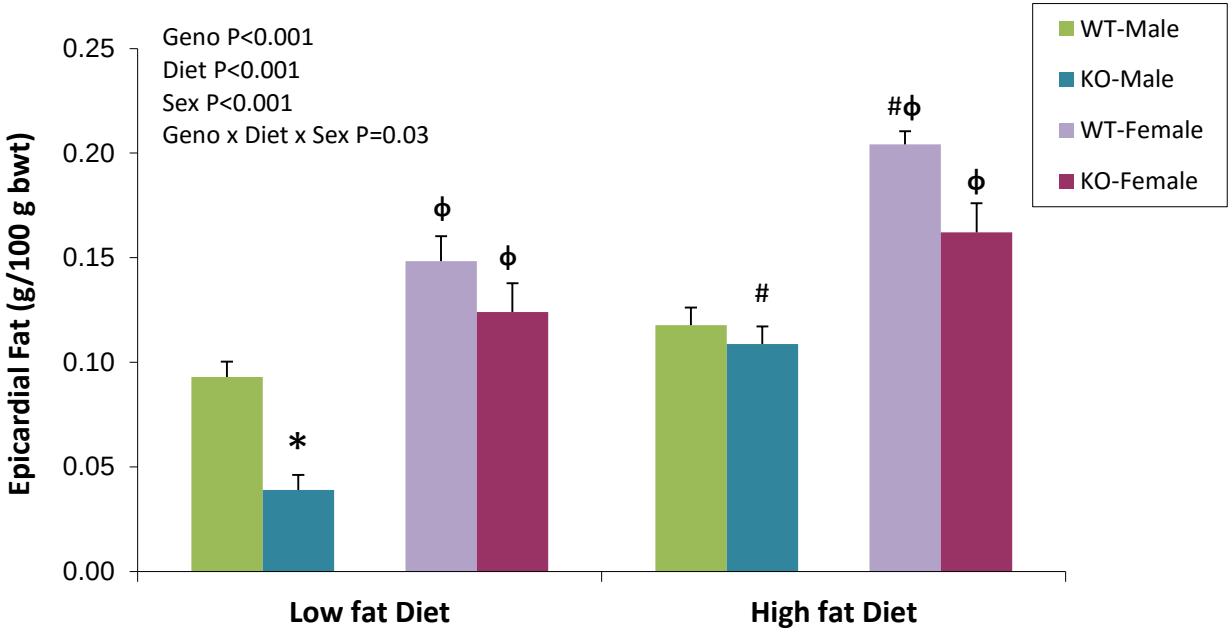


Figure 24: Epicardial white adipose tissue depot weight at week 8 on diet. Epicardial adipose tissue weight relative to body weight, after 8 weeks on low or high fat diet. Data are expressed as means ± SEM (n=10/group). Three-way ANOVA was used with sex, genotype and diet as main effects, and lsmeans with Tukey-Kramer correction for multiple comparisons was used for means testing between specific groups. P<0.05 was considered statistically significant. ϕ indicates differences (P<0.05) between sexes within a diet and genotype; # indicates differences (P<0.05) between diets within a sex and genotype and * indicates differences (P<0.05) between genotypes within a sex and diet. **bwt**: body weight, **KO**: Adiponectin Knockout mice, **WT**: Wild-type APN Knockout mice.

3.2.2. Other Tissue/Organ Weights

All the tissues/organ weights were normalized to 100 g body weight. Brown adipose tissue (BAT) showed a significant genotype × sex interaction (P=0.009) with male APN KO mice having a significantly smaller depot mass than male WT mice on low and high fat diet. Additionally, WT females had lower BAT mass than the WT male mice on both diets. A significant increase in liver weight normalized to 100 g body weight was observed between WT and APN KO in the low fat diet groups of both sexes. In contrast, in high fat diet groups, only the liver of female APN KO weighed significantly more than the WT counterparts. Furthermore, female APN KO had significantly increased pancreas weight relative to the female WT mice on high fat diet. Interestingly, female KO mice on high fat diet showed increased skeletal muscle mass than the male APN KO mice. With respect to heart mass in control low fat diet groups, females of both genotype had significantly higher mass than the male mice. However on high fat diet, only female KO mice had increased heart mass as compared to their WT counterparts (Table 7).

Table 7: Normalized Tissue/Organ weights[#]

Organ (g/100 g bwt)	**LOW FAT DIET**				**HIGH FAT DIET**				**Geno**	**Diet**	**Sex**	**Interactions**
	Male		**Female**		**Male**		**Female**					
	WT	**KO**	**WT**	**KO**	**WT**	**KO**	**WT**	**KO**				
BAT	0.82^{a} ±0.02	0.65^{cbd} ±0.02	0.64^{cbd} ±0.04	0.67^{bc} ±0.03	0.70^{b} ±0.03	0.58^{ced} ±0.03	0.57^{ed} ±0.01	0.51^{e} ±0.02	0.001	<0.0001	0.0004	**Geno x Sex** 0.009
Liver	4.61^{b} ±0.15	5.73^{a} ±0.06	4.93^{b} ±0.24	5.96^{a} ±0.13	3.61^{cd} ±0.14	3.90^{c} ±0.07	3.21^{d} ±0.13	3.96^{c} ±0.18	<0.0001	<0.0001	N.S.	**Geno x Diet** 0.009 **Diet x Sex** 0.03
Pancreas	1.13^{b} ±0.05	1.20^{b} ±0.04	1.50^{a} ±0.08	1.57^{a} ±0.03	0.82^{c} ±0.04	0.91^{c} ±0.06	0.95^{c} ±0.06	1.29^{b} ±0.08	<0.0001	<0.0001	<0.0001	N.S.
Skeletal Muscle	3.50^{ab} ±0.11	3.23^{bc} ±0.10	3.66^{a} ±0.10	3.39^{ab} ±0.10	2.94^{cd} ±0.08	2.76^{d} ±0.08	2.92^{cd} ±0.18	3.29^{b} ±0.15	N.S.	<0.0001	0.02	**Geno x Sex** 0.03
Kidney	1.32^{ab} ±0.06	1.37^{a} ±0.02	1.25^{b} ±0.03	1.33^{ab} ±0.02	1.00^{c} ±0.03	0.97^{c} ±0.02	0.86^{d} ±0.03	1.00^{c} ±0.05	0.02	<0.0001	0.03	**Geno x Sex** 0.04
Heart	0.48^{b} ±0.02	0.48^{b} ±0.02	0.54^{a} ±0.02	0.58^{a} ±0.02	0.40^{c} ±0.02	0.40^{c} ±0.01	0.40^{c} ±0.02	0.46^{b} ±0.03	N.S.	<0.0001	0.0002	N.S.
Lung Fluid wt	0.57^{a} ±0.05	0.54^{ab} ±0.03	0.58^{a} ±0.04	0.63^{a} ±0.03	0.42^{c} ±0.04	0.38^{c} ±0.024	0.44^{bc} ±0.03	0.54^{ab} ±0.04	N.S.	<0.0001	0.004	**Geno x Sex** 0.03

[#]Data are expressed as means ± SEM (n=8-11/group). Three-way ANOVA with genotype, diet and sex as main effects was used and within a row, statistically significant differences among groups ($P<0.05$) are indicated by different lower case letters as superscript. Statistically significant ($P<0.05$) main effects and interactions are reported. There was no significant Geno×Diet×Sex interaction for these variables.

BAT: Brown Adipose Tissue, **Geno**: Genotype, **KO**: Adiponectin Knockout mice, **N.S.**: Not significant, **WT**: Wild-type C57BL/6 mice and **wt**: weight.

3.2.3. Body composition

The finding of a reduction in visceral fat depot weight in female APN KO mice on a high fat diet for 8 weeks was corroborated by whole body composition analysis using EchoMRI™ that showed a significant reduction in % fat mass in female APN KO in comparison with the female WT mice when fed a high fat diet for 8 weeks (Figure 25a - C). A similar trend of reduced % fat mass was found in male APN KO in comparison to WT mice (P=0.08) on control low fat diet was observed. Interestingly, a sex difference was observed in week 0 and 4 but not week 8 on high fat diet with female APN KO having higher % fat mass than male APN KO mice at week 0 and vice versa at week 4 (Figure 25a - A&B), this is illustrated in the time×sex interaction (P=0.002) plot for % fat mass in Figure 25b-A. Additionally, there was significant interaction of genotype (P=0.0002) and diet (P<0.0001) with time (Figure 25b - B&C).

In concordance with the increased % fat mass results, there was a concomitant decrease in % lean mass over time on high fat feeding (Figure 26a) with a significant interaction of time- sex (P=0.002) and -diet(P<0.0001) (Figure 26b - A&B). However, there was no interaction between time and genotype. Additionally, a sex difference similar to % fat mass observed in female versus male APN KO on high fat diet was seen with % lean mass (Figure 25a & 26a).

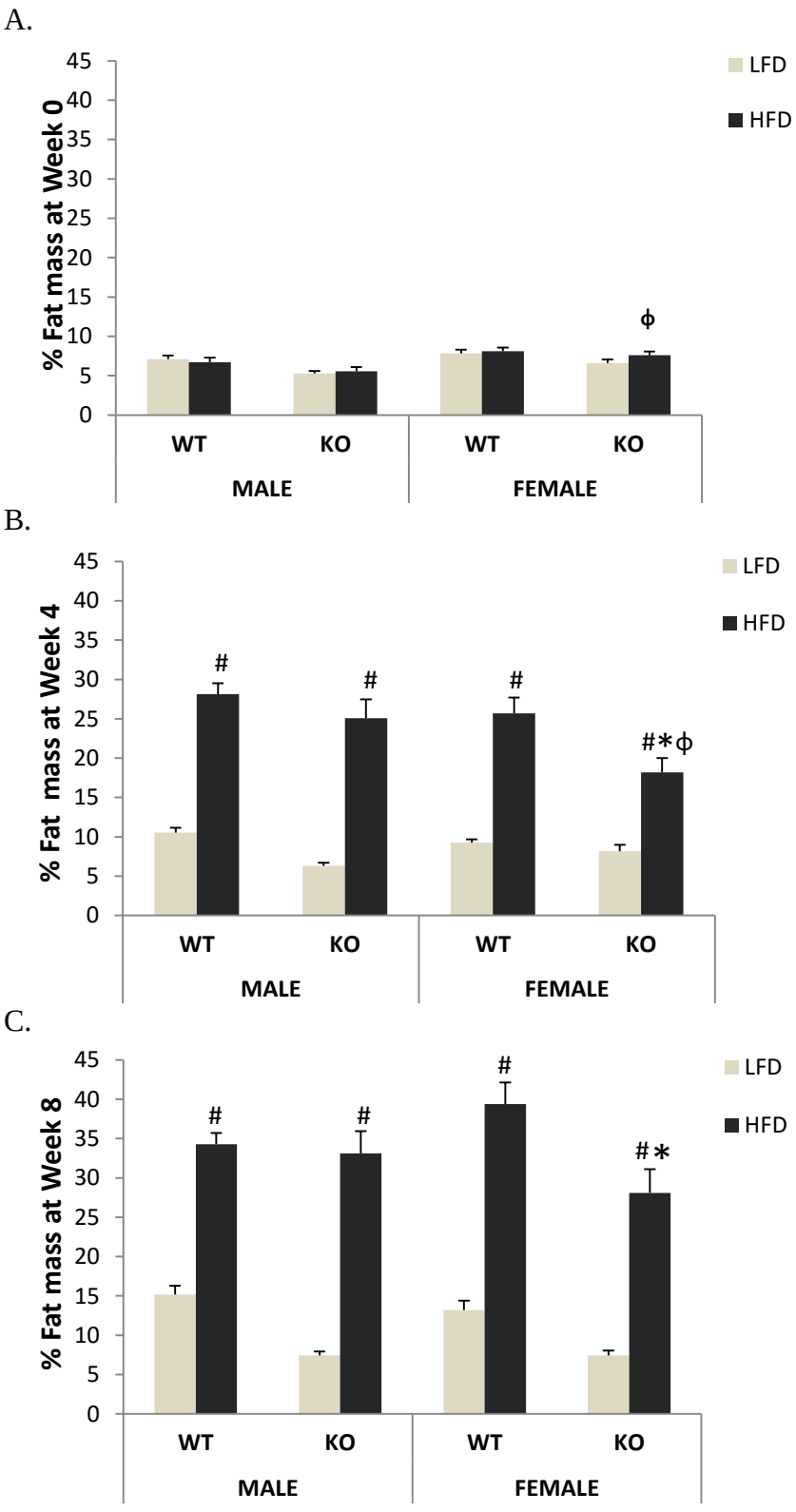
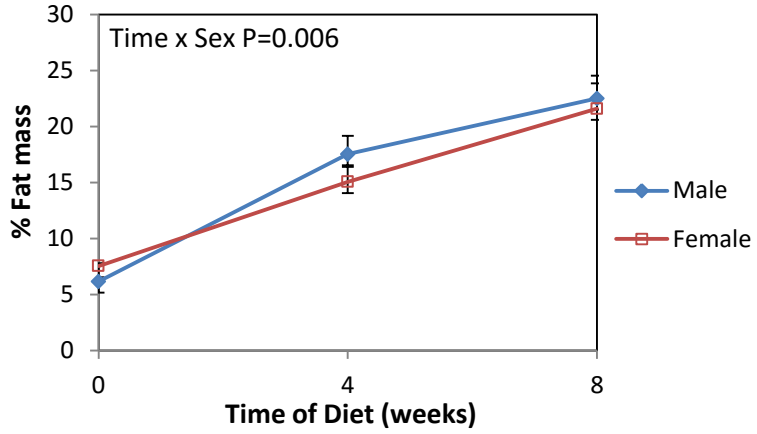


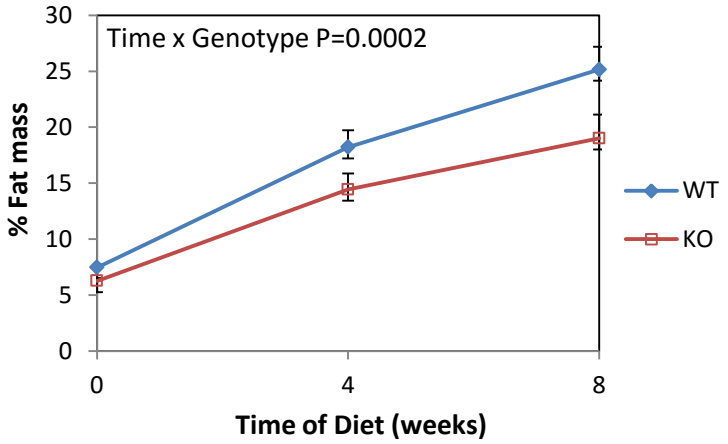
Figure 25a: Comparison of body fat mass in male and female mice fed either a low or a high fat diet. Total fat mass (as of % body weight) of mice fed a low fat diet or a high fat diet

was obtained by whole body composition analysis with an EchoMRI™. Data are expressed as means ± SEM (n=9-10/group). Repeated measures 3-way ANOVA was used to determine significant main effects, and lsmeans with Tukey-Kramer correction for multiple comparisons was used for means testing between specific groups; $P<0.05$ was considered statistically significant. There were significant main effects for time ($P<0.0001$), time×sex ($P=0.0063$), time×genotype ($P=0.0002$), time×diet ($P<0.0001$), time×sex×diet ($P=0.0122$) and time×sex×genotype×diet ($P=0.0224$). Within each timepoint, ϕ indicates differences ($P<0.05$) between sexes within a diet and genotype; # indicates differences ($P<0.05$) between diets within a sex and genotype and * indicates differences ($P<0.05$) between genotypes within a sex and diet. **LFD**: Low fat diet, **HFD**: High fat diet, **KO**: Adiponectin Knockout mice, **WT**: Wild-type C57BL/6 mice.

A.



B.



C.

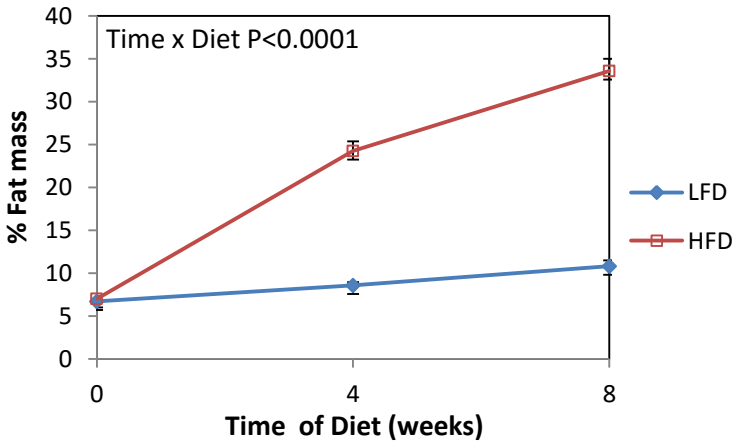


Figure 25b: Body fat mass as a function of time, sex, genotype and diet. Eight-week old wild-type C57BL/6 or adiponectin knockout mice (male and female) were fed either a low fat diet or a high fat diet for 8 weeks. Total fat mass (as of % body weight) is shown as a function of time and sex (A), genotype (B) and diet (C). Data are expressed as means ± SEM (n=39-40/group). Data were analyzed by repeated measures 3-way ANOVA and $P<0.05$ was considered statistically significant. **LFD**: Low fat diet, **HFD**: High fat diet, **KO**: Adiponectin Knockout mice, **WT**: Wild-type C57BL/6 mice.

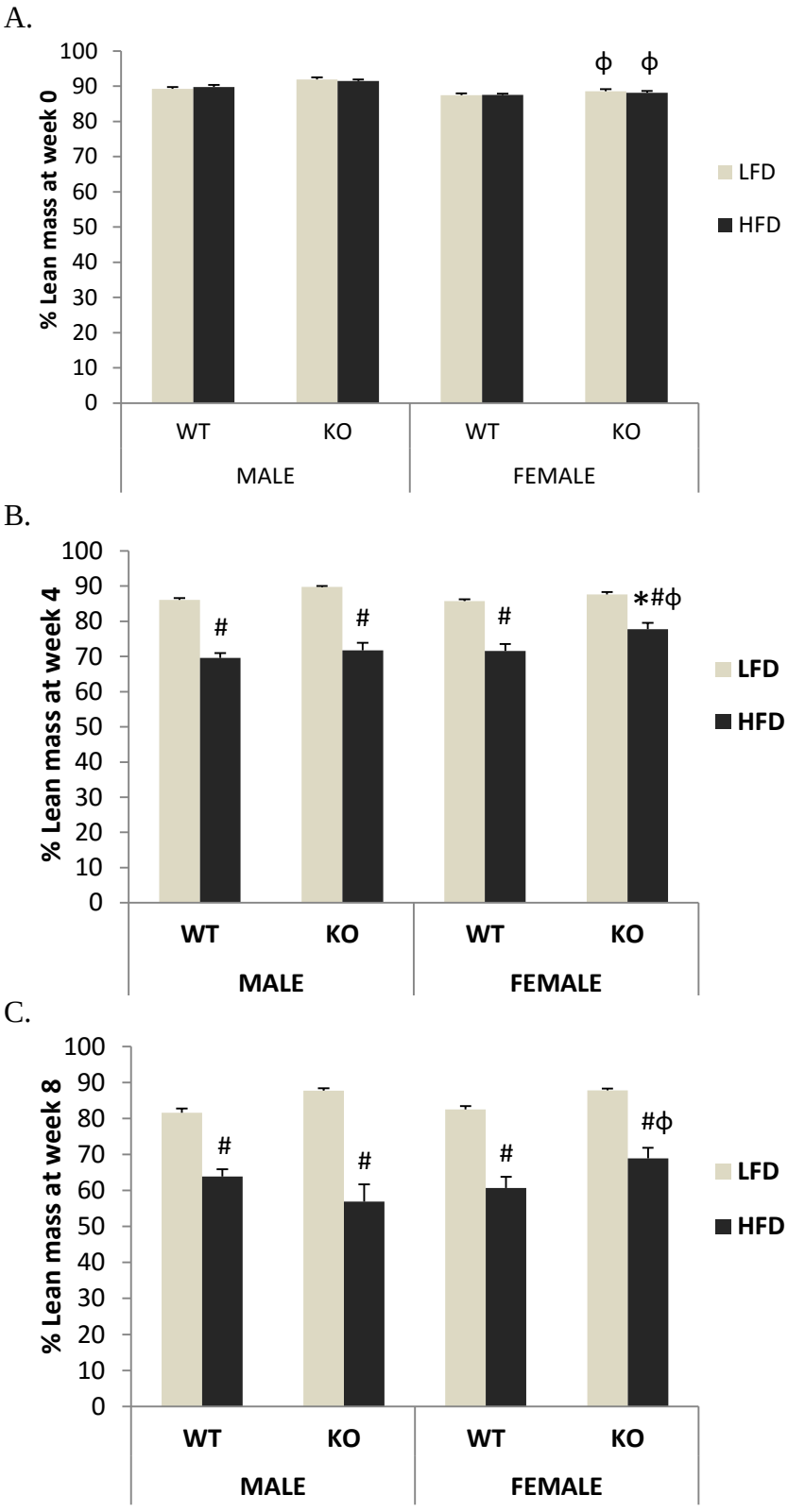
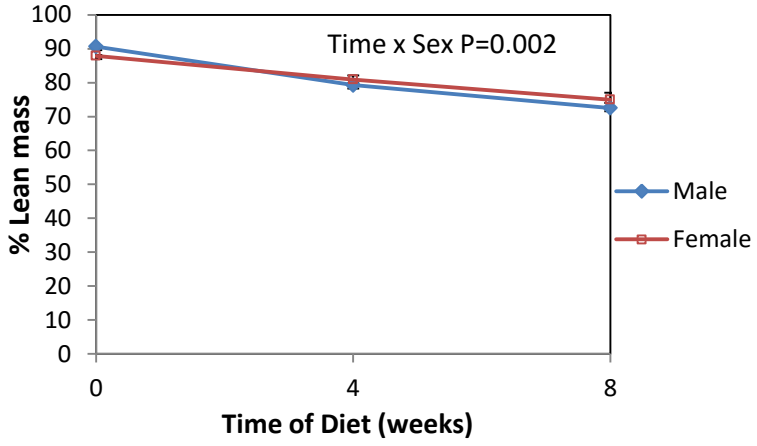


Figure 26a: Comparison of lean body mass in male and female mice fed either a low or a high fat diet. Whole body lean mass (as % body weight) of mice fed low fat diet or high fat diet

for 8 weeks was obtained by whole body composition analysis with an EchoMRI™. Data are expressed as means ± SEM (n=9-10/group). Repeated measures 3-way ANOVA was used to determine significant main effects, and lsmeans with Tukey-Kramer correction for multiple comparisons was used for means testing between specific groups; $P<0.05$ was considered statistically significant. There were significant main effects for time ($P<0.0001$), time×sex ($P=0.0019$), time×diet ($P<0.0001$), time×sex×genotype ($P=0.0194$), time×genotype×diet ($P=0.0303$) and time×sex×genotype×diet ($P=0.0353$). Within each timepoint, ϕ indicates differences ($P<0.05$) between sexes within a diet and genotype; # indicates differences ($P<0.05$) between diets within a sex and genotype and * indicates differences ($P<0.05$) between genotypes within a sex and diet. **LFD**: Low fat diet, **HFD**: High fat diet, **KO**: Adiponectin Knockout mice, **WT**: Wild-type C57BL/6 mice.

A.



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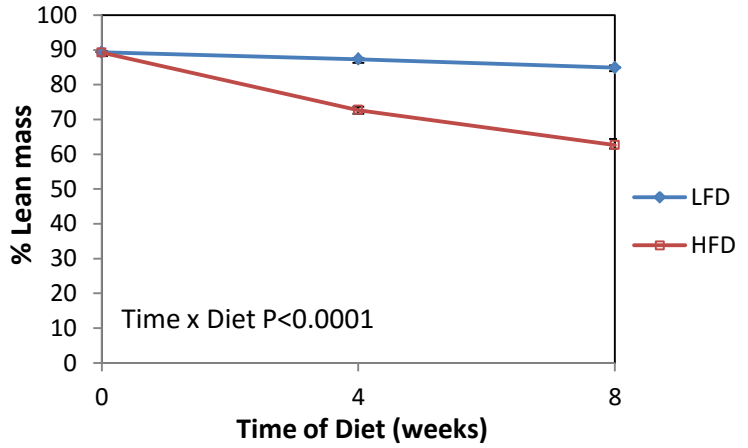


Figure 26b: Lean body mass as a function of time, sex, genotype and diet. Eight-week old wild-type C57BL/6 or adiponectin knockout mice (male and female) were fed either a low fat diet or a high fat diet for 8 weeks. Lean body mass (as % body weight) is shown as a function of time and sex (A), genotype (B) and diet (C). Data are expressed as means ± SEM (n=39-40/group). Data were analyzed by repeated measures 3-way ANOVA and $P<0.05$ was considered statistically significant. **LFD**: Low fat diet, **HFD**: High fat diet, **KO**: Adiponectin Knockout mice, **WT**: Wild-type C57BL/6 mice.

3.3. Insulin Resistance

After 8 weeks on diet, animals were fasted for 6 hours and an oral glucose tolerance test (OGTT) was carried out. Male APN KO and WT mice had significantly higher fasting blood glucose levels than their female counterparts when fed either a control low fat or a high fat diet. Surprisingly, female APN KO mice on the high fat diet had lower fasting glucose levels than the female WT mice. There were main effects of diet ($P<0.001$), sex ($P<0.001$) and genotype but no significant interactions were observed (Figure 27).

Even though the female APN KO had lower fasting glucose concentrations, they showed significant glucose intolerance 30 minutes following a glucose load relative to the low fat diet and WT groups (Figure 28a). After 8 weeks on diet, while male WT mice on both diets were glucose tolerant, male APN KO mice on a high fat diet developed insulin resistance as measured by oral glucose tolerance test. In contrast, female WT mice on high fat diet developed insulin resistance as compared to the control diet group and no diet difference was observed in female APN KO, likely due to a trend of increased glucose intolerance in the control diet group relative to female WT group on same diet (Figure 28b).

Additionally, when the blood glucose concentration during the OGTT was plotted as a function of time, sex, diet and genotype, there were significant interactions between time and sex ($P<0.0001$), genotype ($P<0.0001$) and diet ($P<0.0001$) (Figure 28c). Moreover, significant multiple interactions such as time×sex× diet ($P=0.008$) and time×sex×genotype ($P=0.03$) were observed.

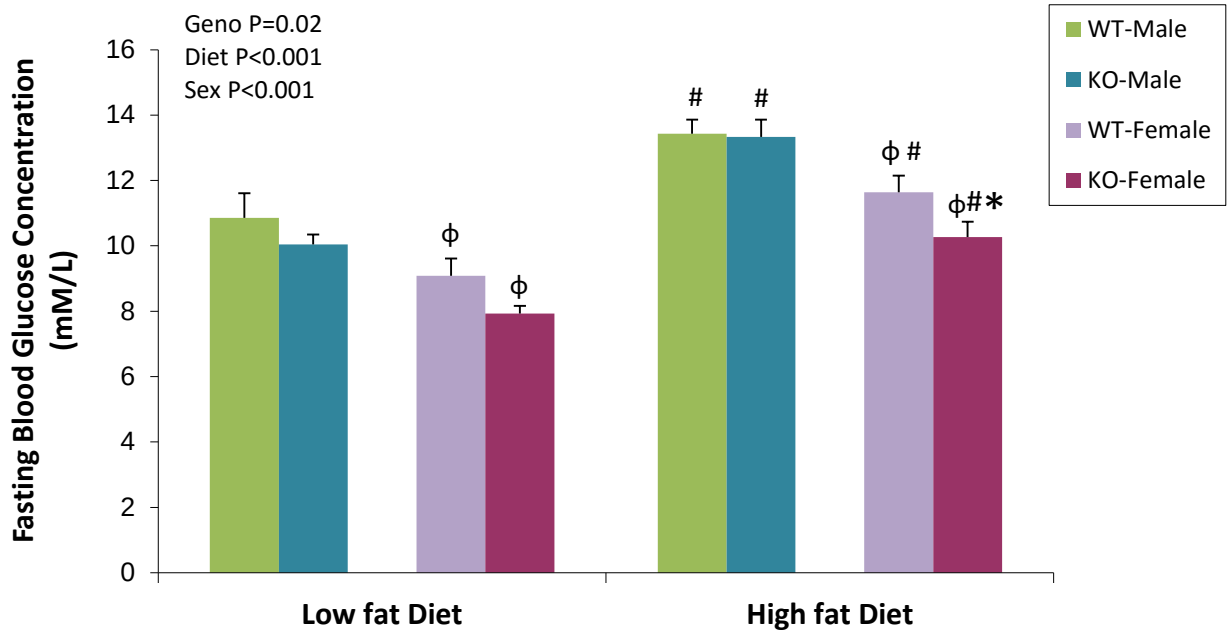


Figure 27: Comparison of fasting blood glucose levels at week 8 on diet. Male and female wild-type C57BL/6 or adiponectin knockout mice were fed either a low fat diet or high fat diet for 8 weeks. Animals were fasted for 6 hours and blood glucose was measured by glucometer strips using tail prick method. Data are expressed as mean ± SEM (n=7-10/group). Three-way ANOVA was used with sex, genotype and diet as main effects. $P<0.05$ was considered statistically significant. ϕ indicates differences ($P<0.05$) between sexes within a diet and genotype; # indicates differences ($P<0.05$) between diets within a sex and genotype and * indicates differences ($P<0.05$) between genotypes within a sex and diet. There were no significant interactions. **KO**: Adiponectin Knockout mice, **WT**: Wild-type C57BL/6 mice.

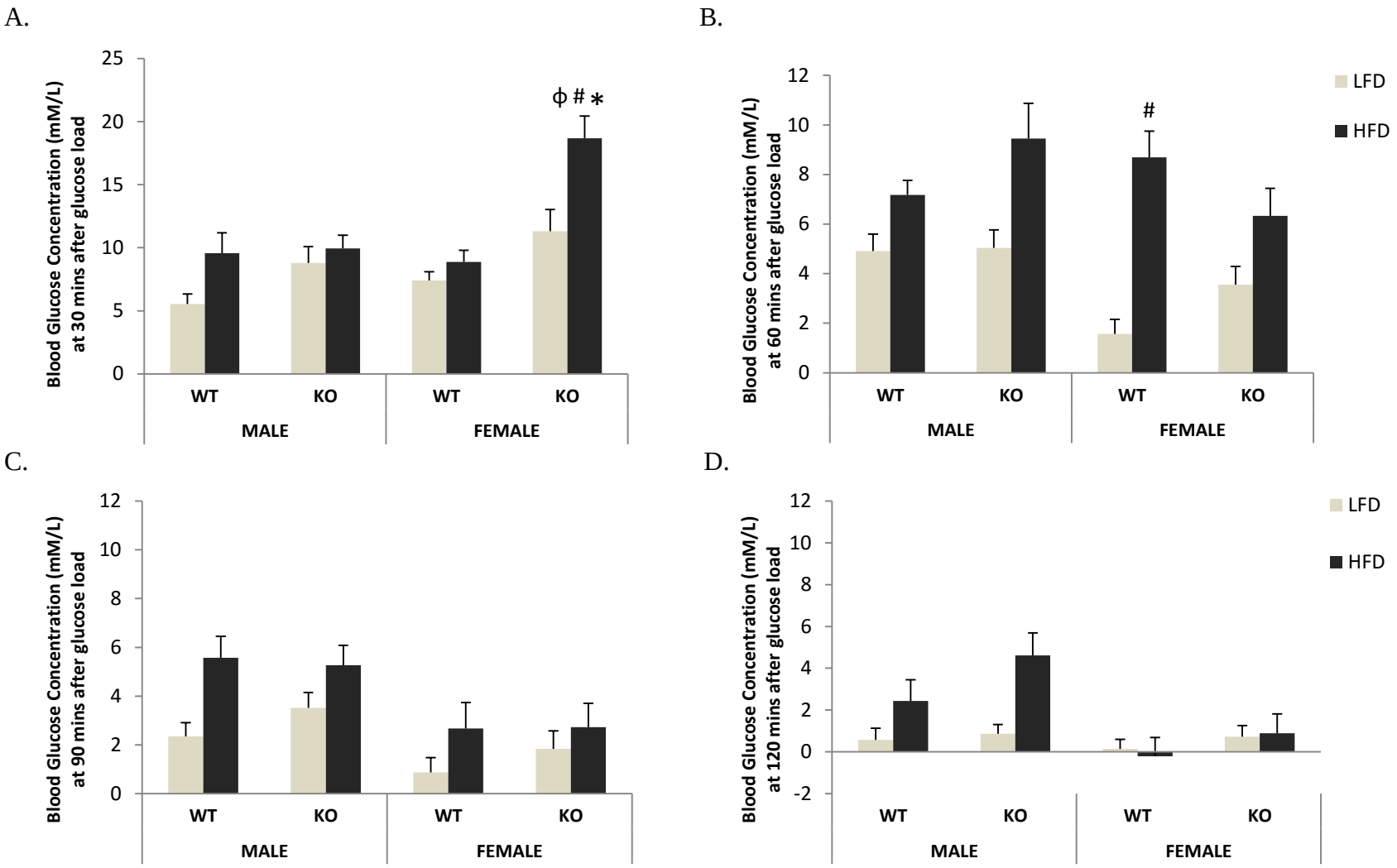


Figure 28a: Comparison of glucose tolerance at week 8 on diet. Oral glucose tolerance test (OGTT) was performed on male and female wild-type C57BL/6 or adiponectin knockout mice fed either a low fat diet or high fat diet for 8 weeks. Animals were fasted for 6 hours and blood glucose was measured at times 0 (pre glucose load) (A), 30 (B), 60 (C), 90 (D) and 120 (E) minutes after an oral

glucose dosage (2.5 mg/g body weight). Data normalized to 0 for baseline are expressed as means ± SEM (n=7-10/group). Repeated measures 3-way ANOVA was used to determine significant main effects, and lsmeans with Tukey-Kramer correction for multiple comparisons was used for means testing between specific groups; $P<0.05$ was considered statistically significant. There were significant main effects for time ($P<0.0001$), time × sex ($P<0.0001$), time × genotype ($P<0.0001$), time × diet ($P<0.0001$), time × sex × genotype ($P=0.0083$), time × sex × diet ($P=0.0343$) and time × sex × genotype × diet ($P=0.0031$). Within each timepoint, ϕ indicates differences ($P<0.05$) between sexes within a diet and genotype; # indicates differences ($P<0.05$) between diets within a sex and genotype and * indicates differences ($P<0.05$) between genotypes within a sex and diet. **LFD**: Low fat diet, **HFD**: High fat diet, **KO**: Adiponectin Knockout mice, **WT**: Wild-type C57BL/6 mice.

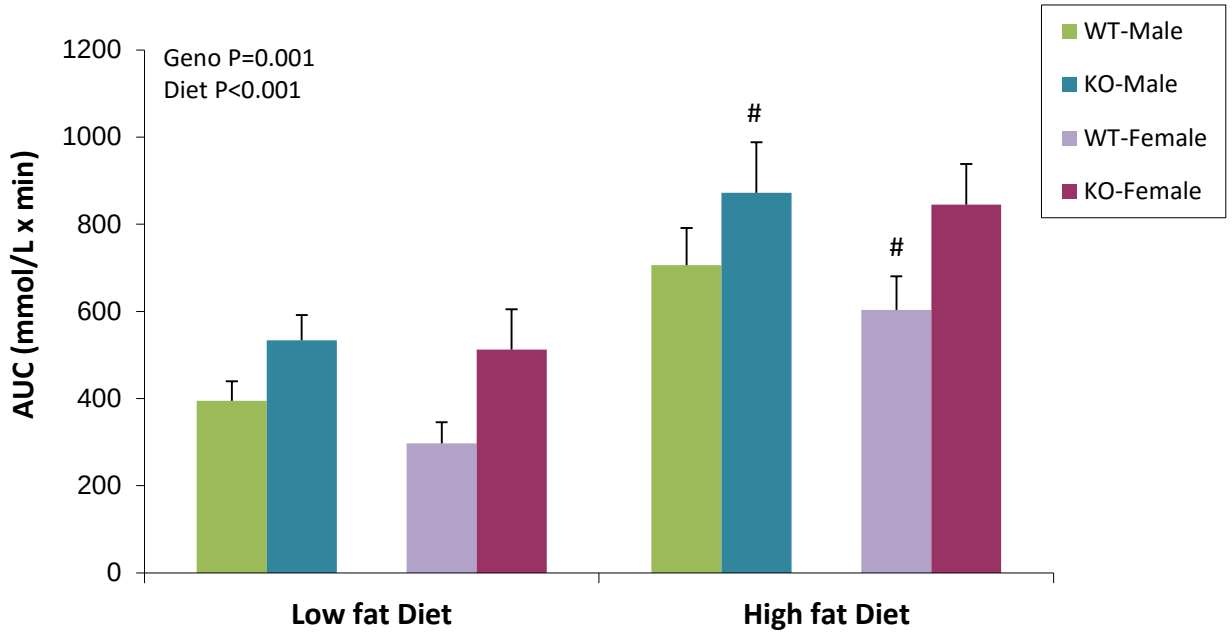
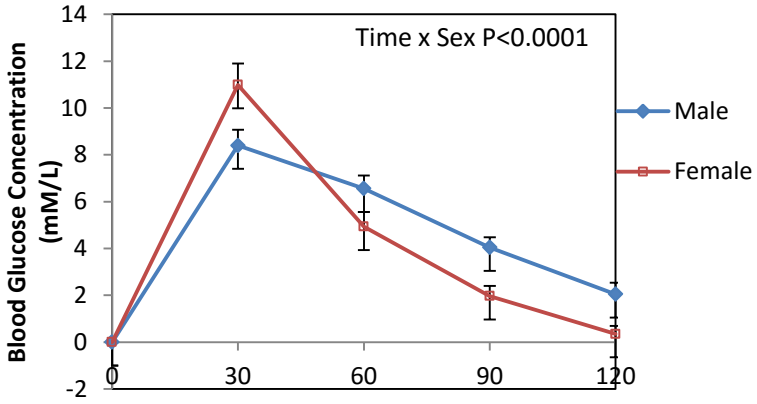


Figure 28b: Area under the curve (AUC) for oral glucose tolerance test. Oral glucose tolerance test was performed after 8 weeks on male and female wild-type C57BL/6 or adiponectin knockout mice fed either a low fat diet or high fat diet. Animals were fasted for 6 hours and blood glucose was measured at times 0 (pre-glucose load), 30, 60, 90 and 120 minutes after glucose dosage (2.5 mg/g body weight) and AUC was calculated. Data are expressed as mean ± SEM (n=7-10/group). 3-way ANOVA was used with sex, genotype and diet as main effects, and lsmeans with Tukey-Kramer correction for multiple comparisons was used for means testing between specific groups. $P<0.05$ was considered statistically significant. # indicates differences ($P<0.05$) between diets within a sex and genotype. **KO**: Adiponectin Knockout mice, **WT**: Wild-type C57BL/6 mice.

A.



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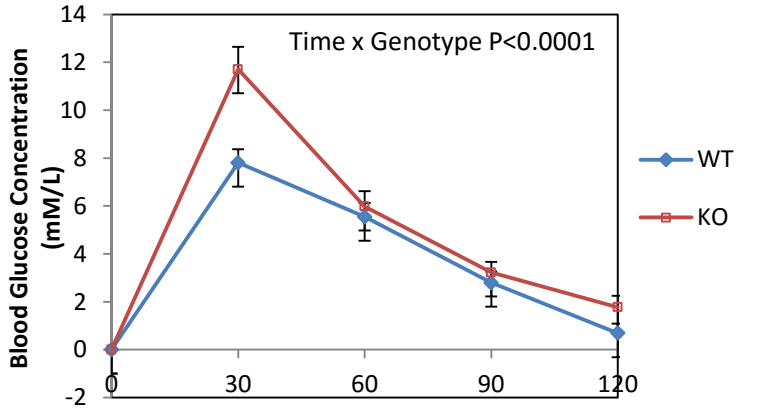

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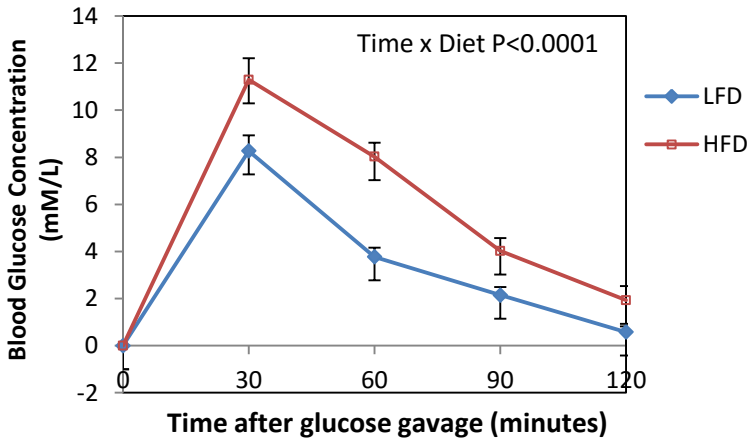
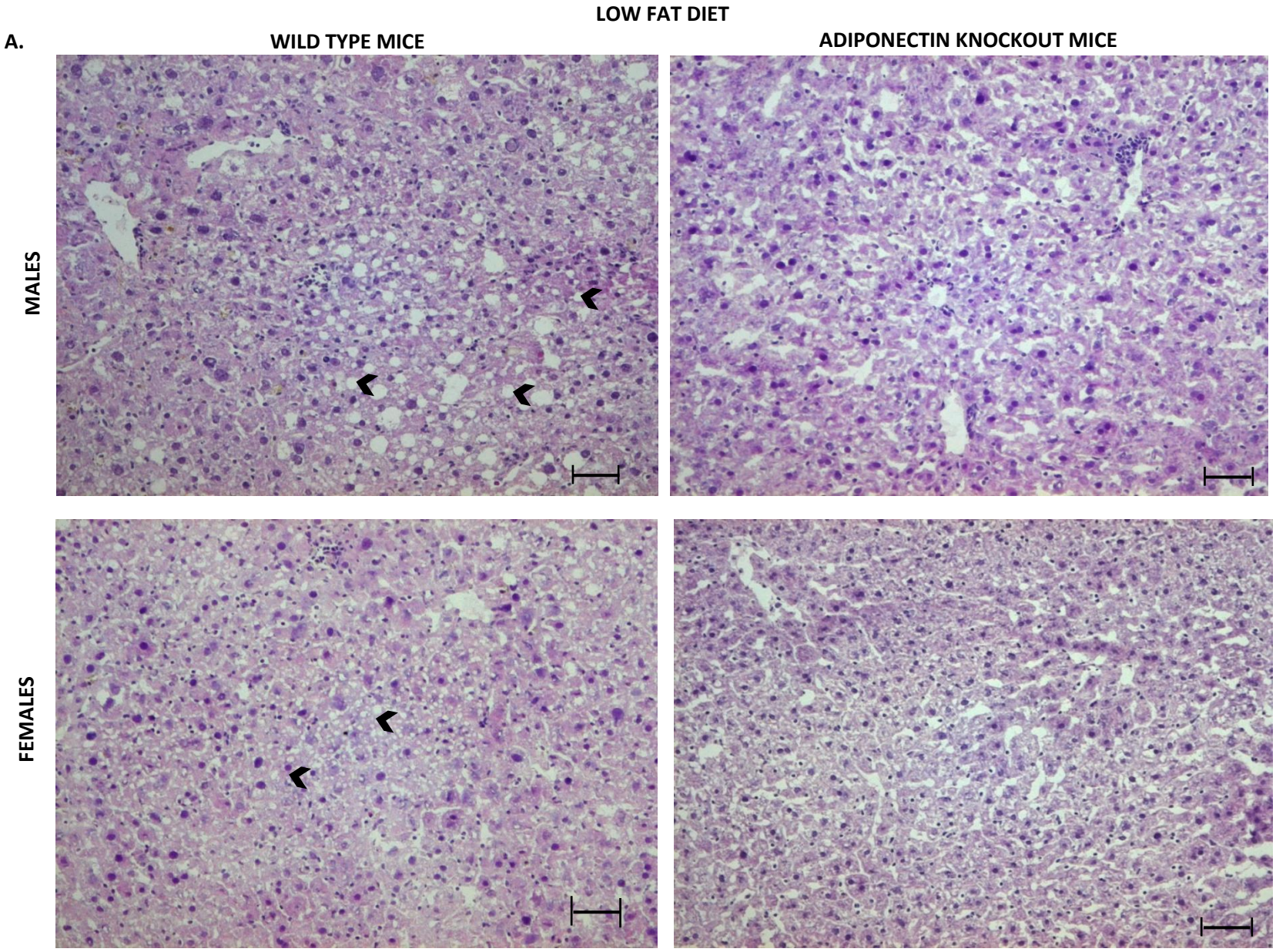


Figure 28c: Blood glucose concentration during the oral glucose tolerance test as a function of time, sex, genotype and diet. Blood glucose concentration measured during OGTT is shown as a function of time and sex (A); genotype (B); and diet (C). Data normalized to 0 for baseline are expressed as means ± SEM (n=34-40/group). Data were analyzed by repeated measures 3-way ANOVA and $P<0.05$ was considered statistically significant. **LFD**: Low fat diet, **HFD**: High fat diet, **KO**: Adiponectin Knockout mice, **WT**: Wild-type C57BL/6 mice.

3.3.1. Histological analysis of hepatic lipid content

We examined hematoxylin and eosin stained and Oil Red O stained liver sections from APN KO and WT mice on a low and high fat diet for morphological changes and lipid content (n=4/group). Both staining techniques demonstrated that the livers of APN KO mice of both sexes had no lipid accumulation relative to the WT groups when on a low fat diet, despite significantly increased liver weight of APN KO mice on LFD, in both sexes (Table 7). However, females had lower lipid content than males in the WT group on control diet (Figure 29A & 30A).

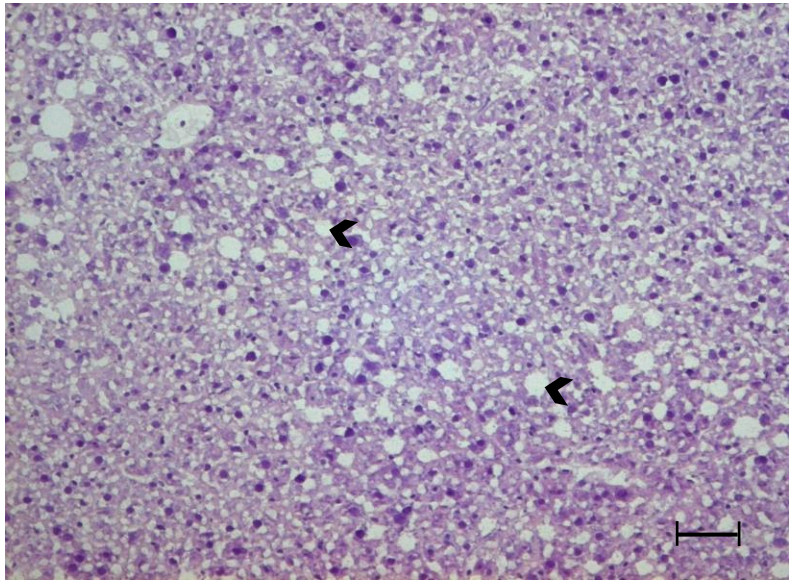
On high fat diet feeding, both male and female APN KO mice had increased hepatic lipid droplet content, with males accumulating more lipids than their female counterparts (Figure 29B & 30B).

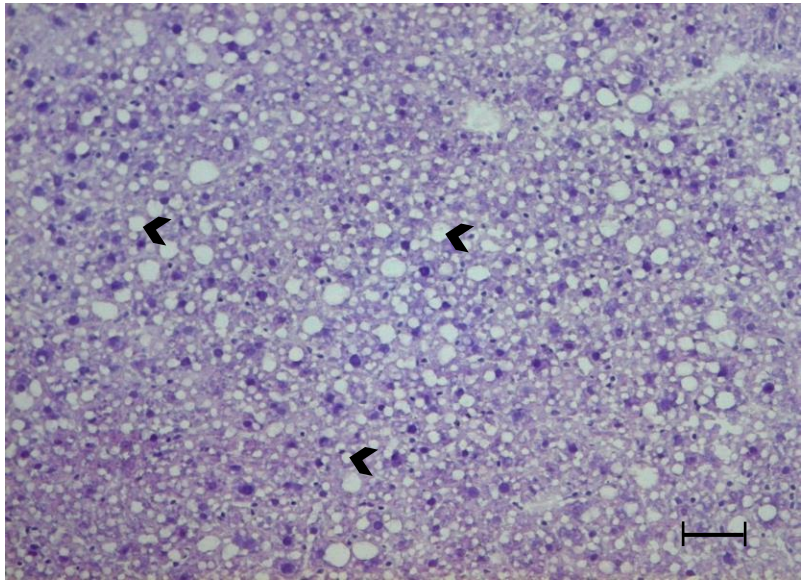

LOW FAT DIET
A.
WILD TYPE MICE
ADIPONECTIN KNOCKOUT MICE
MALES
FEMALES

HIGH FAT DIET

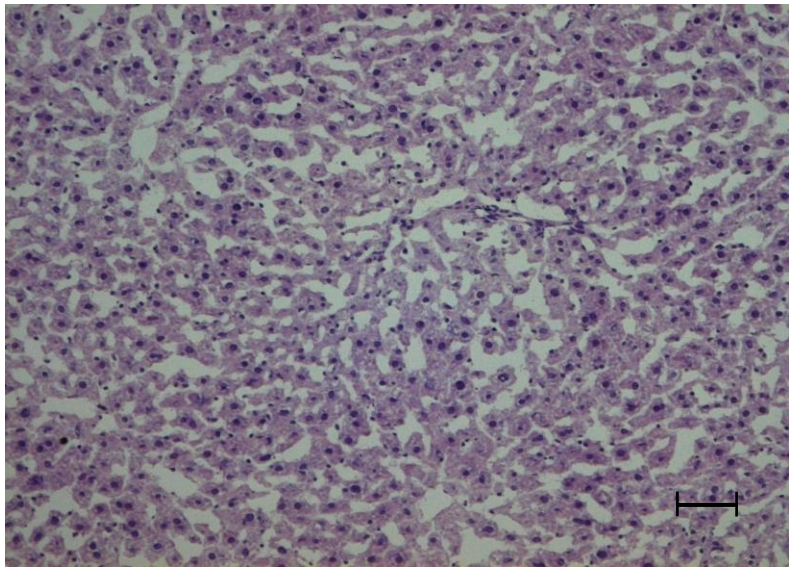
B. **WILD TYPE MICE** **ADIPONECTIN KNOCKOUT MICE**

MALES





FEMALES



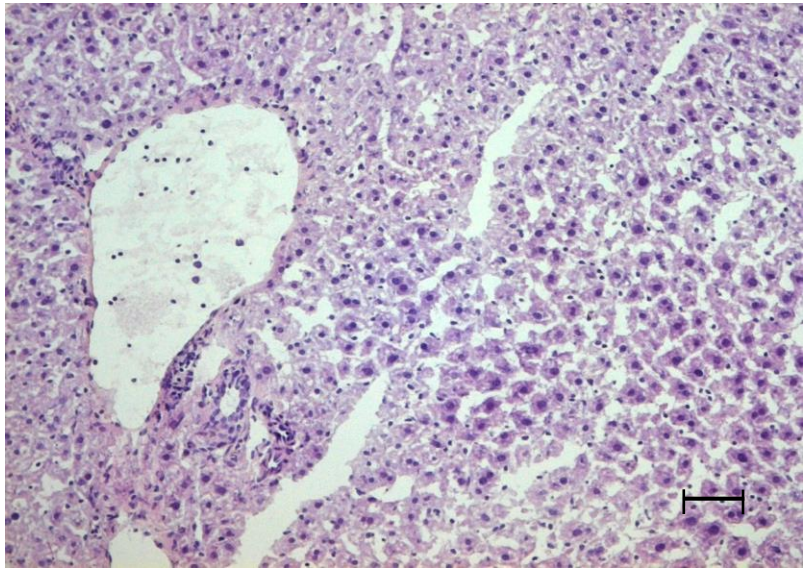


Figure 29: Histological comparison between sexes for lipid droplet content in liver tissue from adiponectin knockout and wild-type mice on diet for 8 weeks. Liver sections from animals fed (A) control low fat diet and (B) high fat diet. Eight μm thick sections of frozen liver in OCT compound were prepared using a cryotome and fixed using 4% paraformaldehyde. Six sections per animal (n=4/group) were stained using hematoxylin and eosin and images were taken by bright field microscopy. A representative micrograph from each group is shown with 20 times magnification. Arrow heads indicate unstained circular lipid-droplets in the liver sections. Scale bar represents 50 μm.

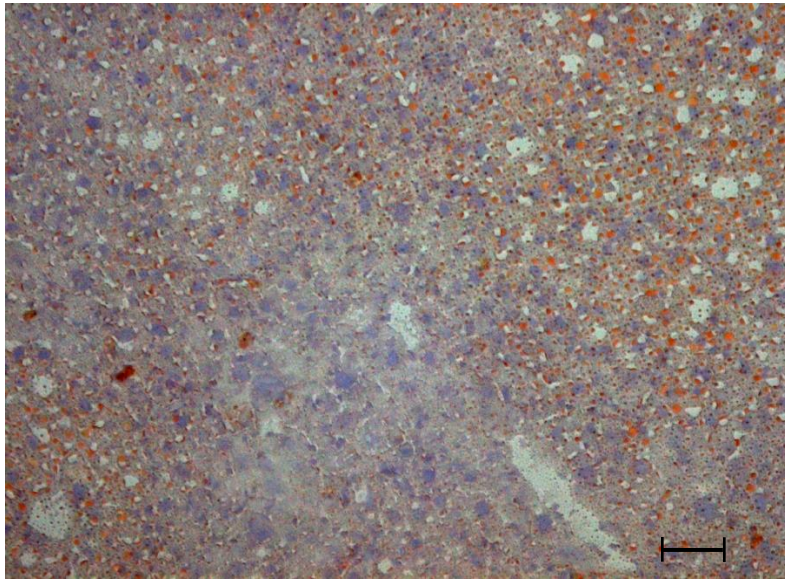
LOW FAT DIET

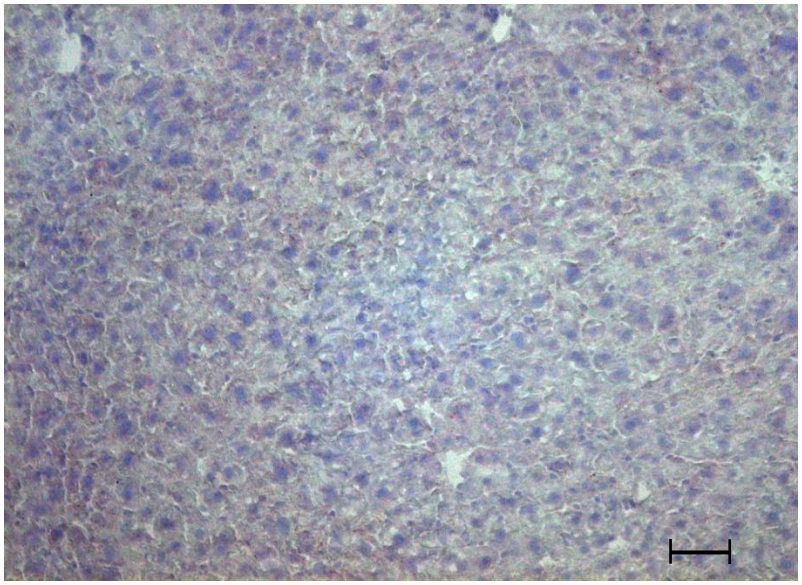
A.

WILD TYPE MICE

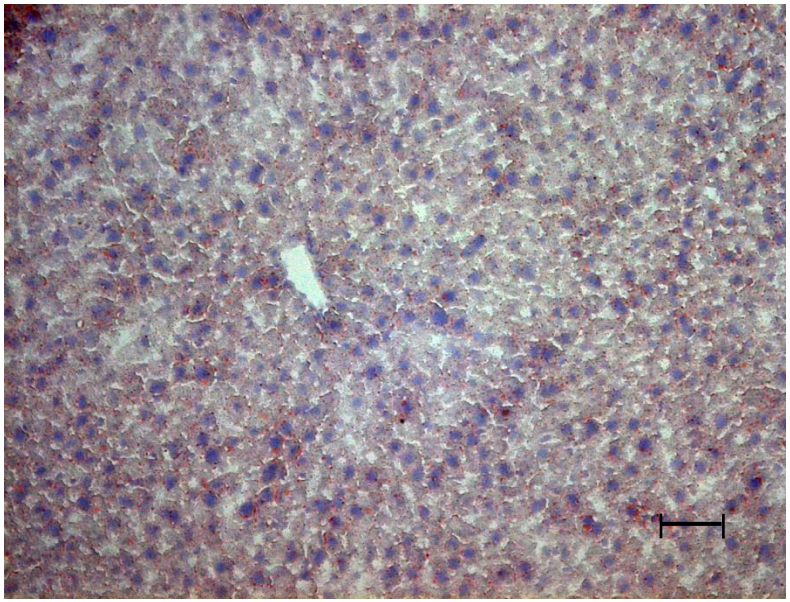
ADIPONECTIN KNOCKOUT MICE

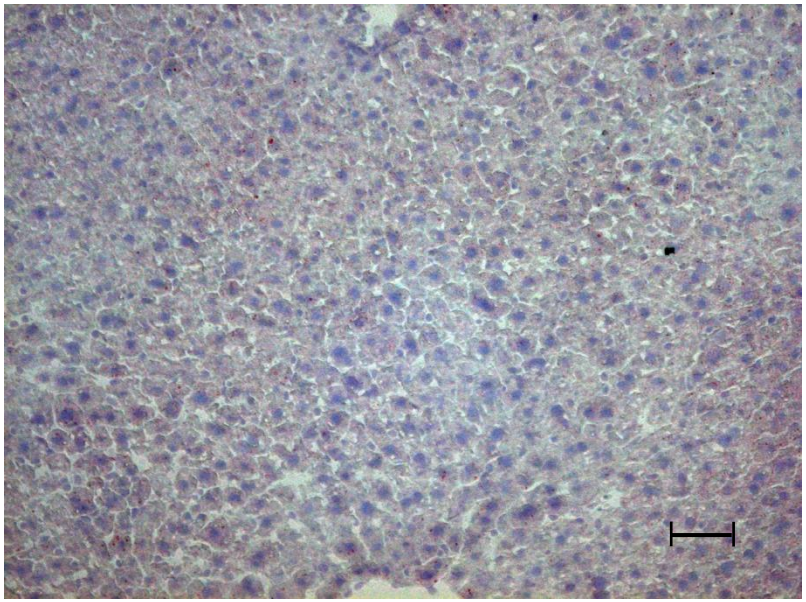
MALES





FEMALES





HIGH FAT DIET

B.

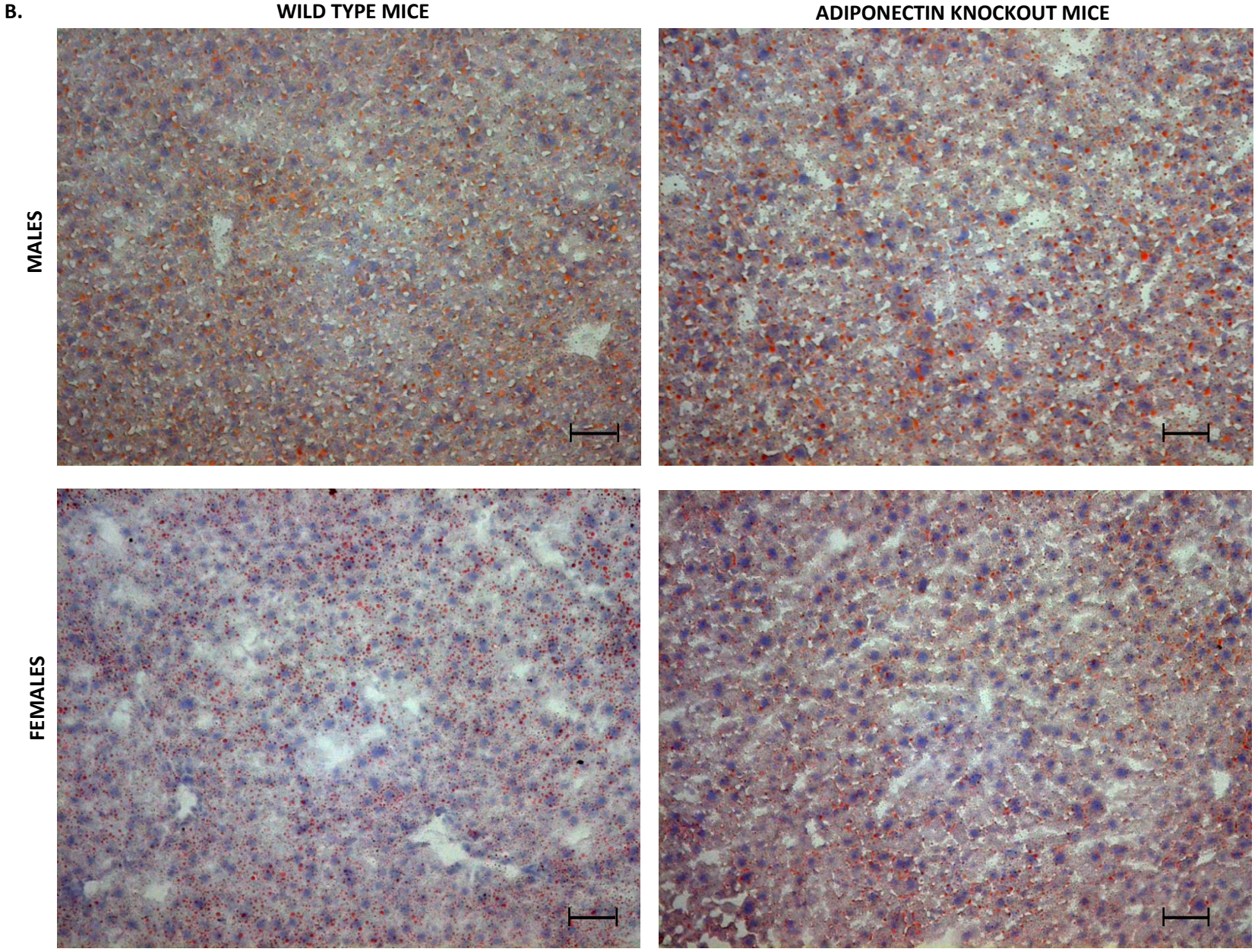


Figure 30: Histological comparison between sexes for lipid droplet content in oil red O stained liver tissue. Liver sections from adiponectin knockout and wild-type mice fed (A) control low fat diet and (B) high fat diet for 8 weeks. Eight μm thick sections of frozen liver in OCT compound were prepared using a cryotome and fixed using 4% paraformaldehyde. Six sections per animal (n=4/group) were stained using oil red O staining followed by hematoxylin counter-staining. Images were taken by bright field microscopy. A representative micrograph from each group is shown with 20 times magnification. Lipid is stained as orange-red regions in the liver sections. Scale bar represents 50 μm.

Table 8: Blinded scores of the lipid droplet content of the hepatic sections from male and female animals on diet for 8 weeks

MALES (8 weeks on diet)	Animal ID #	Blinded observer 1	Blinded observer 2
WT-LFD	#589	+++	+++
	#1143	+	+
	#1167	+	+
	#1168	++	++
WT-HFD	#599	++	+++
	#1146	+++	+++
	#1148	++	++
	#1171	+++	++
KO-LFD	#189	-	-
	#200	-	+
	#214	-	-
	#221	-	-
KO-HFD	#215	++++	+++
	#223	+++	++
	#387	+++	++++
	#388	+++	++++
FEMALES (8 weeks on diet)	**Animal ID #**	**Blinded observer 1**	**Blinded observer 2**
WT-LFD	#618	+	+
	#622	+++	+++
	#624	+	+
	#631	+	+
WT-HFD	#52	+++	+++
	#626	+	+
	#627	+	+
	#633	+++	++
KO-LFD	#262	-	-
	#273	-	-
	#279	-	-
	#289	-	-
KO-HFD	#264	+++	+++
	#274	++	++
	#281	++	+++
	#282	++	++

3.4. Arterial Stiffness

Pulse wave velocity, a determinant of arterial stiffness was unchanged among groups fed a control low fat diet or a high fat diet for 8 weeks. Furthermore, pulse wave velocity was not different between genotypes and sexes. However, a trend was observed with an interaction between genotype and sex (P=0.08) and female APN KO mice on both diets insignificantly trended to have stiffer vessels than the male APN KO mice and female WT mice (Figure 31).

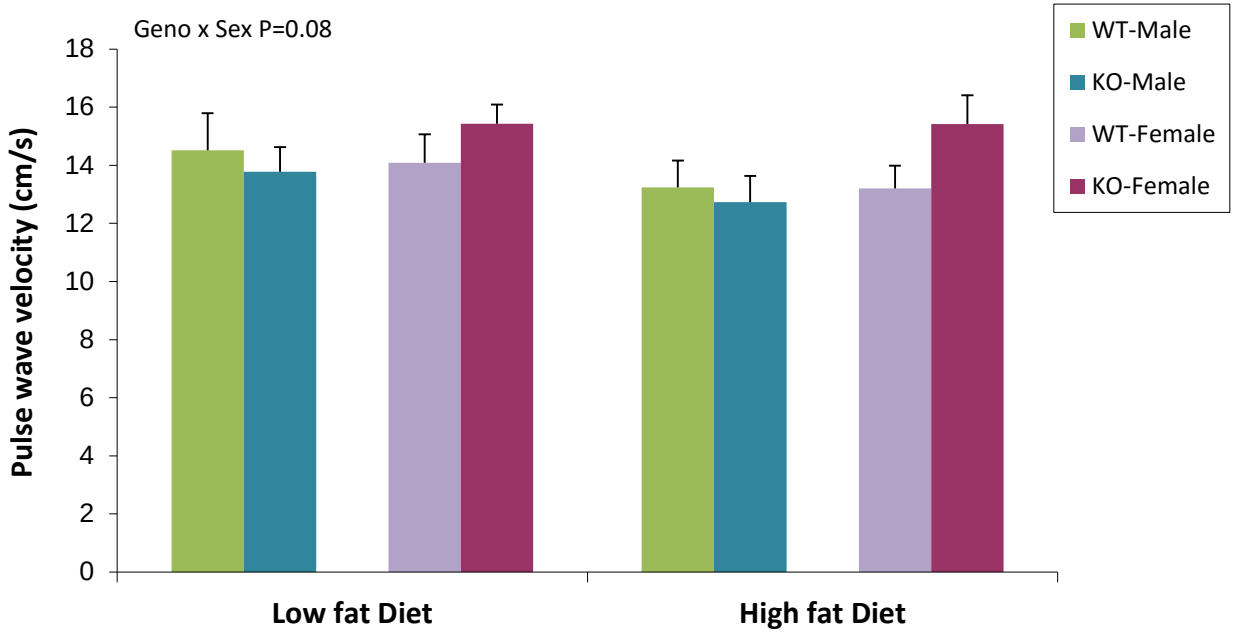


Figure 31: Arterial stiffness as determined by pulse wave velocity. Pulse wave velocity was measured non-invasively by pulse-wave Doppler ultrasound on the femoral artery of mice fed either a low or high fat diet for 8 weeks. Data are expressed as means ± SEM (n=8-10/group). Three-way ANOVA was used with sex, genotype and diet as main effects, and lsmeans with Tukey-Kramer correction for multiple comparisons was used for means testing between specific groups. $P<0.05$ was considered statistically significant and $P<0.1$ was considered as a significant trend. There were no significant main effects for genotype, diet or sex and there were no significant differences among means. **KO**: Adiponectin Knockout mice, **WT**: Wild-type C57BL/6 mice.

DISCUSSION

The rising prevalence of obesity worldwide is recognized as one of the most important risk factors for developing insulin resistance, Type 2 diabetes and cardiovascular diseases. Systemic insulin resistance associated with obesity is exacerbated by adipose tissue dysfunction. Adipose tissue directly influences whole body metabolism by communicating with liver and muscle through the secretion of various adipokines, cytokines and fatty acids, which is adversely affected as adipocytes enlarge during obesity. In the present study we have elucidated the sex-dependent modulation of metabolic and vascular parameters in the absence of adiponectin in a diet-induced obese model. One of the key findings in our study is that the absence of adiponectin in the male APN KO mouse leads to less adipose tissue, both VAT and SCAT when fed a low fat diet. This implies that low adiponectin levels in obese youth may exacerbate the effects of a poor diet as they develop into as adults. Secondly, while female APN KO also had reduced adipose depots on low fat diet, they did not show the same increase in adipose tissue as seen in either the female WT mice or the male APN KO mice when fed a high fat diet. This finding indicates an influence of sex hormones on the actions of adiponectin on adipose tissue development/expansion. The study therefore helps us to better understand the effect of reduced levels of circulating adiponectin in obesity and further findings will shed light on the mechanisms particularly implied to two populations at risk obese youth and post-menopausal women.

1. MODULATION OF ADIPOSE TISSUE EXPANSION AND ARTERIAL STIFFNESS BY ADIPONECTIN

In *in vitro* studies, 3T3-L1 fibroblasts with viral-mediated transgenic adiponectin over-expression showed a faster growth phase and more rapid differentiation into adipocytes (Fu et al.

2005). Interestingly, differentiated adipocytes overexpressing adiponectin were found to accumulate more and larger lipid droplets and exhibited increased insulin stimulated GLUT4 expression and translocation to the plasma membrane. Altogether, these findings corroborate the role of adiponectin as an autocrine element by promoting cell proliferation, pre-adipocyte differentiation, increased expression of genes involved in adipogenesis, lipid accumulation and insulin sensitivity (Fu et al. 2005). Thus our *in vivo* results are in agreement with the literature as adiponectin null mice showed a reduction in all four fat depots *viz*. epididymal, peri-renal, mesenteric and inguinal depots at baseline and on control low fat diet. Whether this difference in depot size is due to reduced adipocyte size or number needs to be addressed further by morphometric analysis. Additionally, dysregulated adipose metabolism involving impaired nutrient uptake and lipid synthesis could be causal factors for the observed reduction in fat pad mass. However, the impairment in adipose tissue expansion in APN KO male mice is overcome when challenged with a high fat diet. Thus, adiponectin may modulate white adipose tissue expansion/development through an autocrine mechanism that promotes either adipocyte hyperplasia or hypertrophy.

Rasmussen et al. (2006) have reported that there is a 10-fold higher expression of AdipoR1 than AdipoR2 in human adipose tissue and in isolated adipocytes with, lower expression in VAT than in SCAT. This indicates that adiponectin may have biological effects in adipose tissue in an autocrine/paracrine manner. However, in obese subjects, AdipoR1/2 expression was reduced, which could further exacerbate the adverse biological effects of low adiponectin levels in obesity (Nannipieri et al. 2007). Another clinical study found similar results with increased AdipoR1 expression in SCAT and improved insulin sensitivity in obese subjects with impaired glucose tolerance upon weight loss by calorie restriction (Kim et al. 2006).

Interestingly they showed a positive correlation between adiponectin receptor expression and PPARγ and plasma adiponectin levels. Besides modulating adipose tissue metabolism, it again alludes to a potential autocrine role of adiponectin on adipose tissue physiology. Thus the current results showing that the lack of adiponectin negatively affects the adipose tissue expansion, in both VAT and SCAT depots on control diet provides the evidence of an effect of adiponectin on adipose tissue physiology.

Whole body composition analysis further validated that total fat mass is reduced and lean mass is concomitantly increased in APN KO mice on control diet in comparison to the wild-type mice. There was no difference in percent fat mass and percent lean mass between genotypes on high fat diet. Yet, some studies have reported that adiponectin blocks pre-adipocyte differentiation and concomitantly increases uncoupling protein 2 (UCP2) levels in chicken adipocytes (Yan et al. 2014; Yan et al. 2013), changes that would limit adipose tissue expansion and decrease the lipid content of adipocytes, respectively. Importantly, since adiponectin increases tissue vascularisation (Aprahamian 2013), it may improve adipose function by overcoming the effects of hypoxia prevalent in obesity due to adipocyte enlargement thereby promoting adipogenesis.

Among various fat depots, increased central visceral adiposity is the best predictive factor of insulin resistance (Pitombo et al. 2006). At week 12, despite their reduced visceral fat mass, adiponectin deficient mice on a control diet exhibited attenuated glucose tolerance as compared to the wild-type mice and were comparable to the glucose intolerance developed by adiponectin deficient mice on high fat diet. However, Nawrocki et al. (2002) had shown glucose intolerance in APN KO mice only on high fat diet and not on a chow diet. This discrepancy could be due to the difference in the duration of the diet. In the present study, the mice were on low fat diet (10%

energy from fat) for 12 weeks and analysed at the age of 20 weeks, whereas in the previous report OGTT was carried out on 10 week old APN KO mice on chow diet (21% energy from fat). However, these 10 week old APN KO mice on chow diet displayed hepatic insulin resistance as shown by the euglycemic-hyperinsulinemic clamp studies with an impaired ability in APN KO to suppress insulin-stimulated hepatic glucose production. APN KO mice therefore required a slightly reduced but insignificant glucose infusion rate to maintain a euglycemic steady state than the wild-type counterparts. The unexpected lack of development of insulin resistance in wild-type mice fed a high fat diet for 12 weeks in the current study in spite of gaining significant body weight could be due to variations in oral delivery of the glucose load during OGTT.

In order to check if the attenuated adipose tissue expansion has resulted in lipids and fatty acids being diverted to other major metabolic organs, liver lipid droplet assessment was done. Liver from adiponectin deficient mice weighed more than the wild-type on both diets. Interestingly, no lipid accumulation was observed in hepatic sections from adiponectin deficient mice on control diet. Unlike the control diet group, deficiency of adiponectin combined with a high fat diet did not protect from lipid accumulation and thus hepatic lipid accumulation was exacerbated as compared to the wild-type genotype on a high fat diet.

Furthermore, Liu et al. (2012) used a APN KO mouse model (Maeda et al 2002) and reported a regulatory role of adiponectin on hepatic genes involved in glucose and lipid metabolism at the transcriptional level via hepatic nuclear factor 4 alpha (HNF4α). Key hepatic lipogenic genes and many transcription factors were found to be downregulated in the absence of adiponectin. Similar to our results they observed reduced epididymal fat mass on chow diet. Although on a high fat diet, an increase in epididymal fat pad mass was not observed, unlike our

current data; this discrepancy could be due to the shorter duration of high fat diet feeding followed in that study (4 week *vs* 8-12 weeks). Moreover, the downregulation of hepatic lipogenic genes resulted in a phenotype of reduced triglyceride content and a remarkably low level of lipid accumulation in adiponectin null hepatocytes. In agreement with these results, another study with female adiponectin transgenic mice exhibited increased adiposity without alteration in food intake (Combs et al. 2004). Thus the observed hepatic lipogenic defect in the absence of adiponectin could be implicated in the adipose tissue as well and may partially account for the reduced adipose expansion due to impaired DNL in adipocytes, besides impaired adipogenesis. However, the AMPK pathway downstream of adiponectin could not explain these findings as the reduction in its activity in the absence of adiponectin should have led to increased lipid synthesis (Liu et al. 2012).

Interestingly, mutations in the HNF4α gene, a master regulator of complex liver functions, which binds to almost 50% of active hepatic genes (Kuo et al. 1992; Odom et al. 2004), are associated with Type 2 diabetes (Hani et al. 1998; Muller et al. 2005; Stoffel and Duncan 1997; Yamagata et al. 1996). Additionally, variants in HNF1α, another major transcription factor regulated by HNF4α, are associated with maturity-onset diabetes of the young (MODY3) (Vaxillaire et al. 1997). Likewise, a specific G319S variant of the HNF1α gene is highly prevalent in the Canadian Oji-Cree aboriginal population (Triggs-Raine et al. 2002). This cohort has the highest rate of Type 2 diabetes in the world (Harris et al. 1997; Hegele et al. 1999) and is characterised by a lower average BMI and insulin resistance (Sellers et al. 2002). This implicates one of the possible pathophysiological mechanisms by which low levels of adiponectin in obesity could modulate insulin sensitivity via hepatic transcriptional regulation.

Altogether, the key findings of impaired adipose tissue depot development in the absence of adiponectin have strong implications for obese youth, who are exposed to adiponectin deficiency at an age where growth is still occurring. This could exacerbate the effects of poor diet on their energy metabolism leading to early onset of cardio-metabolic disorders. Therefore a better understanding of the causal relationship between adiponectin deficiency, adipose tissue development and insulin resistance would help us to map out ways to prevent or treat the rising prevalence of obesity and associated disorders in youths.

The blood vessels of APN KO animals fed a high fat diet for 12 weeks became less elastic based on our pulse wave velocity results while at the same time the high fat diet did not alter the blood vessel elasticity of the wild-type mice. Although studies have shown that the lack of adiponectin leads to impaired vasodilation due to reduced eNOS and thus attenuated NO bioavailability (Ouchi et al. 2003), no study has looked exclusively into the development of arterial stiffness, an increasingly accepted biomarker of cardiovascular diseases. In addition to its protective role against hypertension (DeClercq et al. 2012), adiponectin has been shown to block leukocyte adhesion and restenosis (Matsuda et al. 2002; Ouchi et al. 1999), and decrease atherosclerotic lesion size and number in *ApoE* deficient mice (Okamoto et al. 2002). Adiponectin also promotes the growth of new vasculature in mouse models of hind limb ischemia (Ohashi et al. 2009). Collectively, these studies show a direct effect of adiponectin on the vasculature. Inflammation in obesity involving increased levels of TNFα, IL-6 and high-sensitive C-reactive protein was associated with arterial stiffness (Mahmud et al 2005). Furthermore, the loss of arterial elasticity resulting from the mechanical adaptation of the vessel walls due to hypertension involves elastin fibre degradation, collagen accumulation and reorganization of cellular components (Laurent et al. 2005). Additionally, owing to the presence

of a collagen-like domain in its protein structure, adiponectin could possibly bind extracellular matrix proteins such as fibronectin, which is shown to promote atherosclerotic lesions and fibrous caps (Rohwedder et al. 2012) and perhaps, directly influence the matrix remodelling which renders protection from developing vessel stiffness. Therefore, we propose a novel role for adiponectin in vascular structure through modulation of vessel elasticity via its action on the extra-cellular matrix; this new function warrants further molecular studies.

2. SEX DIMORPHISM IN ADIPONECTIN ACTION

Females have significantly higher levels of circulating adiponectin as compared to males, in both humans and rodents (Bidulescu et al. 2013; Hung et al. 2008; Kamari et al. 2012). The role of sex hormones in controlling adiponectin is evident from studies of women with polycystic ovary syndrome where imbalanced hormone production resulted in dysregulated expression of both adiponectin and its receptors (Krentz et al. 2012; Tan et al. 2006).

In the current study, a sex difference in body weight was observed with females of both genotypes weighing less than males over the 8 week period of study on the control diet. However, on a high fat diet, both male and female adiponectin knockout mice became significantly more obese than their low fat diet counterparts but female adiponectin knockout mice still weighed significantly less than males on both diets. Thus a significant interaction among time, sex, genotype and diet was observed for body weight.

Notably, though the weight of both male and female APN KO mice was comparable to the wild-type mice on control diet, they had significantly less epididymal-parametrial, peri-renal and mesenteric fat pads. This again corroborates the results from our earlier study for 12 weeks, showing impaired adipose tissue development in the absence of adiponectin. Interestingly, wild-

type mice showed a sex difference in epididymal-parametrial and mesenteric fat depots with females having reduced fat mass on control diet. This sex difference was not observed in adiponectin knockout mice; perhaps this was due to APN KO males exhibiting a higher degree of impairment in adipose expansion while females had smaller extent of impairment as compared to their wild-type counterparts. This disparity could be accounted for by the fact that estrogen but not androgen promotes proliferation and differentiation of pre-adipocytes and this effect is depot-specific with subcutaneous fat more responsive than visceral fat (Anderson et al. 2001). Female adipose tissue additionally has expresses more estrogen receptors than male adipose tissue (Grove et al. 2010; Macotela et al. 2009; Payette et al. 2009).

Interestingly, sex dimorphism was observed in the generalised PPARγ knockout mouse model where females develop periuterine (parametrial) and interscapular fat depots, which further increased in size with administration of the PPARγ agonist rosiglitazone. However, males did not develop any fat depots with or without rosiglitazone treatment. Furthermore, the periuterine adipocyte size was greater in PPARγ KO mice than the wild-type and this was further increased by rosiglitazone in KO mice and reduced in wild-type mice (Duan et al. 2010). This study is relevant as PPARγ plays a crucial role in adipogenesis as well as in adiponectin expression and secretion from adipocytes. Thus lack of PPARγ also indicates a lack of adiponectin and the observed fat depot development in females in the absence of PPARγ could be mediated by the action of estrogen. ERα was increased in periuterine fat in female PPARγ KO mice and this could have contributed to adipogenesis of this depot.

In addition to ovary and testes, estrogen is produced in the adipocytes by the action of aromatase on androgens and is increased concomitantly with total adiposity (Schneider et al. 1979; Tchernof et al. 1995). The knockout of aromatase in male mice results in decreased

estradiol levels and correspondingly decreased adiponectin levels (Sinderen et al. 2014). The decreased levels of circulating estrogen in post-menopausal women are observed to increase central obesity and predispose them to metabolic disorders. This is partially protected by estrogen replacement therapy (Gambacciani et al. 1997; Haarbo et al. 1991; Haarbo et al. 1991). Thus we can postulate that the protective effect of estrogen on adiposity during energy surplus could be mediated by adiponectin. This is in agreement with our results showing a significant reduction in SCAT mass in both male and female obese adiponectin knockout mice. Additionally, female obese adiponectin knockout mice had significantly less VAT than the female obese wild-type mice. These results were validated by the whole body composition analysis which showed significantly reduced fat mass in obese females in the absence of adiponectin. Thus it implies that fat expansion is influenced by an interaction between estrogen and adiponectin.

Estrogen signaling via ERα may be the underlying mechanism of the sex difference in adiponectin signaling. Plasma adiponectin levels are decreased in female ERα knockout mice (Bryzgalova et al. 2006). Moreover, serum levels of adiponectin have been associated with polymorphisms in the human ERα gene (Yoshihara et al. 2009). ERα was increased in periuterine fat in female PPARγ KO mice and this could have contributed to adipogenesis in this depot (Duan et al. 2010). Thus the major findings in the current study with respect to adipose tissue is that females did not gain fat mass on high fat diet feeding to the same degree as males in the absence of adiponectin. Whether this mitigation in adipose tissue expansion is protective or is due to adipogenic and/or adipose dysfunction needs to be addressed, along with further research on the cross-talk of estrogen and adiponectin signaling.

Likewise, it is interesting to observe an enlarged epicardial fat mass in female mice but not males on control diet. Notably, the absence of adiponectin impaired epicardial fat mass expansion only in lean males, again suggesting a fat depot specific adipogenic effect of estrogen and adiponectin. However, on high fat diet remarkable sex difference was observed in the depot expansion with a diet effect seen only in male APN KO and female wild-type mice compared to their sex and genotype counterparts. This is in agreement with the findings of the SWAN Cardiovascular Fat Ancilliary Study which shows an increased cardiovascular fat volume with decreased estradiol concentration with respect to menopausal status in women (Khoudary et al. 2015). Our data infers that the epicardial fat expansion in females is negatively affected by adiponectin deficiency on a high fat diet. As enlarged epicardial fat depot is increasingly considered as a source of inflammatory mediators (Mazurek et al. 2003) and a marker for severe visceral obesity (Iacobellis et al. 2003) and CVD (Mookadam et al. 2010); reduced epicardial fat mass in obese adiponectin knockout females may render a cardioprotective effect.

Recently, it has been shown that intra-abdominal adipocytes from females are more insulin sensitive than those from males, and sex hormones play a central role (Adams et al. 1997). Additionally females are less susceptible to metabolic disorders most likely due to the anti-inflammatory effects of estrogen (Ribas et al. 2010; Rogers et al. 2009). Interestingly, independent of sex, ERα KO mice were predisposed to obesity and had increased visceral fat mass, adipocyte hypertrophy, decreased insulin sensitivity and impaired glucose tolerance (Heine et al. 2000), a phenotype similar to humans deficient in ERα and aromatase enzyme (Blüher 2013) and similar to adipocyte and adipose tissue specific ERα KO mice (Davis et al. 2013). We have shown that male APN KO mice are more susceptible to the negative effects associated with obesity with respect to glucose tolerance but both females and males are susceptible with respect

to accumulation of hepatic lipids. At week 8 on diet, both male and female lean APN KO mice did not become glucose intolerant as compared to their wild-type counterparts. This is consistent with the Nawrocki et al (2006) observation where APN KO mice are glucose tolerant on a control diet. Furthermore, significantly increased pancreatic weight in female APN KO mice on high fat diet relative to male APN KO mice on same diet, suggests an increased insulin secretion capacity that may explain the glucose tolerance observed in obese female APN KO mice. However, unlike the previous result where the male APN KO mice were glucose tolerant after being on high fat diet for 8 weeks, here the male APN KO mice were significantly glucose intolerant. This discrepancy could be due to the difference in the technique used for the delivery of glucose load. In the former study, we orally fed the mice which had some inconsistency in delivery whereas in the latter one, oral gavage was carried out.

Importantly, with regard to hepatic lipid droplet content, wild-type females had less lipid accumulation than males on low fat diet. However, adiponectin deficiency was found to protect both males and females from hepatic lipid accumulation relative to their wild-type counterparts on a low fat diet. This is in agreement with the earlier results and the literature showing dysregulation of hepatic lipogenic genes via the downregulation of the master transcriptional factor HNF4α in the absence of adiponectin (Liu et al. 2012). On the other hand, high fat diet feeding for 8 weeks reversed the protective effect of adiponectin deficiency with males accumulating more lipids in liver than females. This suggests a requirement for prolonged feeding of high fat diet for at least 12 weeks to develop significant hepatic fat accumulation in the wild-type mice. Thus the sex dimorphism in fasting glucose levels and hepatic lipid content in mice lacking adiponectin suggests that adiponectin modulates the hepatic response to estrogen

in the context of insulin-mediated glucose production and requires further experiments to tease out the molecular mechanism.

In contrast to the above sex dimorphic results in adiponectin action, there were no difference among groups with respect to the arterial stiffness in our second study. This is in contradiction to our earlier results which showed stiffer artery on obese adiponectin deficient mice. The shorter duration of the latter study (8 vs 12 weeks on high fat diet) could account for this disparity along with the difference in the technical assistance obtained during the pulse wave analysis on the femoral artery in both studies. Furthermore, a lack of association of atherosclerosis with adiponectin levels has been reported in this APN KO mouse model (Nawrocki et al. 2010). However, a study using the same APN KO mouse model had established a sex difference in eNOS activation in response to adiponectin (Durand et al. 2012) but the study duration was 22 weeks and the mice were older than the current study. Overall, a trend was observed with an interaction between genotype and sex alluding to a developing vascular phenotype in this model which could become detectable with increased age, prolonged duration on high fat diet, *etc*.

Based on the current findings, it is possible to propose that the protective effects of estrogen against cardiovascular and metabolic diseases are at least partly mediated by adiponectin. Further research would shed light on the molecular mechanism through which the combined actions of estrogen and adiponectin operate. The proposed integrated action of adiponectin and estrogen is illustrated in Figure 32. The current findings of a sex difference in the impairment of the adipose tissue expansion due to reduced adiponectin levels would have strong implications in two main populations at risk obese youth and post-menopausal women, along with therapeutic development for obesity associated diabetes and CVD management.

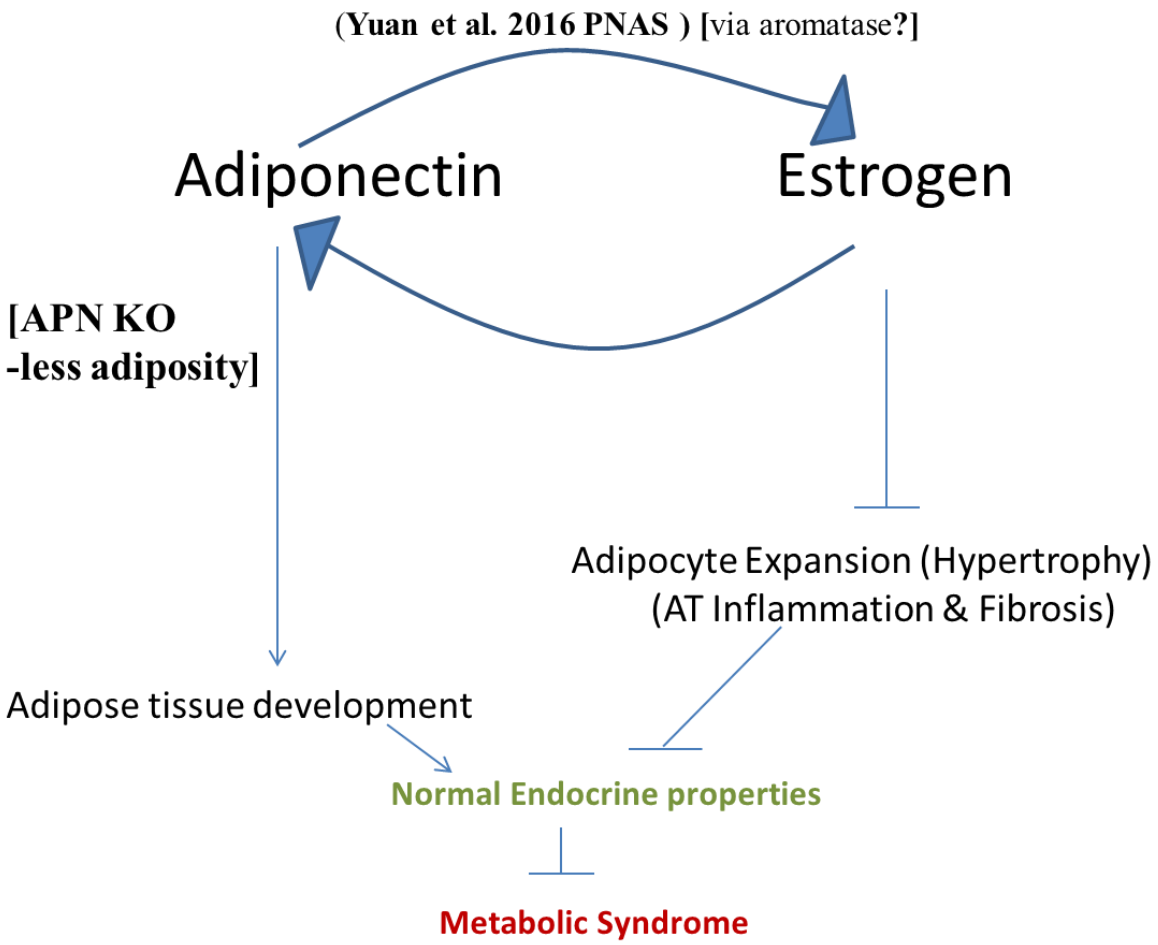


Figure 32: Illustration of proposed integrated action of adiponectin and estrogen on adipose tissue physiology.

CONCLUSIONS

- After 8 and 12 weeks on a low fat diet, despite similar body weights, male adiponectin knockout mice had smaller visceral and subcutaneous fat pad weights relative to wild-type C57BL/6 mice fed low fat diet.
- After 12 weeks on a high fat diet, adiponectin knockout mice weighed significantly more than wild-type C57BL/6 mice but had comparable fat depot weights. Thus from the study we found that although fat pads were lower on LFD, they could expand to the same extent or proportionally more than the WT mice.
- Adiponectin knockout mice had reduced % fat mass with a concomitant increase in % lean mass compared to wild-type C57BL/6 mice when fed a low fat diet for 12 weeks, but there were no differences between genotypes when fed a high fat diet. In conclusion, adiponectin may be modulating white adipose tissue expansion/development through an autocrine mechanism.
- Adipose tissue mass did not increase in adiponectin knockout females to the same degree as adiponectin knockout males or wild-type C57BL/6 mice on a high fat diet suggesting that sex influences the response of white adipose tissue to adiponectin.
- After 8 weeks on high fat diet, only male adiponectin knockout mice were susceptible to the negative effects associated with obesity with respect to glucose tolerance, while both females and males were susceptible with respect to accumulation of liver lipids.
- After 12 weeks on diet, male adiponectin knockout mice exhibited arterial stiffness on a high fat diet compared to low fat diet counterparts, while control wild-type mice showed no changes in arterial stiffness due to diet. We therefore conclude that adiponectin has a

potential role in modulating vascular elasticity in addition to its known effect on eNOS mediated vasoprotection in obesity and insulin resistance.

STRENGTHS AND LIMITATIONS

Strengths

- Whole body physiology study integrating liver and adipose tissue by measuring glucose homeostasis and fat storage.
- Inclusion of multiple control groups
- Use of a diet induced obese mouse model in the absence of adiponectin created a scenario similar to obese human population

Limitations

- Use of 3-way ANOVA enabled the systematic analysis of interaction between main effects. However, for some values, it made physiologically relevant differences statistically insignificant due to lack of freedom.
- In Study I, the oral delivery of a glucose load was inconsistent as mice did not readily take it unlike the rat models and it seemed to be stressful for the animals.
- While OGTT was used to assess insulin resistance, the euglycemic-hyperinsulinemic clamp is the gold standard for assessment of insulin resistance.
- Insulin concentration was not determined during OGTT as blood samples adequate for insulin concentration analysis could not be collected from mice.
- Overnight fasting would have been better than the 6 hour fasting followed as rodents are nocturnal.
- Food intake monitoring was not carried out to check any difference between genotypes or sexes.

- The exact morphological and molecular mechanism underlying the fat pad difference was not addressed.
- The technical inconsistency for the measurement of PWV among the different studies was a major drawback.

FUTURE DIRECTIONS

- Investigate the relationship between reduced fat mass and hepatic lipid accumulation with insulin sensitivity using the standard euglycemic-hyperinsulinemic clamp technique.
- Morphometric analysis of the fat tissue for adipocyte size and number, and measurement of hepatic triglyceride content.
- Examine adipocyte functioning in adiponectin knockout mice fed low fat or high fat diets by measuring pro-/anti-inflammatory adipokine secretome by multiplex assays.
- Study the adipogenic differentiation potential of pre-adipocytes isolated from male and female adiponectin knockout mice by *in vitro* culturing.
- Morphometric analysis of the aortic tissues for lumen size and wall thickness and immunostaining of elastin and collagen proteins to determine the underlying mechanism for arterial stiffness.
- To rescue the alteration in fat mass and vascular function in both lean and diet-induced obese adiponectin knockout mouse model by exogenous administration of adiponectin and use of an intra-peritoneal glucose tolerance test to examine amelioration of glucose tolerance.
- To investigate the combined action of adiponectin and estrogen by using ovariectomized adiponectin knockout mouse models with or without replenishment of adiponectin, estrogen or both.

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