# The Effects of High Protein Diets on Metabolic Syndrome Parameters in the *fa/fa* Zucker Rat

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**ABSTRACT**

Despite inconsistent results in the literature, high protein diets are being promoted for the management of metabolic syndrome parameters primarily due to their proposed favorable effects on weight loss. Therefore, lean and *fa/fa* Zucker rats were given normal and high protein diets with varying protein sources for 12 weeks. A high protein diet with a mixture of animal and plant protein sources was the most effective for improving metabolic syndrome parameters, specifically insulin resistance and hepatic steatosis. A high protein soy diet was the second most effective diet, while a high protein casein diet demonstrated no benefits compared to the other two high protein diets and minimal benefits compared to a normal protein casein diet. Interestingly, high protein diets did not affect body weight regardless of protein source. These findings suggest that the source of protein within a high protein diet is critical for improving metabolic syndrome parameters and that improvements can be observed independent of weight loss.

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**DEDICATION**

I would like to dedicate my thesis to the two most precious people in my life, my children, Dane and Jaxton. You have literally been on this journey with me from day one. I hope one day you will read this and be proud of your mom. I love you two with all my heart, and I hope that my perseverance and hard work will help both of you achieve great things in life.

Finally, I would also like to dedicate my thesis to my dad, who I know is watching over me each and every day, and giving me the strength I need whenever I think I have nothing left to give.

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| | |
|---|---|
| AACE | American Association of Clinical Endocrinologists |
| AHA | American Heart Association |
| AIN | American Institute of Nutrition |
| ALT | Alanine aminotransferase |
| AMDR | Acceptable Macronutrient Distribution Range |
| ANOVA | Analysis of variance |
| aPKC ζ/λ | Atypical protein kinase C ζ/λ |
| AST | Aspartate aminotransferase |
| AUC | Area under the curve |
| $AUC_g$ | Area under the curve for glucose |
| $AUC_i$ | Area under the curve for insulin |
| $AUC_{index}$ | Glucose-insulin index |
| BCA | Bicinchoninic acid |
| BMI | Body mass index |
| BP | Blood pressure |
| BSA | Bovine serum albumin |
| bwt | Body weight |
| CLA | Conjugated linoleic acid |
| CRP | C-reactive protein |
| $ddH_2O$ | Double distilled water |
| DEXA | Dual-energy x-ray absorptiometry |
| DIO | Diet-induced obesity |
| DRI | Dietary Reference Intake |
| eEF2 | Eukaryotic elongation factor 2 |
| EGIR | European Group for the Study of Insulin Resistance |

| | |
|---|---|
| faNPC-wtm | *fa/fa* Zucker rats weight matched group given normal protein casein diet |
| faHPC | *fa/fa* Zucker rats given high protein casein diet |
| faHPM | *fa/fa* Zucker rats given high protein mixed diet |
| faHPS | *fa/fa* Zucker rats given high protein soy diet |
| faNPC | *fa/fa* Zucker rats given normal protein casein diet |
| FFA | Free fatty acids |
| FPG | Fasting plasma glucose |
| GLP-1 | Glucagon-like-peptide-1 |
| GLUT1 | Glucose-transporter-1 |
| GLUT4 | Glucose-transporter-4 |
| HDL | High-density lipoprotein |
| HOMA-IR | Homeostatic model assessment index for insulin resistance |
| HPC | High protein casein |
| HPM | High protein mixed |
| HPS | High protein soy |
| HRP | Horseradish peroxidase |
| IDF | International Diabetes Federation |
| IFG | Impaired fasting glucose |
| IGT | Impaired glucose tolerance |
| IL-6 | Interleukin-6 |
| INTERMAP | International Collaborative Study of Macronutrients, Micronutrients and Blood Pressure |
| IR | Insulin resistance |
| IRS-1 | Insulin-receptor substrate |
| LBM | Lean body mass |
| LDL | Low-density lipoprotein |

| | |
|---|---|
| lnNPC | lean rats given normal protein casein diet |
| MAPK p44/42 | Mitogen-activated protein kinase p44/42 |
| NAFLD | Non-alcoholic fatty liver disease |
| NCEP ATP III | National Cholesterol Education Program Adult Treatment Panel III |
| NHANES | National Health and Nutrition Examination Survey |
| NHLBI | National Heart, Lung, and Blood Institute |
| NASH | Nonalcoholic steatohepatitis |
| NPC | Normal protein casein |
| OGTT | Oral glucose tolerance testing |
| PAI-1 | Plasminogen activator inhibitor-1 |
| pAkt(Ser473) | Phosphorylated Akt at serine 473 |
| pAkt(Thr308) | Phosphorylated Akt at threonine 308 |
| PBS | Phosphate buffered saline |
| PG | Plasma glucose |
| PI | Phosphatidylinositol |
| PI3K | Phosphatidylinositol 3-kinase |
| pIRS-1(Ser636/639) | Phosphorylated insulin receptor substrate-1 at serine 636/639 |
| PVDF | Polyvinylidene difluoride |
| Rx | Receiving treatment |
| SAS | Statistical analysis software |
| SDS-PAGE | Sodium dodecylsulfate polyacrylamide gel electrophoresis |
| SEM | Standard error of the mean |
| SHHF | Spontaneously hypertensive heart failure |
| SREBP-1 | Sterol-regulatory element binding protein-1 |
| T2DM | Type 2 diabetes mellitus |
| TBHQ | *tert*-Butylhydroquinone |

| | |
|---|---|
| TBST | Tris-buffered saline in Tween-20 |
| TEMED | N, N, N', N' –Tetramethylethylenediamine |
| TG | Triglycerides |
| TMB | 3, 3', 5, 5' - Tetramethylbenzidine |
| TNF-$\alpha$ | Tumour necrosis factor-alpha |
| VPR | Volume pressure recording |
| WHR | Waist:hip ratio |
| WHO | World Health Organization |
| ZDF | Zucker diabetic fatty |

# CHAPTER 1 – LITERATURE REVIEW

## 1.1 Metabolic Syndrome

### Definition

The metabolic syndrome is a collection of risk factors including obesity, hyperglycemia, hypertension, elevated triglyceride levels and reduced high-density lipoprotein (HDL) cholesterol levels which lead to a 2-fold increase in cardiovascular risk, cardiovascular mortality, myocardial infarction and stroke (Mottillo et al., 2010), and a 5-fold increase in risk for type 2 diabetes mellitus (T2DM) (Alberti et al., 2009).

The metabolic syndrome was first described in 1988 (Reaven, 1988). Since then, a number of definitions have been published by different organizations including the World Health Organization (WHO) (Alberti & Zimmet, 1998), the European Group for the Study of Insulin Resistance (EGIR) (Balkau & Charles, 1999), the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III) (Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults, 2001), the American Association of Clinical Endocrinologists (AACE) (Einhorn et al., 2003), the International Diabetes Federation (IDF) (Alberti, Zimmet, & Shaw, 2006), and the American Heart Association (AHA) and the National Heart, Lung, and Blood Institute (NHLBI) (Grundy et al., 2005). Most recently, in 2009, the IDF, AHA, NHLBI, World Heart Federation, International Atherosclerosis Society, and International Association for the Study of Obesity published a joint statement with a harmonized definition for the metabolic syndrome (Alberti et al., 2009).

Among these definitions, there are core components that all groups have identified: obesity, insulin resistance, dyslipidemia and hypertension. These core components have remained the same since the WHO created its definition in 1998 (Alberti & Zimmet, 1998; Gallagher, Leroith, & Karnieli, 2011). However, all definitions differ in their clinical criteria used to identify the metabolic syndrome and the levels set

for each component. A summary of the diagnostic criteria for the metabolic syndrome from the above mentioned groups is provided in Table 1.

While these definitions have been proven to be useful, the emergence of all these definitions has also caused uncertainty as to which definition should be used when identifying patients with the metabolic syndrome. The “best” definition is said to be the one that uses clinical criteria that can be easily and accurately measured in an everyday clinical setting, and can be used most successfully in preventing cardiovascular disease and T2DM (Fulop, Tessier, & Carpentier, 2006). Furthermore, there have been many other additional metabolic criteria that have emerged, such as measuring free fatty acids (FFA) to assess insulin resistance or measuring inflammatory cytokines to assess inflammation. Further research is required to determine the predictive power of these additional factors for cardiovascular disease and diabetes, and to potentially lead to further modification of future metabolic syndrome definitions if necessary (Alberti et al., 2006).

For the purpose of this thesis, the most recent harmonized definition will be used to define the clinical criteria for the metabolic syndrome. The metabolic syndrome parameters included and to be discussed further are: obesity, insulin resistance, dyslipidemia, hypertension, inflammation, and non-alcoholic fatty liver disease (NAFLD).

**TABLE 1** Diagnostic criteria for the metabolic syndrome[1]

| Components | **WHO** (Alberti & Zimmet, 1998) | **EGIR** (Balkau & Charles, 1999) | **NCEP ATP III** (Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults, 2001) | **AACE** (Einhorn et al., 2003) |
|---|---|---|---|---|
| Insulin Resistance | Presence | Presence | - | - |
| IFG (FPG) (mmol/L) | - | ≥ 6.1 (but not T2DM) | ≥ 6.1 | 6.1 – 6.9 |
| IGT (2 h PG) (mmol/L) | ≥ 7.8 or T2DM | - | - | 7.8 – 11.1 |
| Waist (cm) | - | M = > 94<br>W = > 80 | M = > 102<br>W = > 88 | - |
| WHR | M = WHR > 0.9<br>W = WHR > 0.85 | - | - | - |
| BMI (kg/m$^2$) | >30 | - | - | ≥ 25 |
| BP (mmHg) | ≥ 140/90 | ≥ 140/90 or Rx | ≥ 130/85 | ≥ 130/85 |
| TG (mmol/L) | ≥ 1.7 | > 2.0 or Rx | ≥ 1.7 | ≥ 1.7 |
| HDL cholesterol (mmol/L) | M = < 0.9<br>W = < 1.0 | < 1.0 or Rx | M = < 1.03<br>W = < 1.29 | M = < 1.03<br>W = < 1.29 |
| Microalbuminuria | ≥ 30 mg/g albumin:creatinine ratio or ≥ 20 µg/min urinary albumin excretion rate | - | - | - |
| Number of components for diagnosis | T2DM, IGT and/or IR plus ≥ 2 other components | IR plus ≥ 2 other components | ≥ 3 of the components above | Diagnosis depends on clinical judgement based on risk factors/ features of IR |

**TABLE 1 (continued)**

| Components | **IDF** (Alberti et al., 2006) | **AHA/NHLBI** (Grundy et al., 2005) | **Harmonized Definition** (Alberti et al., 2009) |
|---|---|---|---|
| Insulin Resistance | - | - | - |
| IFG (FPG) (mmol/L) | ≥ 5.6 or Rx | ≥ 5.6 or Rx | ≥ 5.6 or Rx |
| IGT (2 h PG) (mmol/L) | - | - | - |
| Waist (cm) | Ethnic specific | M = > 120<br>W = > 88 | Population and country-specific |
| WHR | - | - | - |
| BMI (kg/m$^2$) | - | - | - |
| BP (mmHg) | ≥ 130/85 or Rx | ≥ 130/85 or Rx | ≥ 130/85 or Rx |
| TG (mmol/L) | ≥ 1.7 or Rx | ≥ 1.7 or Rx | ≥ 1.7 or Rx |
| HDL cholesterol (mmol/L) | M = ≤ 1.03 or Rx<br>W = ≤ 1.29 or Rx | M = ≤ 0.9 or Rx<br>W = ≤ 1.1 or Rx | M = < 1.0 or Rx<br>W = < 1.3 or Rx |
| Microalbuminuria | - | - | - |
| Number of components for diagnosis | Waist circumference plus ≥ 2 other components | ≥ 3 of the components above | ≥ 3 of the components above |

[1]**Abbreviations:** AACE = Association of Clinical Endocrinologists; AHA/NHLBI = American Heart Association/National Heart, Lung and Blood Institute; BMI = body mass index; BP = blood pressure; EGIR = European Group for the Study of Insulin Resistance; FPG = fasting plasma glucose; HDL = high-density lipoprotein; IDF = International Diabetes Federation; IFG = impaired fasting glucose; IGT = impaired glucose tolerance; IR = insulin resistance; NCEP ATP III = National Cholesterol Education Program Adult Treatment Panel III; PG = plasma glucose; Rx = receiving treatment; TG = triglycerides; T2DM = type 2 diabetes mellitus; WHR = waist:hip ratio; WHO = World Health Organization

## Prevalence

The prevalence of the metabolic syndrome is on the rise worldwide largely due to the current obesity epidemic (Alberti et al., 2009; Grundy et al., 2004). However, the varying diagnostic criteria, as shown in Table 1, can make it difficult to determine the true prevalence of the metabolic syndrome (Day, 2007; Meigs et al., 2003). For example, using data from the National Health and Nutrition Examination Survey (NHANES) (1992 – 2002), the prevalence of the metabolic syndrome in the United States using the NCEP ATP III criteria is 34%. However, using the IDF diagnostic criteria on the same data results in a prevalence of 39% (Ford, Li, & Zhao, 2010).

Using data from the Canadian Health Measures Survey, the prevalence of the metabolic syndrome in Canada ranges from approximately 18% to 23% depending on the criteria used (Riediger & Clara, 2011). In the same study, age was found to be the strongest predictor of the syndrome (17% of participants 18 – 39 years old had metabolic syndrome vs. 39% of those 70 – 79 years), abdominal obesity was the most common component, and the prevalence of the metabolic syndrome was higher among individuals in households with lower education and income levels (Riediger & Clara, 2011).

## Pathogenesis

The pathogenesis of the metabolic syndrome and each of its core components is complex and not fully elucidated. However, the majority of research focuses on abdominal obesity and insulin resistance as potential causative factors (Alberti et al., 2006).

*Obesity*

Obesity is a worldwide epidemic affecting both children and adults in all areas of the world including low, middle and high income countries (World Health Organization, 2013). It is estimated that approximately 1.4 billion adults worldwide are overweight (body mass index [BMI] of 25.0 – 29.9 kg/$m^2$). Of these, over 500 million are obese (BMI of 30 kg/$m^2$ or greater) (World Health Organization, 2013). In Canada, 25% of the adult population (1 in 4) is obese. When obesity is combined with overweight, the prevalence is 62% (Public Health Agency of Canada, 2011). Lack of physical activity and unhealthy eating habits are recognized as two of the main contributors to the rising prevalence of obesity (Gillingham, Harris-Janz, & Jones, 2011).

Obesity is a complex disorder that results from an imbalance of energy intake and energy expenditure, leading to the accumulation of fat in various adipose tissues and organs (Velasquez & Bhathena, 2007). This accumulation of fat contributes to risk factors such as hyperlipidemia, hypertension and hyperglycemia (Grundy et al., 2004). Furthermore, this excess visceral adipose tissue causes alterations in adipokines including leptin, resistin and adiponectin, which leads to the secretion of inflammatory cytokines such as interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α), as well as plasminogen activator inhibitor-1 (PAI-1) and FFA (Grundy et al., 2004; Potenza & Mechanick, 2009). Secretion of these factors leads to insulin resistance in skeletal muscle and liver, resulting in hyperinsulinemia and exacerbating the risk factors mentioned above. This hyperinsulinemia further contributes to endothelial dysfunction and atherogenesis. The increased FFA also impacts hepatic lipid production by decreasing HDL cholesterol and increasing low-density lipoprotein (LDL) cholesterol and triglycerides, which also enhances insulin resistance (Grundy et al., 2004; Potenza & Mechanick, 2009). These alterations caused by an increase in visceral adipose tissue

clearly illustrate why abdominal obesity is one of the main underlying risk factors for the metabolic syndrome (Grundy et al., 2004; Potenza & Mechanick, 2009).

It is important to note that when assessing a patient for the presence of the metabolic syndrome, obesity is generally identified by measuring waist circumference rather than BMI (Gallagher, Leroith, & Karnieli, 2010). This recognizes the importance of abdominal/visceral obesity in the metabolic syndrome. In the most recent harmonized definition, population and country-specific cutoff values have been provided for waist circumference to diagnose obesity. The cutoff values recommended for Canada are ≥ 102 cm for men and ≥ 88 cm for women (Alberti et al., 2009).

*Insulin Resistance*

Insulin resistance is a significant link among the components of the metabolic syndrome (Gallagher et al., 2011). In fact, the metabolic syndrome has alternatively been known as insulin resistance syndrome (Day, 2007). Insulin resistance is a reduced response by insulin target tissues, mainly skeletal muscle, liver and adipose tissue to the physiologic effects of insulin. Insulin is a hormone produced by pancreatic β-cells that controls the amount of glucose in the blood. Glucose is the main fuel used by humans and is the sole source of energy for the brain. As such, normal glucose homeostasis is tightly controlled (Abdul-Ghani, Tripathy, & DeFronzo, 2006). When insulin resistance is present, blood glucose levels increase and the pancreas secretes more insulin in an attempt to maintain normal blood glucose levels and hyperinsulinemia ensues (Abdul-Ghani & DeFronzo, 2010).

When the WHO published its definition of the metabolic syndrome in 1998, it was stated that insulin resistance should be determined by the hyperinsulinemic euglycemic clamp (Alberti & Zimmet, 1998). However, it was quickly recognized that the determination of insulin resistance with the hyperinsulinemic euglycemic clamp was

nearly impossible in a clinical setting (Fulop et al., 2006). As more recent definitions emerged, the presence of insulin resistance was simply determined by measuring blood glucose levels (fasting or 2 hours post-prandial). The harmonized definition of the metabolic syndrome uses impaired fasting glucose (IFG) as its measurement of insulin resistance and defines IFG of having a fasting glucose concentration ≥ 5.6 mmol/L, or receiving treatment to manage blood glucose levels. In addition, other methods such as measuring blood insulin levels, calculating the homeostatic model assessment index for insulin resistance (HOMA-IR), or conducting oral glucose tolerance testing (OGTT) are widely used in clinical settings and in large population studies (Alberti et al., 2006; Fulop et al., 2006).

To further illustrate what is occurring in insulin resistance, the following sections will explain what is happening under normal glucose handling conditions and impaired glucose handling conditions (i.e. insulin resistance) in the fasting and fed states, as well as what is occurring at the skeletal muscle cell level. Finally, progression to T2DM will also be discussed.

a) Insulin Resistance in Fasting State

In the fasting state (10 – 12 hours overnight fast) under normal glucose handling conditions, plasma insulin levels are low. Since insulin is the main factor that inhibits lipolysis and stimulates glucose uptake by skeletal muscle, the low insulin levels during the fasting state results in low glucose uptake by skeletal muscle (25% of glucose use) and elevated plasma FFA. The majority of total body glucose uptake takes place in insulin-independent tissues including the brain (50% of all glucose use), and liver and gastrointestinal tissues (25% of all glucose use). Therefore, in the fasting state, the brain utilizes glucose as its sole energy source, while FFA are the main energy source in skeletal muscle (Abdul-Ghani & DeFronzo, 2010).

In the fasting state, the rate of glucose uptake by insulin-independent and dependent tissues is matched by the rate of endogenous glucose production, with the majority of glucose production being derived from the liver and a small amount being produced by the kidney. Glycogenolysis (breakdown of glycogen to glucose) and gluconeogenesis (formation of glucose from non-carbohydrate molecules) contribute equally to hepatic glucose production (DeFronzo, 2004). Glucagon, a hormone produced by the α-cells of the pancreas, also plays an important role in normal glucose homeostasis (Cherrington, 1999). In the fasting state, approximately half of total hepatic glucose production is dependent on the maintenance of normal glucagon levels (DeFronzo, 2004).

When insulin resistance is present, the liver is the main site of insulin resistance in the fasting state (Abdul-Ghani et al., 2006; DeFronzo, 2004). Since the liver cells become resistant to the action of insulin, hepatic glucose production increases and glucose production becomes greater than glucose uptake. This occurs overnight, resulting in elevated fasting blood glucose levels in the morning (DeFronzo, 2004). According to the 2013 Canadian Diabetes Association Clinical Practice Guidelines, fasting plasma glucose (no caloric intake for at least 8 hours) between 6.1 – 6.9 mmol/L is classified as IFG – an intermediate state in glucose metabolism that exists between normal glucose tolerance and diabetes – whereas a fasting plasma glucose reading ≥ 7.0 mmol/L is classified as diabetes (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee, Goldenberg, & Punthakee, 2013). An individual with IFG and/or impaired glucose tolerance (IGT) is said to have prediabetes and is at an increased risk for developing diabetes and its complications (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee, Goldenberg et al., 2013).

b) Insulin Resistance in Fed State

Following a meal under normal glucose handling conditions, there is an increase in plasma glucose levels which stimulates insulin secretion from pancreatic β-cells. The combination of hyperglycemia and hyperinsulinemia stimulates glucose uptake by liver, gut and peripheral tissues and also suppresses hepatic glucose production. The majority of glucose uptake by peripheral tissues occurs in skeletal muscle (80 – 85%), while a small amount is taken up by adipocytes (4 – 5%) (DeFronzo, 2004). The resultant hyperinsulinemia following a meal suppresses lipolysis, leading to a decline in plasma FFA and a decrease in the rate of lipid oxidation. This decrease in plasma FFA also enhances muscle glucose uptake and contributes to the suppression of hepatic glucose production (Bays, Mandarino, & DeFronzo, 2004; Bergman, 2000; Boden, 2011; Groop et al., 1989). Finally, the resultant hyperinsulinemia following a meal also inhibits glucagon secretion, causing further suppression of hepatic glucose production (DeFronzo, 2004). As plasma glucose levels decrease, the β-cells reduce insulin output, maintaining plasma glucose levels between 4 – 6 mmol/L (Abdul-Ghani et al., 2006).

Under impaired glucose handling conditions in the fed state, the main characteristics of insulin resistance include decreased insulin-stimulated glucose uptake by skeletal muscle, impaired insulin-mediated suppression of hepatic glucose production in liver, and a decreased ability of insulin to inhibit lipolysis in adipose tissue resulting in an increase in plasma FFA. The resultant increase in FFA further contributes to impaired suppression of hepatic glucose production (Schenk, Saberi, & Olefsky, 2008). As such, blood glucose levels increase and the pancreatic β-cells secrete more insulin to try to maintain normal blood glucose levels and hyperinsulinemia ensues (Abdul-Ghani & DeFronzo, 2010). However, when the pancreas can no longer compensate, glucose homeostasis declines and eventually T2DM can develop (Stumvoll, Goldstein, & van Haeften, 2005). In the fed state, an OGTT is used to screen for IGT or diabetes.

According to the 2013 Canadian Diabetes Association Clinical Practice Guidelines, blood glucose levels between 7.8 – 11.0 mmol/L two hours after a 75 gram dose of glucose is classified as IGT, whereas ≥ 11.1 mmol/L is classified as diabetes (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee, Goldenberg et al., 2013).

c) Insulin Signaling Cascade in Skeletal Muscle

As skeletal muscle is a main site of glucose uptake, it is important to understand what is occurring at the level of skeletal muscle cells. Under normal glucose handling conditions, the cellular events by which insulin instigates its stimulatory effect on glucose metabolism start with the binding of insulin to specific insulin receptors that are present on the muscle cell surface (Abdul-Ghani & DeFronzo, 2010; Pessin & Saltiel, 2000; Saltiel & Kahn, 2001). The insulin receptor is a glycoprotein comprised of two α-subunits and two β-subunits linked by disulfide bonds. The binding of insulin to the insulin receptor α-subunits results in phosphorylation of the β-subunit on multiple tyrosine residues (Abdul-Ghani & DeFronzo, 2010). Following the activation of insulin receptor tyrosine kinase, specific intracellular proteins become phosphorylated (Saltiel & Kahn, 2001). In skeletal muscle, insulin-receptor substrate (IRS)-1 serves as the main docking protein and it undergoes tyrosine phosphorylation following activation of the insulin receptor (Abdul-Ghani & DeFronzo, 2010). Phosphorylation of tyrosine residues on IRS-1 results in the activation of phosphatidylinositol 3-kinase (PI3K) – an enzyme composed of an 85-kDa regulatory subunit and a 110-kDa catalytic subunit – by mediating an association with the 85-kDA regulatory subunit of the enzyme (Abdul-Ghani & DeFronzo, 2010; Pessin & Saltiel, 2000; Saltiel & Kahn, 2001). The 110-kDa subunit catalyzes the 3′ phosphorylation of phosphatidylinositol (PI) 4-phosphate and PI 4,5-disphosphate (Abdul-Ghani & DeFronzo, 2010). Upon activation of PI3K, phosphorylation of thr308

and ser473 leads to activation of protein kinase B/Akt, a serine/threonine kinase involved in many of the metabolic actions of insulin. Protein kinase B/Akt is also responsible for the activation of glycogen synthase, an enzyme involved in the conversion of glucose to glycogen (Abdul-Ghani & DeFronzo, 2010).

Activation of the insulin signal transduction system in skeletal muscle eventually results in the stimulation of glucose transport through a process that involves translocation of a large intracellular pool of glucose transporters to the plasma membrane and their resultant activation after insertion into the cell membrane (Hunger & Garvey, 1998; Shepherd & Kahn, 1999). The insulin-dependent glucose-transporter-4 (GLUT4) is the predominant glucose transporter in skeletal muscle. After exposure to insulin, GLUT4 levels in the plasma membrane increase substantially, and this increase is associated with an equal decrease in the intracellular GLUT4 pool. Glucose-transporter-1 (GLUT1) is the predominant glucose transporter in insulin-independent tissues, but is also found in muscle and does contribute to muscle glucose uptake following a meal (Abdul-Ghani & DeFronzo, 2010). Once inside the cells, glucose is then metabolized by a series of enzymatic steps that are under the control of insulin including glucose phosphorylation (catalyzed by hexokinase II), glycogen synthase which controls glycogen synthesis, and phosphofructokinase and pyruvate dehydrogenase which regulate glycolysis and glucose oxidation, respectively (Abdul-Ghani & DeFronzo, 2010).

Insulin resistance at the skeletal muscle cell level is due to a number of factors. The primary cause appears to be impaired insulin signaling with a defect at the level of IRS-1. It is thought that both the elevated plasma FFA and accumulation of triglycerides in muscle cells causes an increase in serine phosphorylation of IRS-1, thereby negating the ability of IRS-1 to undergo tyrosine phosphorylation by the insulin receptor. Serine phosphorylation of IRS-1 results in impaired PI3K activation. If the PI3K pathway is

impaired, there is a decrease in glucose transport, phosphorylation and metabolism (Abdul-Ghani & DeFronzo, 2010). As such, blood glucose levels increase and the pancreas secretes more insulin to try to maintain normal blood glucose levels and hyperinsulinemia ensues.

d) Progression to T2DM

An individual can progress from normal glucose tolerance to prediabetes to T2DM. However, it is important to note that not all individuals with prediabetes will progress to T2DM. In fact, if appropriate lifestyle changes are taken to manage prediabetes, an individual with either IFG or IGT can revert to normoglycemia (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee, Goldenberg et al., 2013). T2DM is characterized by the presence of hyperglycemia because pancreatic β-cells can no longer produce enough insulin or the body does not effectively use the insulin that is produced (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee, Goldenberg et al., 2013). Depending on the severity of the disease at diagnosis, T2DM may initially be treated with lifestyle interventions (nutritional therapy and physical activity). However, over time, β-cell function deteriorates and blood glucose levels worsen, resulting in the need for antihyperglycemic agents, such as insulin, to maintain normal blood glucose levels (DeFronzo, 2004; Gastaldelli et al., 2004; Saad et al., 1989). There are many factors that lead to the progression of β-cell dysfunction including genetics, insulin resistance, increased insulin secretion demand, glucotoxicity, lipotoxicity, impaired incretin secretion and action, amylin accumulation and decreased β-cell mass (Defronzo & Abdul-Ghani, 2011). The reduction in β-cell mass could be due to increased β-cell apoptosis, or decreased β-cell replication, or a combination of both (Cnop et al., 2005). Regardless, interventions that both preserve β-

cell function and improve insulin resistance have been proven to be the most effective in preventing the progression to T2DM (Defronzo & Abdul-Ghani, 2011).

*Dyslipidemia*

Dyslipidemia is simply an abnormal amount of lipids in the blood including cholesterol, triglycerides, phospholipids and FFA. In the metabolic syndrome, the clinical criteria for dyslipidemia include elevated triglycerides and reduced HDL cholesterol (Grundy et al., 2004). The most recent harmonized definition defines hypertriglyceridemia as ≥ 1.7 mmol/L or receiving treatment to control triglyceride levels, and reduced HDL cholesterol as < 1.0 mmol/L in males and < 1.3 mmol/L in females or receiving treatment (Alberti et al., 2006). Excess visceral adipose tissue and insulin resistance both cause hyperlipidemia due to the presence of elevated FFA which impacts hepatic lipid production by decreasing HDL cholesterol and increasing LDL cholesterol and triglycerides (Grundy et al., 2004; Potenza & Mechanick, 2009). Further analysis of lipoproteins usually reveals other abnormalities such as elevated apolipoprotein B and small LDL particles, both of which have been implicated in the development of atherogenesis (Grundy et al., 2004).

*Hypertension*

Blood pressure is a measure of the pressure or force of blood against artery walls. High blood pressure (i.e. hypertension) is diagnosed when blood pressure is consistently more than 140/90 mmHg. However, individuals with diabetes or kidney disease are at a greater risk of developing chronic diseases associated with hypertension; therefore, high blood pressure for these individuals is diagnosed when blood pressure is more than 130/80 mmHg (Heart and Stroke Foundation, 2011). The most recent harmonized definition of the metabolic syndrome defines hypertension as a

blood pressure value ≥ 130/85 mmHg, or receiving treatment for hypertension (Alberti et al., 2009). Hypertension is common among individuals with the metabolic syndrome due to its strong association with obesity and insulin resistance (Grundy et al., 2004).

*Inflammation*

Inflammation is commonly present in the metabolic syndrome; however, it is currently not part of the clinical criteria (Alberti et al., 2006). Clinically, inflammation in the metabolic syndrome would be recognized by elevated C-reactive protein (CRP) levels (Grundy et al., 2004). There is a significant positive relationship between plasma CRP levels and measures of obesity and insulin resistance (Alberti et al., 2006; Lemieux et al., 2001). In addition, a linear increase in plasma CRP levels has been found with the increasing number of components of the metabolic syndrome (Festa et al., 2000; Hak et al., 1999; Santos, Lopes, Guimaraes, & Barros, 2005). As previously mentioned, inflammation is present in the metabolic syndrome due to the secretion of inflammatory cytokines that occurs as a result of excess visceral adipose tissue. Secretion of these factors further contribute to insulin resistance, endothelial dysfunction and atherogenesis (Grundy et al., 2004; Potenza & Mechanick, 2009).

*Non-Alcoholic Fatty Liver Disease*

While NAFLD is currently not part of the clinical criteria used to diagnose the metabolic syndrome, it has been suggested that NAFLD should be included as another feature of the metabolic syndrome and it is often referred to as the hepatic manifestation of the metabolic syndrome (Brunt, 2010; Marchesini et al., 2003). Hepatic steatosis, the accumulation of fat in liver cells, is one of the first stages of NAFLD which can progress to cirrhosis and end-stage liver disease (Liu, Bengmark, & Qu, 2010). Furthermore, the presence of diabetes, obesity, dyslipidemia and hypertension can further contribute to

severe liver disease (Marchesini et al., 2003). It is estimated that the prevalence of NAFLD in the obese population is as high as 95% (Basaranoglu et al., 2010), with the criteria for diagnosis of NAFLD being the presence of hepatic fat accumulation in hepatocytes that is greater than 5% of liver weight (Liu et al., 2010).

While an association between obesity, insulin resistance and NAFLD is well established, it is unknown whether insulin resistance causes the accumulation of fat in liver cells, or whether the accumulation of fat itself causes the development of insulin resistance (Postic & Girard, 2008). Increased delivery of FFA via the portal vein directly to the liver, inadequate fatty acid oxidation, and increased *de novo* lipogenesis also seem to play a role (Bugianesi, Moscatiello, Ciaravella, & Marchesini, 2010). Furthermore, the secretion of pro-inflammatory cytokines associated with insulin resistance may further contribute to liver damage (Garg & Misra, 2002). Clinically, high serum levels of liver enzymes, mainly alanine and aspartate aminotransferases (ALT and AST) are common markers of NAFLD (Bugianesi et al., 2010). Data from the NHANES III study demonstrated a significant association between increased ALT levels and insulin resistance, T2DM and the metabolic syndrome (Liangpunsakul & Chalasani, 2005).

### 1.2 Management of Metabolic Syndrome

There is full agreement that healthy lifestyle changes focused primarily on weight loss (5 – 10% of body weight), followed by weight maintenance, are the first-line approach in managing the metabolic syndrome in order to reduce the risk of cardiovascular disease and T2DM (Muzio, Mondazzi, Harris, Sommariva, & Branchi, 2007). The Diabetes Prevention Program demonstrated that healthy lifestyle changes (diet and physical activity to achieve 7% weight loss) reduced the incidence of the metabolic syndrome by 41% compared with placebo (Orchard et al., 2005). However, the optimal diet to achieve this remains unclear (Muzio et al., 2007). The conventional method supports an energy-restricted, low fat, high carbohydrate diet containing < 35% energy from fat, 45 – 65% energy from carbohydrate and approximately 15% energy from protein (Wycherley, Moran, Clifton, Noakes, & Brinkworth, 2012). However, dietary strategies of this nature are associated with only modest weight loss and poor long-term compliance (Brinkworth et al., 2004). As such, this conventional method has been repeatedly challenged over the years by supporters of other diets (Te Morenga, Levers, Williams, Brown, & Mann, 2011).

One diet that has been gaining attention from both researchers as well as the general public is a diet high in protein. A high protein intake is considered in the range of 25 – 35% of energy as protein, compared to normal protein intake which is considered to be 12 – 18% of energy as protein (Clifton & Keogh, 2007). Advocators claim that a high protein diet has more favorable effects on weight loss and improves an array of cardiovascular disease risk factors, including glucose homeostasis (Te Morenga & Mann, 2012; Wycherley et al., 2012). Furthermore, the 2006 Canadian Clinical Practice Guidelines on the Management and Prevention of Obesity in Adults and Children (Lau et al., 2007), and health organizations such as the American Diabetes Association (American Diabetes Association et al., 2008) are now starting to state that high protein

diets are an appropriate strategy for weight management. Finally, the Institute of Medicine established new Dietary Reference Intake (DRI) recommendations which gave an Acceptable Macronutrient Distribution Range (AMDR) for protein of 10 – 35% of energy (Institute of Medicine, 2005), giving the impression that a higher level of protein in the diet is acceptable.

However, results pertaining to the effects of high protein diets on metabolic syndrome parameters are varied and not all studies have reported beneficial outcomes (Santesso et al., 2012; Te Morenga & Mann, 2012). For instance, in a recent systematic review of long-term randomized controlled trials, high protein diets exerted no specific beneficial effects on outcomes of obesity, cardiovascular disease or glycemic control (Schwingshackl & Hoffmann, 2013). In addition, the 2013 Canadian Diabetes Association Clinical Practice Guidelines stated that the usual intake of 15 – 20% of energy from protein does not need to be modified for people with diabetes (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee et al., 2013). Some studies have actually demonstrated detrimental effects of high protein intake on insulin resistance (Linn et al., 2000; Tremblay, Lavigne, Jacques, & Marette, 2007; Weickert et al., 2011). Different sources of protein also appear to have differing effects on health outcomes (Gannon, Nuttall, Neil, & Westphal, 1988; Westphal, Gannon, & Nuttall, 1990). For example, some studies have reported positive effects of soy protein, complete dairy protein and whey protein on weight loss, glucose homeostasis and hepatic steatosis (Ascencio et al., 2004; Davis et al., 2005; Eller & Reimer, 2010; Mezei et al., 2003; Tovar et al., 2005), while others have reported no effects (Pal, Ellis, & Dhaliwal, 2010; Yamashita, Sasahara, Pomeroy, Collier, & Nestel, 1998). Potential adverse effects of high protein intake on kidney and bone health have also lead to concerns regarding the safety of high protein diets (Jia et al., 2010; Johnstone, 2012; Wakefield, House, Ogborn, Weiler, & Aukema, 2011). Therefore, more research is necessary before health

care professionals can make concrete recommendations regarding the best way to manage obesity and other metabolic syndrome parameters.

To further illustrate the research in the area of high protein diets on metabolic syndrome parameters, the following section will provide a review of human and animal studies that have examined the effects of high protein intake on the metabolic syndrome parameters included in this thesis (obesity, insulin resistance, dyslipidemia, hypertension, inflammation and NAFLD), the role of different sources of protein, as well as possible mechanisms.

## Effects of High Protein Intake on Metabolic Syndrome Parameters

### *High Protein Intake and Obesity*

It is thought that the positive effects seen on metabolic syndrome parameters are mainly related to the reduction in body weight associated with high protein intake (Westerterp-Plantenga, Lemmens, & Westerterp, 2012). In the first weight loss study that was said to compare high protein diets with other diets in the most comprehensive manner, 65 healthy, overweight and obese adults were randomly assigned to a high carbohydrate group (12% energy as protein, 58% as carbohydrate), a high protein group (25% energy as protein, 45% as carbohydrate), or a control group. Total fat in the diets was 30% of energy. Protein sources were primarily dairy products and lean meats. After six months, weight loss was 5.1 kg in the high carbohydrate group and 8.9 kg in the high protein group and fat loss was 4.3 kg and 7.6 kg, respectively. The researchers contributed the greater weight and fat loss to the lower energy intake seen in subjects in the high protein group, perhaps as a result of the satiating effects of high protein intake. The high protein diet also significantly decreased fasting plasma triglycerides and FFA (Skov, Toubro, Ronn, Holm, & Astrup, 1999).

An important aspect of the study mentioned above was that diets were provided *ad libitum* as opposed to energy-restricted (Skov et al., 1999). It has been proposed that *ad libitum* diets are more likely to detect unique properties of protein in facilitating weight loss compared to energy-restricted diets, since strict control of energy will achieve clinically important weight loss that will conceal any relevant metabolic effects of protein (Te Morenga & Mann, 2012). Several other randomized intervention trials using *ad libitum* diets with high protein content (25 – 30% of energy) have also demonstrated weight and fat loss to a significantly greater extent than high carbohydrate diets with protein content around 15% of energy (Claessens, van Baak, Monsheimer, & Saris, 2009; Due, Toubro, Skov, & Astrup, 2004; McAuley et al., 2005; McAuley et al., 2006; Samaha et al., 2003). The proposed reason for this is that high protein diets have a more satiating effect compared to high carbohydrate diets. As such, under *ad libitum* conditions, subjects tend to have reduced energy intake when consuming high protein diets (Weigle et al., 2005).

When comparing energy-restricted diets, the evidence related to weight loss is not as convincing and often, weight loss from the high protein diets is similar to the weight loss seen from the high carbohydrate diets (Te Morenga & Mann, 2012). For instance, four studies comparing energy-restricted, high protein diets to high carbohydrate diets found that weight loss was the same between both dietary interventions (Brinkworth et al., 2004; Farnsworth et al., 2003; Layman et al., 2003; Noakes, Keogh, Foster, & Clifton, 2005). Conversely, two other studies demonstrated that while both diet groups lost weight, subjects in the high protein diet groups lost significantly more weight compared to subjects in the high carbohydrate diet groups (Flechtner-Mors, Boehm, Wittmann, Thoma, & Ditschuneit, 2010; Te Morenga et al., 2011). In addition, a recent meta-analysis of randomized controlled trials that compared energy-restricted, isocaloric, high protein, low fat diets with standard protein, low fat

diets, found that high protein diets provided more favorable changes on weight loss and body composition by increasing loss of fat mass while mitigating the reduction in fat-free mass compared to standard protein diets (Wycherley et al., 2012). The ability of high protein diets to reduce fat mass while preserving lean body mass (LBM) is particularly relevant in regards to the metabolic syndrome (Claessens et al., 2009; Farnsworth et al., 2003; Skov et al., 1999). For instance, the preservation of LBM during weight loss may enhance insulin sensitivity (Baba et al., 1999; Piatti et al., 1994).

There are several theories as to why high protein intake can assist with weight loss. The first explanation is regarding the high thermic effect of protein. The thermic effect of a food is the energy required for digestion, absorption and disposal of consumed nutrients. The thermic effect of protein is estimated to be between 20 – 25% of energy consumed (Halton & Hu, 2004), while the thermic effect of both carbohydrate and fat is estimated to be between 5 – 15% (Nair, Halliday, & Garrow, 1983; Westerterp, Wilson, & Rolland, 1999). This heightened thermal effect of protein helps promote a negative energy balance by increasing energy expenditure (Tremblay et al., 2007).

As previously mentioned, consumption of foods high in protein is also known to increase satiety. In a current review that looked at 14 studies that compared higher protein meals to either higher carbohydrate or higher fat meals, 11 of the studies showed that the protein preload significantly increased subjective ratings of satiety (Halton & Hu, 2004). Satiety may also help explain why body weight loss is greater when high protein diets are given *ad libitum,* as under *ad libitum* conditions subjects tend to eat less (Westerterp-Plantenga et al., 2012).

The type of protein consumed also appears to have an influence on weight loss. In one study, 100 obese subjects were randomized to either a soy-based meal replacement treatment group (240 g/day, 1200 kcal/day) or the control group for 12 weeks. Subjects consuming the soy-based meal replacement formula lost more weight

(7.0 kg vs. 2.9 kg) and had significantly greater reductions in body fat mass (Allison et al., 2003). Similarly, in another study, Wistar fatty rats and their lean counterparts were given either casein or soy protein isolate diet containing hydrogenated fat (4% hydrogenated fat plus 1% corn oil) or corn oil (5%) for 3 weeks. The rats given soy protein had lower body weight compared to those given casein (Iritani, Hosomi, Fukuda, Tada, & Ikeda, 1996). There are several theories as to why soy protein exerts beneficial effects on obesity including its favorable effects on lipid absorption, insulin resistance, fatty acid metabolism and other hormonal, cellular or molecular changes related to adiposity (Velasquez & Bhathena, 2007). However, results on the effects of soy have been varied. For example, in one study, subjects were given either a weight-loss diet based on meat (150 g/day) or a weight-loss diet based on soy protein (130 g/day) with protein providing 25% of energy. However, no differences were seen between these diets over a 16 week period in weight loss, lipids, blood pressure, insulin or arterial compliance (Yamashita et al., 1998).

Studies such as one based on the NHANES (Beydoun et al., 2008) and the Coronary Artery Risk Development in Young Adults (Pereira et al., 2002) have proposed that higher dairy intake also reduces the risk of obesity. It is thought that specific components of dairy, such as casein (approximately 80% of total protein in milk) or whey proteins (approximately 20% of total protein in milk), may be responsible for the beneficial effects of dairy on metabolic risk factors due to their high concentrations of branched-chain amino acids and their superior effect on appetite control compared to other proteins (Anderson, Tecimer, Shah, & Zafar, 2004; Bowen, Noakes, & Clifton, 2005; Hall, Millward, Long, & Morgan, 2003; Pal et al., 2010). However, studies examining dairy proteins on weight loss do not report consistent findings. For example, a recent study that examined the effects of whey protein supplementation in comparison to casein and glucose (control) supplementation in overweight and obese subjects for 12

weeks found no significant change in body weight, BMI, waist circumference, total body fat, or LBM among groups. Interestingly, despite no differences in body composition, the whey group did have improved fasting lipids and insulin levels at the end of the study (Pal et al., 2010).

*High Protein Intake and Insulin Resistance*

Research pertaining to the effects of high protein diets on insulin resistance is controversial, as both positive and negative effects have been demonstrated. The majority of studies that have demonstrated positive effects support the theory that improvements in insulin resistance are related to a reduction in weight loss and/or fat mass while sparing LBM, rather than the high protein diet itself (Farnsworth et al., 2003). Studies have also shown similar results for both high protein and high carbohydrate diets when weight loss has been achieved.

For instance, in one study, obese women were given either a high protein diet (34% energy as protein, 46% as carbohydrate) or a high carbohydrate diet (17% energy as protein, 64% as carbohydrate) and the results indicated that both groups had reduced fasting glucose and insulin concentrations and that these reductions were attributed to the decrease in body weight of both groups (Noakes et al., 2005). Similarly, another study also illustrated that a high protein diet (30% energy as protein, 40% as carbohydrate) provides similar results compared to a high carbohydrate diet (15% energy as protein, 55% as carbohydrate) as both diets produced weight loss and improved insulin resistance in obese, hyperinsulinemic men and women and these improvements were not significantly different between the two groups (Brinkworth et al., 2004). Finally, in a more recent study that compared high protein to high carbohydrate diets, researchers found that BMI, fat mass, fasting insulin levels and HOMA-IR decreased similarly between both groups, and that the reductions in body weight were

responsible for the improvements in the insulin resistance measurements (Soenen, Martens, Hochstenbach-Waelen, Lemmens, & Westerterp-Plantenga, 2013).

Only a limited number of studies have been able to demonstrate improvements in insulin resistance due to the actual high protein diet and not attributed to any corresponding weight loss. For example, the effects of high protein and high carbohydrate diets on weight loss and insulin sensitivity were examined in obese women. After three weeks, insulin sensitivity, as measured with the euglycemic clamp, was maintained in the high protein diet group but had decreased by 30% in the high carbohydrate diet group. There were no significant differences in weight loss among the two groups, and the improvements in insulin sensitivity were attributed to a greater retention of LBM in the high protein group (Piatti et al., 1994).

One possible reason why high protein intake has favorable effects on insulin resistance is due to a lower glycemic load because of a reduced carbohydrate intake (Layman et al., 2003; Layman, Shiue, Sather, Erickson, & Baum, 2003; Noakes et al., 2005). As previously mentioned, another explanation is that a higher protein intake during weight loss may also prevent the inevitable loss of LBM and thus may lead to improved insulin sensitivity (Baba et al., 1999; Piatti et al., 1994). High protein diets also supply increased amounts of branched-chain amino acids, particularly leucine. Research has shown leucine to improve glycemic control due to its unique regulatory actions on muscle protein synthesis, modulation of the insulin signaling pathway, and sparing of glucose use by stimulation of the glucose-alanine cycle (Layman & Baum, 2004).

The type of protein consumed also appears to have differing effects on insulin resistance. For instance, Wistar rats were given a high fat diet for nine weeks to induce obesity, and then switched to one of four diet groups for six weeks: a) low protein, whey diet (8% energy as protein); b) low protein, red meat diet (8% energy as protein); c) high

protein, whey diet (32% energy as protein); and d) high protein, red meat diet (32% energy as protein). Diets were isoenergetic, contained equal amounts of fat, and were given *ad libitum*. Both high protein groups had reduced energy intake, and visceral and subcutaneous fat compared to the low protein groups. However, rats given the whey protein (both 8% and 32%) had lower plasma insulin levels and a reduced insulin:glucose ratio compared to both red meat groups. This led to the conclusion that while high protein diets reduced energy intake and adiposity, whey protein was more effective than red meat for increasing insulin sensitivity (Belobrajdic, McIntosh, & Owens, 2004).

As previously mentioned, whey protein is a component of dairy and there have been several studies that have demonstrated improvements in insulin sensitivity with dairy consumption. For instance, the prospective Coronary Artery Risk Developing in Young Adults study found a 21% reduction in risk of insulin resistance in overweight individuals with each daily serving of low-fat dairy food (Pereira et al., 2002). In another study, complete dairy protein was shown to improve body composition and insulin sensitivity in a diet-induced obesity (DIO) rat model (Eller & Reimer, 2010).

Studies have also demonstrated that soy protein positively impacts glucose homeostasis. For example, male lean spontaneously hypertensive heart failure (SHHF +/cp) rats, a unique rat model that exhibits the early features of the metabolic syndrome, were given casein or soy protein for 36 weeks. Rats given soy protein had significantly lower body weight, liver weight, fasting blood glucose, and plasma insulin concentrations compared to rats given casein (Davis et al., 2005). Another study also demonstrated that consumption of a high-isoflavone soy protein diet improved glucose tolerance, insulin resistance, and hepatic cholesterol and triglyceride levels in obese Zucker rats (Mezei et al., 2003).

However, as mentioned above, results pertaining to the effects of high protein diets are not conclusive as some studies have actually demonstrated negative effects. In one study, consumption of a high protein diet for 6 months by healthy, non-obese subjects resulted in a higher glucose-stimulated insulin secretion, increased fasting glucose concentrations, impaired suppression of hepatic glucose output by insulin, and enhanced gluconeogenesis (Linn et al., 2000). Another study also reported that a high protein diet, compared to a diet high in cereal fiber, had detrimental effects on insulin sensitivity which may have been due to increased protein expression in adipose tissue of the translation initiation factor serine-kinase-6-1, a protein shown to mediate amino acid-induced insulin resistance (Weickert et al., 2011). In a large prospective cohort with a 10 year follow-up, consuming 5% of energy from both animal and total protein at the expense of carbohydrates or fat increased diabetes risk by 30% (Sluijs et al., 2010). In a review, it was concluded that high protein intake has detrimental effects on glucose homeostasis by promoting insulin resistance and increasing gluconeogenesis (Tremblay et al., 2007). Finally, studies have also reported a rapid onset of insulin resistance in humans exposed to amino acid infusions (Krebs et al., 2002; Tremblay et al., 2005). As such, research reporting these negative effects have made it difficult for both researchers and health care professionals to make concrete conclusions regarding the effects of high protein diets on insulin resistance.

a) High Protein Intake at the Skeletal Muscle Cell Level

As skeletal muscle is a main site of insulin-stimulated glucose uptake, it is important to examine what is occurring at the skeletal muscle cell level during high protein intake. However, research examining the effects of high protein diets on components of the insulin signaling pathway (for example, protein levels of insulin receptor, IRS-1, PI3K and Akt) in skeletal muscle is limited.

In one study, rats were given a standard chow diet or a high fat diet in which the protein source was casein, soy or cod (15% energy from protein in all diets) for 4 weeks. The dietary cod protein prevented obesity-linked muscle insulin resistance by normalizing insulin activation of the PI3K/Akt pathway and by improving GLUT4 translocation to the T-tubules (Tremblay, Lavigne, Jacques, & Marette, 2003). In another study, healthy subjects were given either a lower protein (12% protein) or higher protein (18% protein) diet and performed resistance training 3 times per week for 12 weeks. At baseline and at the end of the study, a muscle biopsy sample was obtained in the fasting state and Western blotting was used to analyze changes in insulin receptor, IRS-1, Akt and atypical protein kinase C ζ/λ (aPKC ζ/λ). Skeletal muscle insulin receptor, IRS-1, and Akt were unchanged whereas the amount of aPKC ζ/λ increased with resistance training in both diet groups (Iglay, Thyfault, Apolzan, & Campbell, 2007). Resistance training also improved oral glucose tolerance as evident by decreased area under the curve (AUC) for glucose in both groups; however, this was not significantly different between the two groups. Finally, infusion of amino acids in humans under physiological hyperinsulinemia increased the phosphorylation of IRS-1 on Ser312 and Ser636/639 and completely blunted the activation of PI3K in skeletal muscle (Tremblay et al., 2005). As previously mentioned, the primary cause of insulin resistance appears to be due to an increase in serine phosphorylation of IRS-1 which negates the ability of IRS-1 to undergo tyrosine phosphorylation by the insulin receptor. The increased serine phosphorylation of IRS-1 results in impaired PI3K activation (Abdul-Ghani & DeFronzo, 2010).

*High Protein Intake and Dyslipidemia*

Only a few studies have shown that high protein diets improve blood lipids independent of weight loss (Wolfe & Giovannetti, 1991). Rather, the majority of studies

have shown improvements in blood lipids in conjunction with weight loss, making it difficult to determine specific effects of high protein diets on blood lipids (Layman, Clifton, Gannon, Krauss, & Nuttall, 2008). In addition, researchers have difficulty concluding whether it is the increase in protein or a reduction in carbohydrates that produces the favorable effects, as it is well established that dietary carbohydrates, particularly simple sugars, promote atherogenic dyslipidemia (Grundy, 1997; Layman et al., 2008).

When comparing high protein diets to high carbohydrate diets, some studies have shown no differences between the two on blood lipids, while others have shown high protein diets to be superior. For example, overweight and obese females had reductions in total cholesterol, LDL cholesterol and triglycerides when consuming either a high protein diet (30% energy as protein, 40% as carbohydrate) or a high carbohydrate diet (20% energy as protein, 50% as carbohydrate) over eight weeks. These reductions were attributed to weight loss and there was no significant effect of diet (Te Morenga et al., 2011). Conversely, three other human studies all demonstrated that high protein diets produced statistically greater reductions in triglyceride levels compared to high carbohydrate diets even with weight loss being similar between both diet groups (Farnsworth et al., 2003; Layman et al., 2003; Noakes et al., 2005). In fact, the most consistent effect of high protein, weight loss diets on blood lipids appears to be a reduction in triglyceride levels (Layman et al., 2008).

The type of protein consumed also appears to play a role in improving blood lipids. For example, in a meta-analysis of 38 controlled clinical trials, soy protein intake was effective in reducing total cholesterol by 9.3%, LDL cholesterol by 12.9% and triglycerides by 10.5%, and in increasing HDL cholesterol by 2.4% among adults and children with or without hypercholesterolemia (Anderson, Johnstone, & Cook-Newell, 1995). In another more recent meta-analysis of 41 prospective, randomized controlled

trials, an overall benefit of soy preparations on lipid profiles was demonstrated including reductions in total cholesterol, LDL cholesterol and triglycerides as well as increases in HDL cholesterol among adults with or without hypercholesterolemia (Reynolds et al., 2006). However, the authors concluded that replacing saturated fat with soy protein contributed to the positive effects on lipid profile, leaving the question as to whether it is soy protein that affects the lipid profile, limiting saturated fat, or a combination of both (Potenza & Mechanick, 2009). There is also controversy over whether it is soy protein or the isoflavone content of soy that has the lipid lowering effects. In a meta-analysis that attempted to clarify the importance of isoflavones compared to soy protein, 11 studies that used matched amounts of isoflavone-enriched soy and isoflavone-depleted soy protein found that the beneficial effect on lipids was lost without isoflavones (Taku et al., 2007).

*High Protein Intake and Hypertension*

Studies regarding the effects of high protein diets on hypertension have shown conflicting results. For instance, in the OmniHeart study diet (Appel et al., 2005), compared with the high carbohydrate diet (15% energy as protein, 58% as carbohydrate), the high protein diet (25% energy as protein, 48% as carbohydrate) further decreased mean systolic blood pressure by 1.4 mm Hg among normal individuals and by 3.5 mm Hg among those with hypertension. In another study, diastolic blood pressure decreased more in overweight and obese females given a high protein diet (30% energy as protein, 40% as carbohydrate) compared to those given a high carbohydrate diet (20% energy as protein, 50% as carbohydrate), with the improvements likely due to greater reductions in body weight and total body fat in the high protein diet group (Te Morenga et al., 2011). However, in two recently conducted meta-analyses comparing low protein to high protein diets, no significant differences were observed for

diastolic or systolic blood pressure (Schwingshackl & Hoffmann, 2013; Wycherley et al., 2012).

The type of protein may play a role in the effects of protein on hypertension. In a recent study that compared the effects of soy protein, milk protein and carbohydrate supplementation on blood pressure among adults with prehypertension or stage 1 hypertension, both soy and milk protein intake reduced systolic blood pressure compared with the carbohydrate-supplemented diet (He et al., 2011). In the INTERMAP Study (International Collaborative Study of Macronutrients, Micronutrients and Blood Pressure) a significant inverse association between total vegetable protein intake and blood pressure was found, but there was no significant association between total animal protein and blood pressure (Elliott et al., 2006).

*High Protein Intake and Inflammation*

As previously mentioned, inflammation in the metabolic syndrome would be recognized by elevated CRP levels in a clinical setting (Grundy et al., 2004). Studies have shown CRP levels to improve following weight loss, independent of dietary composition (Azadbakht, Izadi, Surkan, & Esmaillzadeh, 2013). For example, two studies reported similar reductions in CRP levels in subjects consuming high protein and high carbohydrate diets, with the decreased levels attributed to weight loss (Clifton, Keogh, & Noakes, 2008; Noakes et al., 2005). Conversely, there were differences in CRP levels in a recent study when obese, premenopausal women without diabetes received either a high protein diet (30% energy as protein, 40% as carbohydrate) or high carbohydrate diet (15% energy as protein, 55% as carbohydrate) for six months. Both groups lost weight, but there was no difference in weight loss or BMI between the two groups at the end of the study. However, the high protein diet group had significantly decreased CRP levels compared to the high carbohydrate diet at the end of the study,

suggesting that the high protein intake was superior to the high carbohydrate diet at improving CRP levels (Kitabchi et al., 2013).

Since altering the source of protein has been shown to produce varying effects on other metabolic syndrome parameters, studies have tried to determine whether different sources of protein also have varying effects on CRP levels. For instance, a recent study evaluated the effects of whey protein supplementation compared to casein and glucose (control) supplementation on inflammatory markers in overweight individuals for 12 weeks. However, at the end of the study there were no significant changes in circulating IL-6, TNF-$\alpha$, or CRP levels (Pal & Ellis, 2010). In another study, the effects of soybean protein, milk protein and complex carbohydrate (control) supplementation on inflammatory biomarkers were examined. However, no significant changes in circulating IL-6, TNF-$\alpha$, or CRP levels were observed among groups (Rebholz et al., 2013). It is important to note that the two studies mentioned above were not weight loss studies and hence no weight loss was observed. Both studies also used supplements rather than whole foods and the diets were also not high protein diets. These factors may explain why no improvements in inflammatory markers were seen in these studies.

*High Protein Intake and Non-Alcoholic Fatty Liver Disease*

As previously mentioned, NAFLD is the hepatic manifestation of the metabolic syndrome (Brunt, 2010; Marchesini et al., 2003). While the study of high protein intake on NAFLD is relatively new, the majority of studies thus far have shown favorable effects of high protein diets on the liver. For instance, a high protein diet (35% energy as protein) prevented and reversed hepatic steatosis, the first stage of NAFLD, in male C57Bl/6 mice independently of fat and carbohydrate intake and more efficiently than a

20% reduction in energy intake (Garcia-Caraballo et al., 2013). In a human study, a high protein intake (25% energy as protein) significantly prevented intrahepatic fat deposition induced by a hypercaloric, high fat diet (Bortolotti et al., 2009).

As with the other metabolic syndrome parameters, the source of protein appears to play a role in the effects of high protein intake on NAFLD. In animal studies, a soy protein diet ameliorated fatty liver and hepatic cholesterol and triglyceride levels to a greater extent compared to a casein diet in Zucker diabetic fatty (ZDF) rats (Tovar et al., 2005). In another study, a high fat diet containing soy as the protein source prevented the development of fatty liver in Wistar rats, while rats given a high fat diet containing casein as the protein source developed fatty liver (Ascencio et al., 2004). In a human study, the effects of whey protein supplementation on intrahepatocellular lipids and blood lipids in obese, nondiabetic women were examined. After four weeks, whey protein supplementation significantly decreased intrahepatocellular lipids, fasting triglycerides and total cholesterol. However, no changes were observed for visceral fat, total liver volume, glucose tolerance or insulin sensitivity (Bortolotti et al., 2011).

**Additional Potential Adverse Health Effects of High Protein Intake**

Possible adverse effects of high protein diets have focused mainly on bone health and kidney function; however, results have been inconclusive (Clifton & Keogh, 2007). In regards to kidney function, reported adverse effects of high protein diets include glomerular hyperfiltration and hyperemia, acceleration of chronic kidney disease, increased proteinuria and increased risk for nephrolithiasis (Friedman, 2004). It appears that high protein diets cause these detrimental effects mainly in individuals with chronic kidney disease or other susceptible groups such as those with diabetes (Calvez, Poupin, Chesneau, Lassale, & Tome, 2012; Johnstone, 2012). Obese individuals may also be susceptible as there is a high prevalence of glomerular hyperfiltration in obese

individuals (Bosma, Krikken, Homan van der Heide, de Jong, & Navis, 2006; Friedman et al., 2010) which may increase their susceptibility to diet-associated hyperfiltration-related kidney damage (Friedman et al., 2012). As such, obese individuals with the metabolic syndrome who are consuming high protein weight loss diets need to be aware of any potential risks and discuss these with their healthcare practitioner (Friedman, 2004).

Conversely, there are currently no clear kidney-related contraindications for high protein diets in individuals with healthy kidney function (Calvez et al., 2012; Friedman, 2004). In two recently conducted meta-analyses that compared the effects of high protein diets to low protein diets on health outcomes, no significant effects on markers for kidney health were observed (Santesso et al., 2012; Schwingshackl & Hoffmann, 2013). In addition, in another long-term study that examined the effects of a high protein weight loss diet over two years, no harmful effects on glomerular filtration rate, albuminuria, or fluid and electrolyte balance were observed in healthy, obese subjects (Friedman et al., 2012).

It has even been suggested that in individuals with healthy kidney function, increasing dietary protein intake may cause adaptive alterations in kidney size and function without adverse effects, and that these alterations are a normal adaptive mechanism (Hoy, Hughson, Bertram, Douglas-Denton, & Amann, 2005; Martin, Armstrong, & Rodriguez, 2005; Soenen et al., 2013). However, a major limitation of these human studies is that they do not allow for histological analysis on the kidney which is an important assessment when examining changes in kidney health. For example, in an animal study conducted in our laboratory, pigs who received a high protein diet (35% energy as protein) had enlarged kidneys at both 4 and 8 months compared to pigs who received a normal protein diet (15% energy as protein). Histological analysis showed that the enlarged kidneys in the high protein diet group had

55% more fibrosis and 30% more glomerulosclerosis compared to those in the normal protein diet group. The high protein diet group also had higher renal and glomerular volumes, renal monocyte chemoattractant protein-1 levels and plasma homocysteine levels compared to the normal protein diet group (Jia et al., 2010). In a follow-up study, rats who received a high protein diet (35% energy as protein) for 17 months had higher kidney weights, proteinuria and creatinine clearance, larger glomeruli and more glomerulosclerosis compared with rats who received a normal protein diet (15% energy as protein) (Wakefield et al., 2011). Therefore, both of these suggest that an increase in kidney size due to long-term high protein intake may be indicative of renal damage. As such, additional long-term studies on the effects of high protein diets on kidney function are required before making conclusive statements on this issue.

In regards to bone health, it has been argued that high protein diets can have a negative effect on bone health via increased calciuria (Johnstone, 2012). The reason for this is because a high protein diet creates a higher acid load which cannot be neutralized by the kidneys. To compensate, the body pulls calcium from bones to balance the pH, and calcium is then excreted in the urine (Johnstone, 2012). However, few studies support this theory (Calvez et al., 2012; Johnstone, 2012; Pye et al., 2009). In fact, some studies have actually shown a positive effect of high protein diets on bone health via promotion of bone growth and retardation of bone loss. For example, a recent meta-analysis of randomized controlled trials indicated a positive effect of protein supplementation on lumbar spine bone mineral density (Darling, Millward, Torgerson, Hewitt, & Lanham-New, 2009). In another study, a high protein diet reduced the risk of fractures in post-menopausal women (Munger, Cerhan, & Chiu, 1999). In a study conducted in our laboratory, a high protein diet (35% energy as protein) lowered body fat content without hindering the mechanical and weight-bearing properties of bone in adult female Sprague-Dawley rats (Pye et al., 2009). In the Framingham Osteoporosis Study

(Hannan et al., 2000), individuals in the highest quartile of protein intake had the smallest loss of bone mineral density over four years. The only clinical data that appears to support the hypothesis of a detrimental effect of high protein diets on bone health is in the context of inadequate calcium supply (Calvez et al., 2012). Other than that, the majority of research has concluded that high protein diets do not lead to calcium bone loss and thus do not cause adverse effects on bone health (Calvez et al., 2012; Johnstone, 2012).

### 1.3 Obese (*fa/fa)* Zucker Rat Model

Obese (*fa/fa*) Zucker rats are the most widely used animal model of genetic obesity (Aleixandre de Artinano & Miguel Castro, 2009). They are also the most representative rat strain to study the metabolic syndrome as the *fa/fa* Zucker rat exhibits hyperlipidemia, mild glucose intolerance and hyperinsulinemia compared to their lean counterparts, changes similarly observed in human metabolic syndrome (Aleixandre de Artinano & Miguel Castro, 2009). The *fa/fa* Zucker rat also exhibits elevated levels of pro-inflammatory markers such as TNF-$\alpha$ (Aleixandre de Artinano & Miguel Castro, 2009), elevated blood pressure (Kurtz, Morris, & Pershadsingh, 1989) and elevated hepatic lipid concentration (Stringer et al., 2010) compared to their lean counterparts.

The *fa/fa* Zucker rat becomes obese between the third and fifth week of life due to a mutation in the leptin receptor (Aleixandre de Artinano & Miguel Castro, 2009). Leptin is a hormone produced by adipose tissue that plays a critical role in energy balance (Zhang et al., 1994). Normally, when released into the circulatory system by the adipose tissue, leptin acts in the brain on leptin receptors causing a reduction in food intake and an increase in energy expenditure (Ahima & Flier, 2000; Himms-Hagen, 1999). However, due to the mutation in the leptin receptor, *fa/fa* Zucker rats have increased circulating leptin levels compared with their lean counterparts (Hardie, Rayner, Holmes, & Trayhurn, 1996; Pico, Sanchez, Oliver, & Palou, 2002), and elevated levels of other orexigenic peptides such as neuropeptide Y and ghrelin (Beck, 2000; Beck, Richy, & Stricker-Krongrad, 2003; Beck, Richy, & Stricker-Krongrad, 2004). As such, *fa/fa* Zucker rats develop severe obesity associated with hyperphagia, as well as preferential deposition of energy in adipose tissue (Chua et al., 1996). By 14 weeks of life, the body composition of *fa/fa* Zucker rats is approximately 50% lipid, whereas their lean counterparts are only 20% lipids (Cleary, Vasselli, & Greenwood, 1980).

## 1.4 Study Rationale

### Summary and Limitations of Published research

The metabolic syndrome is a collection of risk factors including obesity, insulin resistance, dyslipidemia, hypertension and NAFLD that lead to an increased risk of cardiovascular disease and T2DM (Alberti et al., 2009; Brunt, 2010; Marchesini et al., 2003; Mottillo et al., 2010). There is full agreement that healthy lifestyle changes focused primarily on weight loss are the first-line approach in managing the metabolic syndrome. However, the optimal diet to achieve this remains unclear (Muzio et al., 2007).

One diet that has been gaining increasing attention is a high protein diet (25 – 35% of energy as protein). While some advocators claim that a high protein diet has favorable effects on weight loss as well as other metabolic syndrome parameters (Te Morenga & Mann, 2012; Wycherley et al., 2012) other studies have shown no specific beneficial effects (Schwingshackl & Hoffmann, 2013), while some studies have actually demonstrated detrimental effects particularly on insulin resistance (Linn et al., 2000; Tremblay et al., 2007; Weickert et al., 2011). There is also concern regarding potential adverse effects of high protein diets on bone health and kidney function (Johnstone, 2012). Therefore, due to these concerns and the inconclusive evidence concerning the effects of high protein diets on metabolic syndrome parameters, more research is required before health care professionals can confidently recommend high protein diets as a tool to manage the metabolic syndrome.

There are several limitations in the literature. Firstly, several studies have examined the effects of high protein diets on metabolic syndrome parameters at protein levels well above the current AMDR for protein (Baba et al., 1999; Lacroix et al., 2004; Soenen et al., 2013). These studies are often challenged due to the potential adverse effects of high protein intake (Clifton & Keogh, 2007; Johnstone, 2012), the poor long-

term compliance on such a diet (Brinkworth et al., 2004), as well as due to the fact that protein intake above 35% of energy results in carbohydrate intake being well below its current AMDR of 45 – 65% of energy and/or fat intake being well above its current AMDR of 20 – 35% of energy (Clifton et al., 2008; Dansinger, Gleason, Griffith, Selker, & Schaefer, 2005; Foster et al., 2003; Institute of Medicine, 2005).

A second limitation is that high protein, weight loss studies are often energy-restricted studies (Baba et al., 1999; Brinkworth et al., 2004; Farnsworth et al., 2003; Flechtner-Mors et al., 2010; Layman et al., 2003; Muzio et al., 2007; Noakes et al., 2005; Soenen et al., 2013; Te Morenga et al., 2011), as opposed to providing experimental diets *ad libitum* (Claessens et al., 2009; McAuley et al., 2005; McAuley et al., 2006; Skov et al., 1999). It has been argued that *ad libitum* diets are more likely to detect unique properties of protein in facilitating weight loss compared to energy-restricted diets since strict control of energy will achieve clinically important weight loss that will conceal any relevant metabolic effects of protein (Te Morenga & Mann, 2012). In fact, when weight loss is achieved in energy-restricted studies that compare high protein diets to high carbohydrate diets, improvements in metabolic syndrome parameters are usually similar between both groups and are due entirely to weight loss with no diet effect (Brinkworth et al., 2004; Noakes et al., 2005; Soenen et al., 2013). There is actually minimal evidence for high protein diets to improve metabolic syndrome parameters independent of weight loss.

Thirdly, studies regarding the effects of high protein diets on NAFLD and hepatic steatosis are lacking. This may be due to the fact that NAFLD is currently not part of the clinical criteria used to diagnose the metabolic syndrome. However, due to the high prevalence of NAFLD in the obese population (Basaranoglu et al., 2010) as well as due to the fact that NAFLD is considered the hepatic manifestation of the metabolic

syndrome (Brunt, 2010; Marchesini et al., 2003), more research in this area is necessary.

Finally, the varying results in the literature regarding the effects of high protein diets on the metabolic syndrome – with positive, negative and no effects being reported – are a major limitation. One main reason why different effects are reported is due to the fact that different sources of protein appear to have differing effects on health outcomes (Noakes et al., 2005). For example, some studies have reported positive effects of soy protein, complete dairy protein, and whey protein on weight loss, glucose homeostasis, and hepatic steatosis (Ascencio et al., 2004; Davis et al., 2005; Eller & Reimer, 2010; Mezei et al., 2003; Tovar et al., 2005); while other studies have reported no effects (Pal et al., 2010; Yamashita et al., 1998). These discrepancies make it very difficult for health care professionals to provide recommendations regarding the use of high protein diets to manage obesity and other metabolic syndrome parameters. As such, more research is needed to determine the effect of high protein diets and the role of different sources of protein.

**Significance of Research**

This study will provide further insight into the effects of high protein diets on the metabolic syndrome due to the following components:

- All components of the metabolic syndrome will be evaluated using a variety of methods to ensure a complete representation on the effects of high protein diets on the metabolic syndrome.
- The experimental diet will be provided *ad libitum* to increase the probability to detect unique metabolic effects of protein that would likely be masked if diets were energy-restricted.

- Experimental diets will be representative of the current AMDRs for protein, carbohydrates and fat.
- Different sources of protein in a high protein diet will be evaluated to determine how critical the source of protein is in modulating metabolic syndrome parameters.
- As Canadians generally consume protein from a variety animal- and plant-based sources, a high protein diet containing a mixture of animal and plant-based protein sources will be used.
- The most representative rat strain to study the metabolic syndrome, the *fa/fa* Zucker rat, will be used as the rodent model.

## 1.5 General Hypotheses and Objectives

### General Hypotheses

High protein intake (35% of energy) will have positive effects on metabolic syndrome parameters compared to normal protein intake (15% of energy). The extent in which high protein intake improves metabolic syndrome parameters will vary depending on the source of protein. The high protein soy diet will show the most positive effects, followed by the high protein mixed diet. The high protein casein diet will also show positive effects but to a lesser extent compared to the high protein soy and mixed diets.

### Objectives

To investigate the hypotheses, the objectives of this study are:

1) To examine the effects of a normal protein diet (15% of energy) compared to a high protein diet (35% of energy) on metabolic syndrome parameters in an animal model of genetic obesity.
2) To examine the effects of different sources of protein in a high protein diet (35% of energy) on metabolic syndrome parameters in an animal model of genetic obesity.

For both objectives, the following metabolic syndrome parameters will be evaluated:

- Obesity – body weight, weight gain, food intake, adiposity, LBM
- Insulin resistance – fasting serum glucose concentrations (weeks 6 and 12), fasting serum insulin concentrations (weeks 6 and 12), OGTT serum glucose and insulin concentrations, AUC calculations, HOMA-IR, insulin:glucose ratio, pancreas weight, pancreatic islet cell size, skeletal muscle insulin signalling

molecules [protein levels of IRS-1, pIRS-1(Ser636/639), Akt, pAkt(Ser473), pAkt(Thr308)]

- Dyslipidemia – fasting serum FFA concentrations (weeks 6 and 12), fasting serum triglyceride concentrations (weeks 6 and 12)
- Hypertension – systolic and diastolic blood pressure, mean arterial pressure (weeks 3, 7 and 11)
- Inflammation – fasting serum haptoglobin concentrations (weeks 6 and 12)
- NAFLD – liver weight, total liver lipid concentrations, hepatic steatosis rating, number and size of liver lipid droplets, size distribution of lipid droplets, liver lipid droplet distribution rating, liver lipid size distribution

# CHAPTER 2 – MANUSCRIPT: SOURCE OF PROTEIN IN A HIGH PROTEIN DIET MODULATES REDUCTIONS IN INSULIN RESISTANCE AND HEPATIC STEATOSIS IN *fa/fa* ZUCKER RATS

## 2.1 Contributions of Co-authors to Chapter 2

Jennifer Wojcik, Jessay Gopuran Devassy, Yinghong Wu, Carla Taylor, Peter Zahradka, Harold Aukema

**Jennifer Wojcik** – Assisted with study design; prepared diet; fed animals; weighed animals weekly; recorded food intake weekly; performed oral glucose tolerance testing; assisted with terminations; performed all assays for serum biochemistry and calculations pertaining to insulin sensitivity; performed insulin immunostaining and quantification of islet cell size; performed total liver lipid concentration; analyzed DEXA results; performed all Western immunoblotting experimentation analyses including optimizations; performed all calculations and statistical analyses for all results; wrote Chapter 2 and created all tables and figures.

**Jessay Gopuran Devassy** – Assisted with study design; prepared diet; fed animals; weighed animals weekly; recorded food intake weekly; performed oral glucose tolerance testing; assisted with terminations.

**Yinghong Wu** – Performed image analysis of number, size and size distribution of lipid droplets in liver; qualitative assessment for hepatic steatosis and distribution rating.

**Carla Taylor** – Was a co-applicant on the grant application; assisted with study design; provided direction for the experimental analysis; provided revisions and editing for Chapter 2.

**Peter Zahradka** – Was a co-applicant on the grant application; assisted with study design; provided training and facilities for Western immunoblotting.

**Harold Aukema** – Wrote the grant application and allocated funds for the investigation; assisted with study design; provided direction for the experimental analysis; provided revisions and editing for Chapter 2.

## 2.2 Abstract

High protein diets are being promoted for the management of metabolic syndrome parameters including obesity, insulin resistance and hepatic steatosis, despite inconsistent results in the literature. Therefore, the objective was to determine the effects of a 12 week intervention with high protein diets (35% energy as protein) containing varying protein sources compared to a normal protein diet (15% energy as protein) on obesity, insulin resistance and hepatic steatosis in 13-week-old male *fa/fa* Zucker rats. A high protein mixed diet containing animal and plant protein sources was the most effective for modulating reductions in insulin resistance as indicated by improvements in area under the curve for insulin during glucose tolerance testing, fasting serum insulin and free fatty acid concentrations, homeostatic model assessment index, insulin:glucose ratio and pancreatic islet cell area. Both the high protein mixed and high protein soy diets demonstrated reductions in hepatic steatosis as indicated by lower hepatic lipid concentrations, liver to body weight ratio and hepatic steatosis rating compared to the high protein casein diet. These improvements were observed despite no differences in body weight, food intake or adiposity among the high protein diet groups. The high protein casein diet demonstrated no benefits on any measurements compared to the other two high protein diets and minimal benefits compared to the normal protein casein diet. In conclusion, the source of protein within a high protein diet is critical for the management of certain metabolic syndrome parameters. Specifically, a high protein mixed diet was the most effective for modulating reductions in both insulin resistance and hepatic steatosis independent of weight loss.

## 2.3 Introduction

The metabolic syndrome is a collection of risk factors which lead to an increased risk of cardiovascular disease and T2DM (Alberti et al., 2009; Mottillo et al., 2010). While the exact pathogenesis of the metabolic syndrome is unknown, obesity and insulin resistance appear to be the main causative factors (Alberti, Zimmet, & Shaw, 2006). In addition, NAFLD is strongly associated with obesity and insulin resistance and is often referred to as the hepatic manifestation of the metabolic syndrome (Brunt, 2010). There is full agreement that moderate weight loss is the first-line approach for managing the metabolic syndrome and preventing the progression to cardiovascular disease or T2DM. However, the optimal diet to achieve this remains unclear (Muzio, Mondazzi, Harris, Sommariva, & Branchi, 2007).

Recently, there has been increasing interest in the consumption of high protein diets for weight loss. A high protein intake is considered in the range of 25 – 35% of energy as protein, compared to normal protein intake which is considered to be approximately 15% of energy as protein (Clifton & Keogh, 2007). Advocators claim that a high protein diet not only has more favorable effects on weight loss, but also improves an array of metabolic syndrome parameters including glucose homeostasis (Brinkworth et al., 2004; Farnsworth et al., 2003; Layman et al., 2003; Layman, Shiue, Sather, Erickson, & Baum, 2003; Wycherley, Moran, Clifton, Noakes, & Brinkworth, 2012) and hepatic steatosis, the accumulation of fat in liver cells and one of the first stages of NAFLD (Bortolotti et al., 2009; Flechtner-Mors, Boehm, Wittmann, Thoma, & Ditschuneit, 2010; Garcia-Caraballo et al., 2013; Tovar et al., 2005). However, results pertaining to the effects of high protein diets on metabolic syndrome parameters are varied and not all studies have reported beneficial outcomes (Santesso et al., 2012; Te Morenga & Mann, 2012; Tremblay, Lavigne, Jacques, & Marette, 2007; Weickert et al., 2011). Different sources of protein also appear to have varying effects on metabolic syndrome

parameters. For instance, some studies have reported positive effects of soy protein, complete dairy protein and whey protein on weight loss, glucose homeostasis and hepatic steatosis (Ascencio et al., 2004; Davis et al., 2005; Eller & Reimer, 2010; Mezei et al., 2003; Tovar et al., 2005), while other studies have reported no effects (Pal, Ellis, & Dhaliwal, 2010; Yamashita, Sasahara, Pomeroy, Collier, & Nestel, 1998).

The varying effects of high protein diets on the metabolic syndrome that have been reported thus far may in part be due to several important limitations in the literature. Firstly, several studies that have compared high protein diets to the more conventional high carbohydrate diets provide energy-restricted experimental diets as opposed to *ad libitum* diets. It has been proposed that *ad libitum* diets are more likely to detect unique properties of protein compared to energy-restricted diets, since strict control of energy will result in clinically important weight loss that will conceal any relevant metabolic effects of protein (Te Morenga & Mann, 2012). Secondly, several studies have examined the effects of high protein diets at protein levels well above the current AMDR for protein which is set at 10 – 35% of energy (Baba et al., 1999; Institute of Medicine, 2005; Lacroix et al., 2004; Soenen, Martens, Hochstenbach-Waelen, Lemmens, & Westerterp-Plantenga, 2013). These studies are often challenged due to the potential adverse effects of high protein intake (Clifton & Keogh, 2007; Johnstone, 2012), the poor long-term compliance on such a diet (Brinkworth et al., 2004), as well as due to the fact that protein intake above 35% of energy usually results in carbohydrate intake being well below, and fat intake being well above their respective AMDRs (Clifton, Keogh, & Noakes, 2008; Dansinger, Gleason, Griffith, Selker, & Schaefer, 2005; Foster et al., 2003; Institute of Medicine, 2005). As such, due to these limitations as well as the contradictory conclusions that exist, more research is necessary before health care professionals can make concrete recommendations regarding the most effective way to manage obesity and other metabolic syndrome parameters.

Therefore, the objectives of this study were to first examine the effects of a normal protein diet (15% energy as protein) compared to a high protein diet (35% energy as protein) offered *ad libitum* on obesity, insulin resistance and hepatic steatosis; and secondly to examine the effects of different sources of protein in a high protein diet offered *ad libitum* on obesity, insulin resistance and hepatic steatosis in the *fa/fa* Zucker rat. The *fa/fa* Zucker rat is the most widely used animal model of genetic obesity and the most representative rat strain to study insulin resistance and other metabolic syndrome parameters (Aleixandre de Artinano & Miguel Castro, 2009).

## 2.4 Materials and Methods

### Animals and Diets

After a 7 – 10 day acclimatization period, male *fa/fa* (fa) and lean (ln) 13-week-old Zucker rats (Charles River, St Constant Quebec, Canada) were randomly assigned to one of five groups (n = 12 per group) for 12 weeks: lean rats given normal protein casein diet (lnNPC), *fa/fa* rats given normal protein casein diet (faNPC), *fa/fa* rats given high protein casein diet (faHPC), *fa/fa* rats given high protein soy diet (faHPS), and *fa/fa* rats given high protein mixed diet (faHPM). The protocol for the animal care procedures was approved by the University of Manitoba Animal Care Committee.

The diet formulations were based on the standard laboratory AIN-93M diet (Reeves, 1997), with the source of protein changing depending on the diet (**Table 2**). Casein was the protein source for the normal protein casein (NPC) and high protein casein (HPC) diets, soy protein isolate was the protein source for the high protein soy (HPS) diet, and a mixture of soy protein isolate, wheat gluten, complete milk protein and egg white were the protein sources for the high protein mixed (HPM) diet. Carbohydrate was replaced with protein in the high protein diets, with the fat content remaining the same in all diets. As such, the macronutrient distribution of the NPC diet was 15% energy from protein, 64% energy from carbohydrate and 21% energy from fat, whereas the high protein diets contained 35% energy from protein, 44% energy from carbohydrate and 21% energy from fat. Macronutrient distribution of both the normal and high protein diets were representative of the current AMDRs set for all three macronutrients. Fresh, 5-kg batches of the diet were prepared bi-weekly. The NPC, HPC and HPM diets were stored at 4$^{o}$C. The HPS diet was stored at -20$^{o}$C until used. Rats were given new feed cups once per week and given fresh feed 3 times per week. Rats had free access to food and water. Rats were weighed weekly. Feed intake (corrected for spillage) was recorded once per week.

**TABLE 2** Diet formulations and macronutrient composition[1,2]

| | NPC | HPC | HPS | HPM |
|---|---|---|---|---|
| **Ingredients (g/1000 g)** | | | | |
| Cornstarch[3] | 396 | 174 | 183 | 157 |
| Casein (87% protein)[4] | 174 | 395 | - | - |
| Soy protein (87% protein)[4] | - | - | 406 | 100 |
| Wheat gluten (76% protein)[4] | - | - | - | 115 |
| Complete milk protein (89% protein)[4] | - | - | - | 100 |
| Egg white (82% protein)[4] | - | - | - | 106 |
| Dextrinized cornstarch | 132 | 132 | 132 | 132 |
| Sucrose | 100 | 100 | 100 | 100 |
| Soybean oil with TBHQ[5] | 98 | 96 | 82 | 93 |
| Fiber | 50 | 50 | 50 | 50 |
| AIN-93M mineral mix | 35 | 35 | 35 | 35 |
| AIN-93VX vitamin mix | 10 | 10 | 10 | 10 |
| L-cystine | 3 | 6 | - | - |
| Choline bitartarate | 3 | 3 | 3 | 3 |
| **Macronutrient Composition** | | | | |
| Protein (% energy) | 15 | 35 | 35 | 35 |
| Carbohydrate (% energy) | 64 | 44 | 44 | 44 |
| Fat (% energy) | 21 | 21 | 21 | 21 |

[1]Abbreviations: HPC = high protein casein; HPM = high protein mixed; HPS = high protein soy; NPC = normal protein casein; TBHQ = *tert*-Butylhydroquinone
[2]All ingredients purchased from Dyets Inc. (Bethlehem PA) except cornstarch which was purchased from Moonshiner Unlimited (Winnipeg MB).
[3]Cornstarch was adjusted for the carbohydrate content of the protein sources, ensuring that the % energy from carbohydrates remained the same for the high protein diets and that all diets were isocaloric.
[4]The protein sources were adjusted for the protein content, ensuring that the % energy from protein remained the same for the high protein diets and that all diets were isocaloric.
[5]Soybean oil was adjusted for the fat content in the protein sources, ensuring that the % energy from fat was the same for all four diets and that all diets were isocaloric.

**Oral Glucose Tolerance Testing**

During week 11 of the study, OGTT was completed after a 5-hour fast. Initial blood samples (t = 0) were collected from the saphenous vein, followed by administration of an oral glucose dose (1 gram glucose per kilogram body weight). Additional blood samples were collected at t = 15, t = 30, t = 60 and t = 120 minutes. Blood samples were stored on ice until centrifuged at 2000 g for 10 minutes at 4$^{o}$C. Serum was aliquoted and stored at -80$^{o}$C until glucose and insulin concentrations were determined. Rats were pre-exposed to the OGTT procedure without blood collection to reduce the stress response during the actual testing period. Total area under the curve was calculated for both glucose ($AUC_g$) and insulin ($AUC_i$) from OGTT data (Brouns et al., 2005). The glucose-insulin index ($AUC_{index}$) was calculated as $AUC_g$ x $AUC_i$ (Cortez, Torgan, Brozinick, & Ivy, 1991; Myllynen, Koivisto, & Nikkila, 1987) and used as a surrogate marker for insulin sensitivity.

**Tissue Collection**

After 12 weeks, rats were fasted overnight (12 hours) and killed under isoflurane anaesthesia according to the Canadian Council on Animal Care Guidelines. Blood was collected via cardiac puncture and immediately placed on ice until centrifuged at 2000 g for 20 minutes at 4$^{o}$C to separate the serum fraction, which was stored at -80$^{o}$C. Dissected tissues were weighed, immediately frozen in liquid nitrogen and subsequently stored at -80$^{o}$C. A portion of the pancreas was fixed in phosphate buffered formalin and paraffin blocks were prepared (CancerCare Manitoba, Winnipeg MB). A portion of the liver was embedded in optimal cutting temperature medium, frozen in a dry-ice ethanol bath and then stored at -80$^{o}$C until sectioning.

**Body Composition**

Dual-Energy X-ray Absorptiometry (DEXA; software version 11.2.3, Hologic QDR 4500A [S/N 45923], Hologic Inc, Bedford MA) was performed on frozen carcasses (without internal organs) to assess LBM (Manitoba Institute of Child Health, Winnipeg MB). Visceral adipose tissue was calculated by weighing epididymal, mesenteric and perirenal adipose tissue at termination.

**Serum Biochemistry**

Enzymatic colorimetric kits were used to determine OGTT serum glucose as well as termination fasting serum glucose (Genzyme Diagnostics, Charlottetown PE), free fatty acids (Roche Diagnostics, Mannheim, Germany), haptoglobin (Tridelta Development Ltd., Maynooth, Ireland) and triglycerides (Genzyme Diagnostics, Charlottetown PE). ELISA kits were used for OGTT serum insulin and termination fasting serum insulin (ALPCO Diagnostics, Salem NH). Termination fasting serum glucose and insulin were used to calculate the fasting insulin:glucose ratio as well as HOMA-IR (fasting glucose [mmol/L]*fasting insulin [µU/mL] / 22.5) (Matthews et al., 1985).

**Pancreatic Islet Size**

Insulin immunostaining for pancreatic islets was determined using the STAT-Q Peroxidase-DAB Staining System (Innovex Biosciences, Richmond CA). Briefly, pancreas sections were incubated with Cyto Q Background Buster for 20 minutes, monoclonal mouse anti-insulin antibody (1:75 dilution) for 20 minutes, secondary linking antibody for 10 minutes, peroxidase-streptavidin tertiary antibody for 10 minutes, 3,3' – diaminobenzidine tetrahydrochloride substrate for 5 minutes, and 3% hematoxylin for 1 minute. Specificity of staining was confirmed by omission of the anti-insulin antibody

(phosphate buffered saline buffer only). Computer images of immunostained sections were visualized with a Zeiss Axioskop 2 Mot Plus microscope and images were captured with a Zeiss Axiocam digital camera using Axio Vision 4.6 software (Carl Zeiss Canada Ltd, Toronto, ON). Insulin-positive islet cell area was then quantified using ImageJ software (NIH, Bethesda, MA).

**Western Blot Analysis**

Frozen gastrocnemius muscle was ground with a mortar and pestle under liquid nitrogen and suspended in 3X sodium dodecyl sulfate sample buffer. The solution was then centrifuged at 13 000 g for 20 minutes at 4$^{o}$C and then sonicated for 10 seconds. Protein content was determined using a Pierce Bicinchoninic Acid (BCA) Assay Kit (Thermo Fisher Scientific, Ottawa ON). SDS-PAGE and Western blotting were carried out as previously described (Yau, Elliot, Lalonde, & Zahradka, 1998). Briefly, protein extracts (7.5 – 10 µg protein per well) were separated by SDS-PAGE in a 6% or 7.5% gel and transferred to PVDF membranes. Membranes were then blocked in 3% BSA-TBST followed by overnight incubation at 4$^{o}$C with primary antibodies (all 1:1000 dilutions) for IRS-1, pIRS-1(Ser636/639), Akt, pAkt(Ser473) and pAkt(Thr308) obtained from Cell Signaling (Danvers MA). Membranes were then incubated with an anti-rabbit immunoglobin G conjugated to horseradish peroxidase secondary antibody (1:10 000 dilution; Cell Signaling, Danvers MA). A chemiluminescent reagent was applied to the membranes and detected using a FluorChem®Q gel scanning system with a charge-coupled device camera (Proteinsimple) and AlphaView®Software (version 1.3.0.6; Alpha Innotech Corporation). A reference muscle sample was run on every gel for comparison of samples from different immunoblots, and MAPK p44/42 (1:2000 dilution; Cell Signaling, Danvers MA) and eEF2 (1:1000 dilution; Cell Signaling, Danvers MA) were used as loading controls.

**Hepatic Steatosis Assessment**

Total liver lipid was separated by chloroform/methanol extraction (Lepage, Giesbrecht, & Taylor, 1999) and total lipid was calculated by weight.

Liver sections (7 µm) were fixed in 4% paraformaldehyde and stained with Harris modified hematoxylin and eosin Y for histological examination. Sections were visualized with a Zeiss Axioskop 2 Mot Plus microscope and images were captured with a Zeiss Axiocam digital camera using Axio Vision 4.6 software (Carl Zeiss Canada Ltd, Toronto ON). Quantification of lipid droplet number and size was carried out using ImageJ software (NIH, Bethesda MA). For lipid droplet size measurement, 2 lipid droplets were randomly selected from 25 different sections of liver tissue for a total of 50 lipid droplets for each rat. An average was then taken for each rat. For quantification of lipid droplet number, liver sections were divided into 0.01 $mm^2$ and all lipid droplets within this area were counted. Distribution rating, a qualitative assessment used to determine uniformity of lipid droplet size, was conducted by rating images in a blinded manner on scale of 1 – 4, with 1 representing mostly even lipid droplets, 2 representing small degree of unevenness, 3 representing moderate degree of unevenness, and 4 representing highest degree of unevenness in lipid droplet size. For hepatic steatosis rating, images were rated in a blinded manner on a scale of 1 – 4, with 1 representing low degree of hepatic steatosis, 2 representing low to medium degree, 3 representing medium to high degree, and 4 representing high degree of hepatic steatosis.

**Statistical Analysis**

Statistical analysis was performed using Statistical Analysis Software (SAS; Version 9.3, SAS Institute Inc., Cary NC). Time course and end-point data were analyzed by repeated measures ANOVA and one-way ANOVA, respectively. Significant differences among means were determined using Duncan's multiple range test. Data

was transformed to achieve normality when necessary. Data that did not follow a normal distribution or were not homogenous were analyzed by Kruskal-Wallis, a non-parametric test, followed by Duncan's multiple range test. The chi-squared test was used for liver lipid size distribution. Data are presented as means ± standard error of the mean (SEM). All differences were considered significant at $P < 0.05$.

## 2.5 Results

### Altering the source of protein in a high protein diet affects glucose and insulin concentrations during glucose tolerance testing.

The faHPC group had similar serum glucose and insulin concentrations at all time points compared to the faNPC group, except at baseline when the faHPC group had lower serum glucose concentrations. This translated into no differences in $AUC_g$, $AUC_i$, and $AUC_{index}$ between the faNPC and faHPC groups (**Fig 1A, B and C**), suggesting that simply increasing the amount of casein protein in the diet has no effect on glucose tolerance. However, altering the source of protein in a high protein diet did affect serum glucose and insulin concentrations. While there were no differences in serum glucose concentrations among the high protein diet groups at baseline, the faHPM group had higher serum glucose concentrations at t = 15, t = 30 and t = 60 compared to the faHPC group, resulting in a higher $AUC_g$ for the faHPM group compared to the faHPC group (Fig 1A). However, at t = 120, glucose concentrations in the faHPM group returned to baseline levels and no differences among the high protein diet groups were observed. The faHPM group, however, had lower serum insulin concentrations at all time points compared to the faHPC group, with statistical differences observed at t = 0, t = 60 and t = 120 (Fig 1B). This resulted in a lower $AUC_i$ for the faHPM group compared to the faHPC group. There were no significant differences among the high protein diet groups for $AUC_{index}$ (Fig 1C). However, the faHPM group was the only group that had similar $AUC_i$ and $AUC_{index}$ values compared to the lnNPC group (Fig 1B and C). A genotype effect was observed as the faNPC group had higher serum insulin concentrations at all time points compared to the lnNPC group while serum glucose concentrations remained similar between both groups except at baseline, resulting in a higher $AUC_i$ and $AUC_{index}$ in the faNPC group but no differences in $AUC_g$ (Fig 1A, B and C).

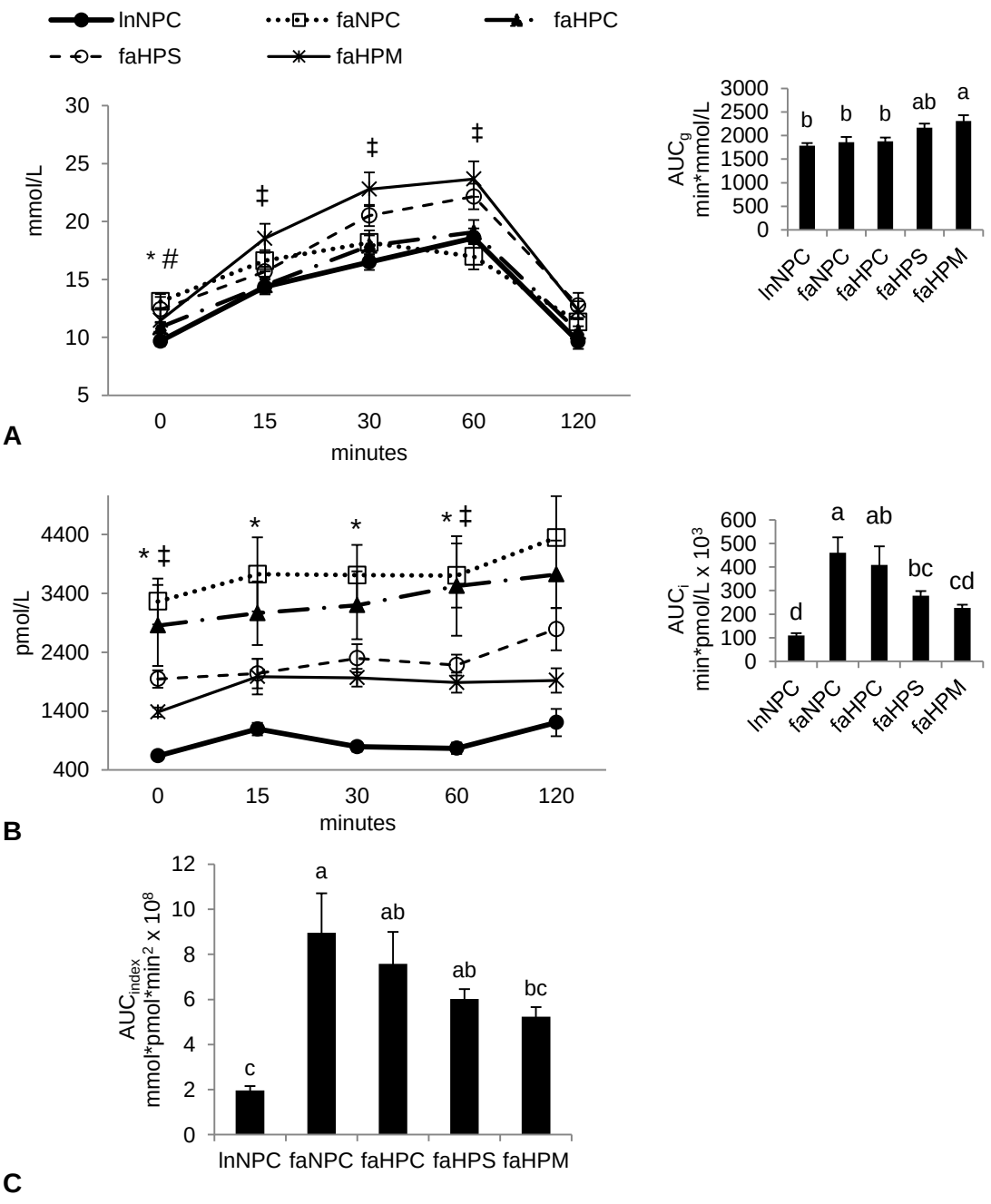


**FIGURE 1** Oral glucose tolerance testing – serum glucose time-course changes and area under the curve for glucose ($AUC_g$) (A), serum insulin time-course changes and area under the curve for insulin ($AUC_i$) (B), and glucose-insulin index ($AUC_{index}$) (C) in lean and *fa/fa* Zucker rats given normal or high protein diets for 12 weeks. Data are presented as means ± SEM (n = 7 – 10 per group). For time-course changes in serum glucose and serum insulin, * denotes lnNPC significantly different from faNPC group ($P < 0.05$); # denotes faNPC significantly different from faHPC ($P < 0.05$); ‡ denotes faHPC significantly different from faHPM ($P < 0.05$). For $AUC_g$, $AUC_i$ and $AUC_{index}$, values with different superscript letters are significantly different ($P < 0.05$) from each other.

**High protein diets have higher absolute LBM regardless of protein source, but have no effect on weight, food intake or adiposity in *fa/fa* rats.**

The lnNPC group had lower final body weights, total food intake, visceral adipose to body weight ratio, and perirenal adipose to body weight ratio compared to the *fa/fa* groups; however, there were no differences between the faNPC and faHPC groups or among the faHPC, faHPS and faHPM groups (**Table 3**). Total weight gain, epididymal adipose and mesenteric adipose to body weight ratios did not differ among groups. Interestingly, absolute LBM was higher in the high protein diet groups compared to the faNPC group. However, when LBM was adjusted for body weight, there were no significant differences. As expected, the lnNPC group had higher LBM (absolute and adjusted for body weight) compared to the *fa/fa* groups.

**A high protein mixed diet is the most effective at enhancing insulin sensitivity and preserving pancreatic function.**

After 12 weeks, there were no differences for fasting serum glucose concentrations among the groups (**Table 4**). However, the faHPM group had 44% and 59% lower fasting serum insulin concentrations compared to the faHPS and faHPC groups, respectively, with the values not being different than the lnNPC group (Table 4). This resulted in the faHPM group having a 47% and 58% reduction in HOMA-IR compared to the faHPS and faHPC groups, respectively, as well as a 60% lower insulin:glucose ratio compared to the faHPC group (Table 4). The reduced circulating insulin concentrations in the faHPM group was reflected in a reduction in pancreatic islet cell area (**Fig 2A and B**), demonstrating pancreatic preservation. The islet cell area in the faHPM group was 61% lower compared to the faHPC group, and was not different than the islet cell area of the lnNPC group. The faHPS and faHPM groups had higher pancreas to body weight ratios compared to the faHPC group, with the values not being

**TABLE 3** Body weight, food intake and body composition data of lean and *fa/fa* Zucker rats given normal or high protein diets for 12 weeks[1]

| | lnNPC | faNPC | faHPC | faHPS | faHPM |
|---|---|---|---|---|---|
| Final body weight (g) | 558 ± 15[c] | 682 ± 12[b] | 726 ± 24[ab] | 777 ± 37[a] | 755 ± 31[ab] |
| Total weight gain (g) | 236 ± 10 | 184 ± 12 | 220 ± 14 | 278 ± 29 | 253 ± 23 |
| Total food intake (g) | 1784 ± 75[b] | 2344 ± 109[a] | 2158 ± 68[a] | 2401 ± 160[a] | 2399 ± 119[a] |
| Visceral adipose tissue (g/100 g bwt)[2] | 7.02 ± 0.23[b] | 11.9 ± 0.7[a] | 10.9 ± 0.8[a] | 10.7 ± 0.6[a] | 12.0 ± 0.5[a] |
| Epididymal adipose (g/100 g bwt) | 2.74 ± 0.09 | 2.55 ± 0.20 | 2.22 ± 0.15 | 2.78 ± 0.18 | 2.90 ± 0.19 |
| Perirenal adipose (g/100 g bwt) | 2.62 ± 0.10[b] | 7.16 ± 0.50[a] | 6.85 ± 0.66[a] | 6.15 ± 0.46[a] | 7.07 ± 0.44[a] |
| Mesenteric adipose (g/100 g bwt) | 1.66 ± 0.12 | 2.20 ± 0.19 | 1.78 ± 0.15 | 1.76 ± 0.09 | 2.05 ± 0.16 |
| Lean body mass (g)[3] | 333 ± 6[a] | 194 ± 7[c] | 232 ± 13[b] | 223 ± 9[b] | 224 ± 7[b] |
| Lean body mass (g/100 g bwt) | 60.9 ± 1.2[a] | 28.9 ± 1.3[b] | 30.3 ± 2.0[b] | 29.3 ± 1.5[b] | 29.9 ± 1.0[b] |

[1]Data are presented as means ± SEM (n = 8 – 11 per group). Values in the same row with different superscript letters are significantly different ($P < 0.05$) from each other; bwt = body weight.
[2]Visceral adipose = epididymal + perirenal + mesenteric adipose tissue.
[3]Lean body mass assessed using dual-energy x-ray absorptiometry.

**TABLE 4** Fasting serum biochemistry of lean and *fa/fa* Zucker rats given normal or high protein diets for 12 weeks[1]

| | lnNPC | faNPC | faHPC | faHPS | faHPM |
|---|---|---|---|---|---|
| Glucose (mmol/L) | 13.1 ± 0.6 | 16.2 ± 0.7 | 15.7 ± 1.6 | 18.8 ± 2.3 | 14.1 ± 0.6 |
| Insulin (pmol/L) | 75 ± 16$^{c}$ | 663 ± 75$^{a}$ | 597 ± 68$^{ab}$ | 440 ± 99$^{b}$ | 246 ± 25$^{c}$ |
| HOMA-IR (mmol/L*µU/mL) | 6.89 ± 1.58$^{d}$ | 66.7 ± 8.0$^{a}$ | 56.7 ± 9.3$^{ab}$ | 44.7 ± 8.9$^{b}$ | 23.6 ± 2.3$^{c}$ |
| Insulin:glucose ratio (pmol/mmol) | 5.23 ± 1.25$^{c}$ | 43.0 ± 5.4$^{a}$ | 42.3 ± 4.7$^{a}$ | 29.4 ± 9.6$^{ab}$ | 16.9 ± 2.1$^{b}$ |
| Free fatty acids (mmol/L) | 0.273 ± 0.03$^{ab}$ | 0.313 ± 0.04$^{a}$ | 0.282 ± 0.03$^{ab}$ | 0.395 ± 0.07$^{a}$ | 0.167 ± 0.04$^{b}$ |
| Triglycerides (mmol/L) | 1.31 ± 0.20$^{c}$ | 6.90 ± 0.94$^{b}$ | 11.1 ± 2.5$^{ab}$ | 10.2 ± 1.8$^{ab}$ | 12.9 ± 2.1$^{a}$ |
| Haptoglobin (mg/mL) | 1.13 ± 0.12$^{b}$ | 2.84 ± 0.30$^{a}$ | 2.17 ± 0.28$^{a}$ | 2.12 ± 0.35$^{a}$ | 2.46 ± 0.21$^{a}$ |

[1]Data are presented as means ± SEM (n = 8 – 11 per group). Values in the same row with different superscript letters are significantly different ($P < 0.05$) from each other; HOMA-IR = homeostatic model assessment index for insulin resistance.

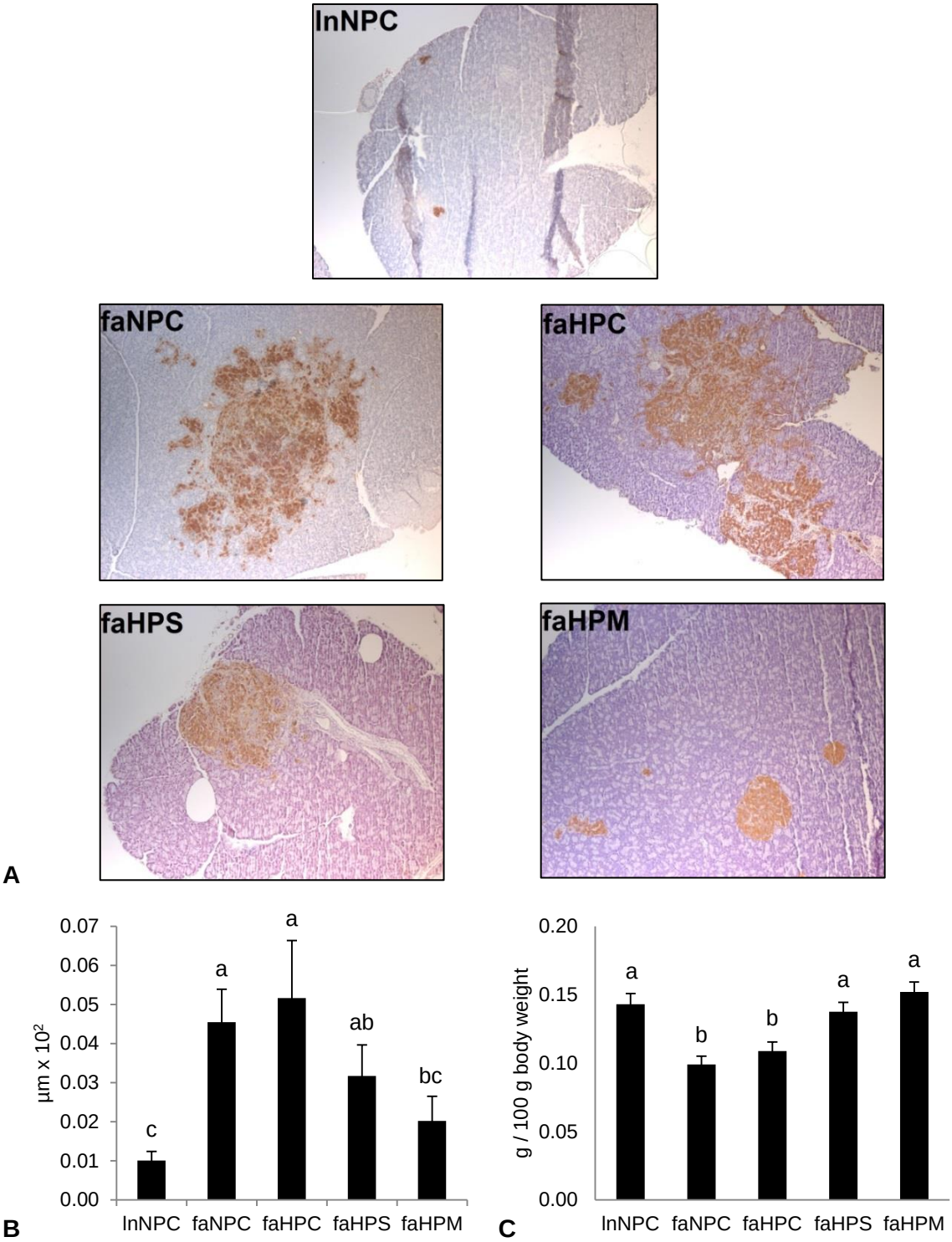


**FIGURE 2** Insulin immunostaining of pancreatic islets (A), pancreatic islet size (B) and pancreas weight per body weight (C) of lean and *fa/fa* Zucker rats given normal or high protein diets for 12 weeks. Data are presented as means ± SEM (n = 8 – 10 per group). Values with different superscript letters are significantly different ($P < 0.05$) from each other.

different than the pancreas to body weight ratio in the lnNPC group (**Fig 2C**). Conversely, compared to the lnNPC group, the faNPC group had significantly higher fasting serum insulin concentrations, insulin to glucose ratio, HOMA-IR (Table 4), islet cell area, and significantly lower pancreas to body weight ratio (Fig 2). However, there were no differences between the faNPC and faHPC groups for all these parameters suggesting that simply increasing casein protein content in the diet has no effect on insulin sensitivity and pancreas preservation.

**A high protein mixed diet is more effective than a high protein soy diet at lowering fasting serum FFA concentrations, but high protein diets have no effect on fasting serum triglycerides or haptoglobin concentrations regardless of protein source.**

Serum FFA and triglycerides were measured to assess the effects of high protein diets on dyslipidemia while haptoglobin was used as a marker of inflammation. In the high protein diet groups, the faHPM group had 58% lower fasting serum FFA concentrations compared to the faHPS group (Table 4). There were no differences between the lnNPC and faNPC groups, or among the faNPC, faHPC and faHPS groups in fasting serum FFA concentrations. While all *fa/fa* groups had higher fasting serum triglyceride concentrations compared to the lnNPC group, there were no differences among the *fa/fa* groups (Table 4). Finally, the lnNPC group had lower fasting serum haptoglobin concentrations compared to all *fa/fa* groups with no differences among the *fa/fa* groups (Table 4).

**High protein diets have no effect on insulin signaling proteins in skeletal muscle regardless of protein source.**

No differences among groups were observed for pAkt(Ser473), pAkt(Thr308), total Akt (**Fig 3**), pIRS-1(Ser636/639) and total IRS-1 (**Fig 4**).

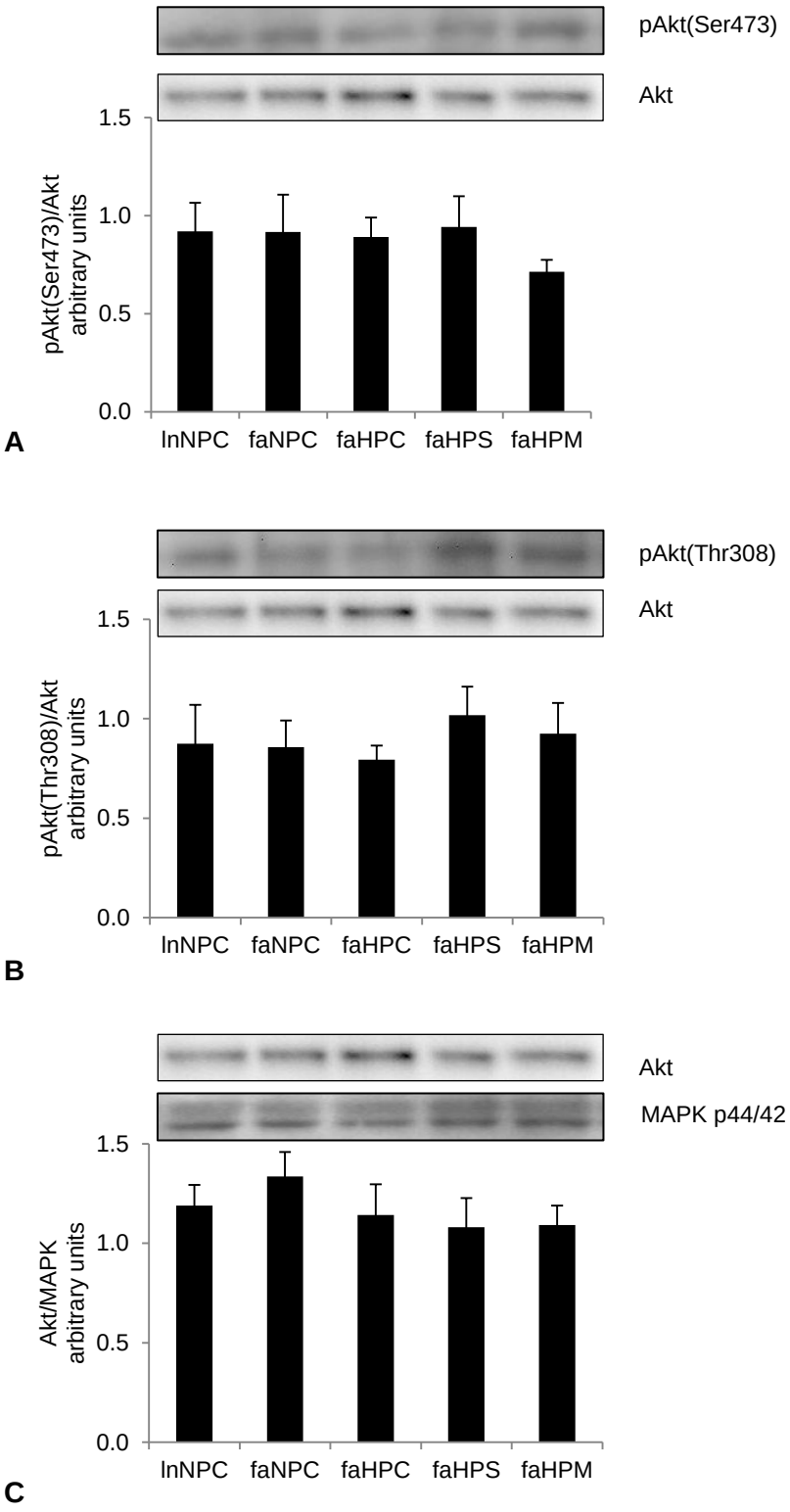


**FIGURE 3** Western immunoblotting for protein levels of pAkt(Ser473) (A), pAkt(Thr308) (B) and Akt (C) in gastrocnemius muscle of lean and *fa/fa* Zucker rats given normal or high protein diets for 12 weeks. Data are presented as means ± SEM (n = 8 per group).

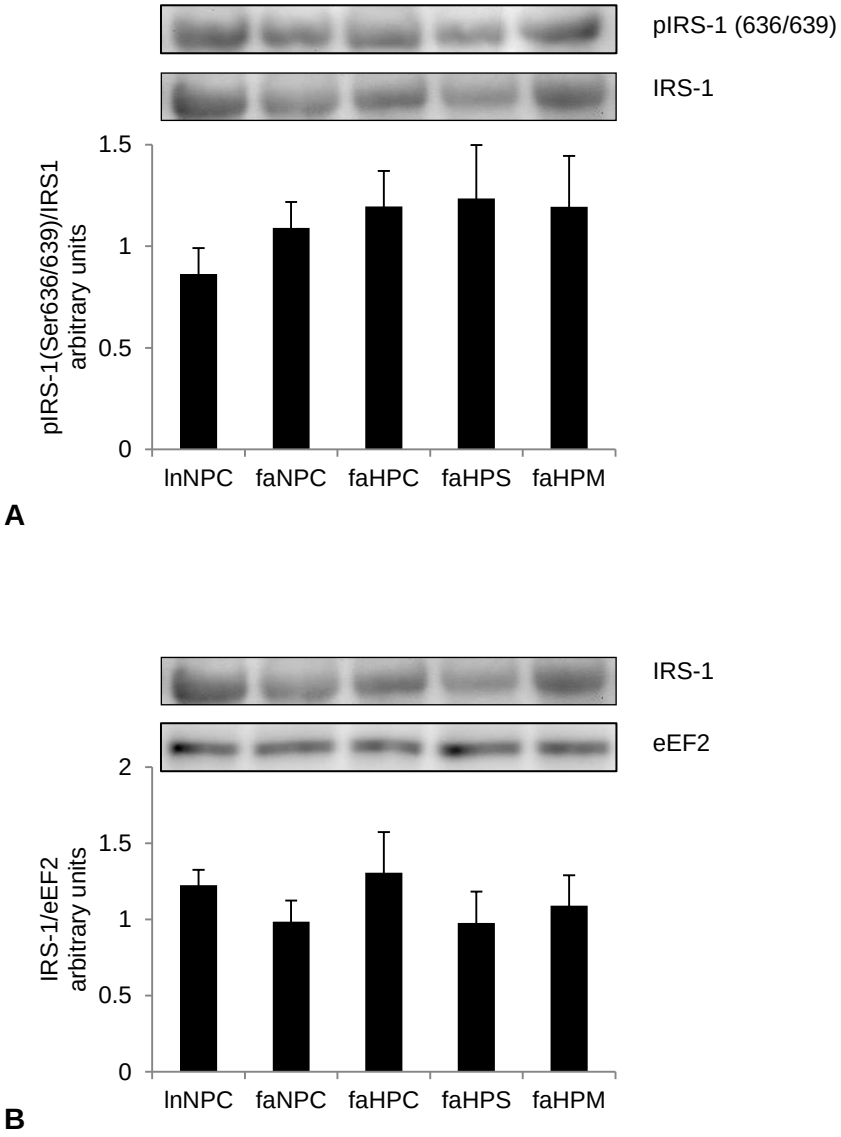


**FIGURE 4** Western immunoblotting for protein levels of pIRS-1(Ser 636/639) (A) and IRS-1 (B) in gastrocnemius muscle of lean and *fa/fa* Zucker rats given normal or high protein diets for 12 weeks. Data are presented as means ± SEM (n = 8 per group).

**High protein mixed and high protein soy diets have a profound effect on reducing hepatic steatosis.**

Hepatic steatosis was present in the faNPC group as the liver lipid concentration was 61% higher than the lnNPC group, and this was reflected in the faNPC group having a 61% higher liver to body weight ratio compared to the lnNPC group (**Fig 5A and B**). Hepatic steatosis rating, which was qualitatively assessed after liver sections were stained with hematoxylin and eosin for histological examination (**Fig 6**), was also higher in the faNPC group compared to the lnNPC group (**Fig 5C**). There were no differences between the faNPC and faHPC groups for liver lipid concentrations or hepatic steatosis rating (Fig 5A and C). However, altering the source of protein in a high protein diet had a profound effect on liver lipid concentrations. The faHPS and faHPM groups had 53% and 62% lower liver lipid concentrations, respectively, compared to the faHPC group and the values were not different than the lnNPC group (Fig 5A). The reduced liver lipid concentrations were reflected in the liver to body weight ratios as the faHPS and faHPM groups had a 28% and 32% reduction in liver to body weight ratios, respectively, compared to the faHPC group (Fig 5B) even though there were no differences in final body weights (Table 3). Interestingly, the faHPC group also had a 16% reduction in liver to body weight ratio compared to the faNPC group (Fig 5B) despite no difference in final body weight (Table 3), liver lipid concentrations or hepatic steatosis rating (Fig 5A and C). The hepatic steatosis rating was also significantly reduced in the faHPS and faHPM groups compared to the faHPC group, with the values not being different than the lnNPC group (Fig 5C).

Liver sections stained with hematoxylin and eosin (Fig 6) were also used to assess the effects of high protein diets on intrahepatocellular lipid accumulation. The faNPC group had significantly less liver lipid droplets compared to the lnNPC group, but there were no differences between the faNPC and faHPC groups (**Fig 7A**). However,

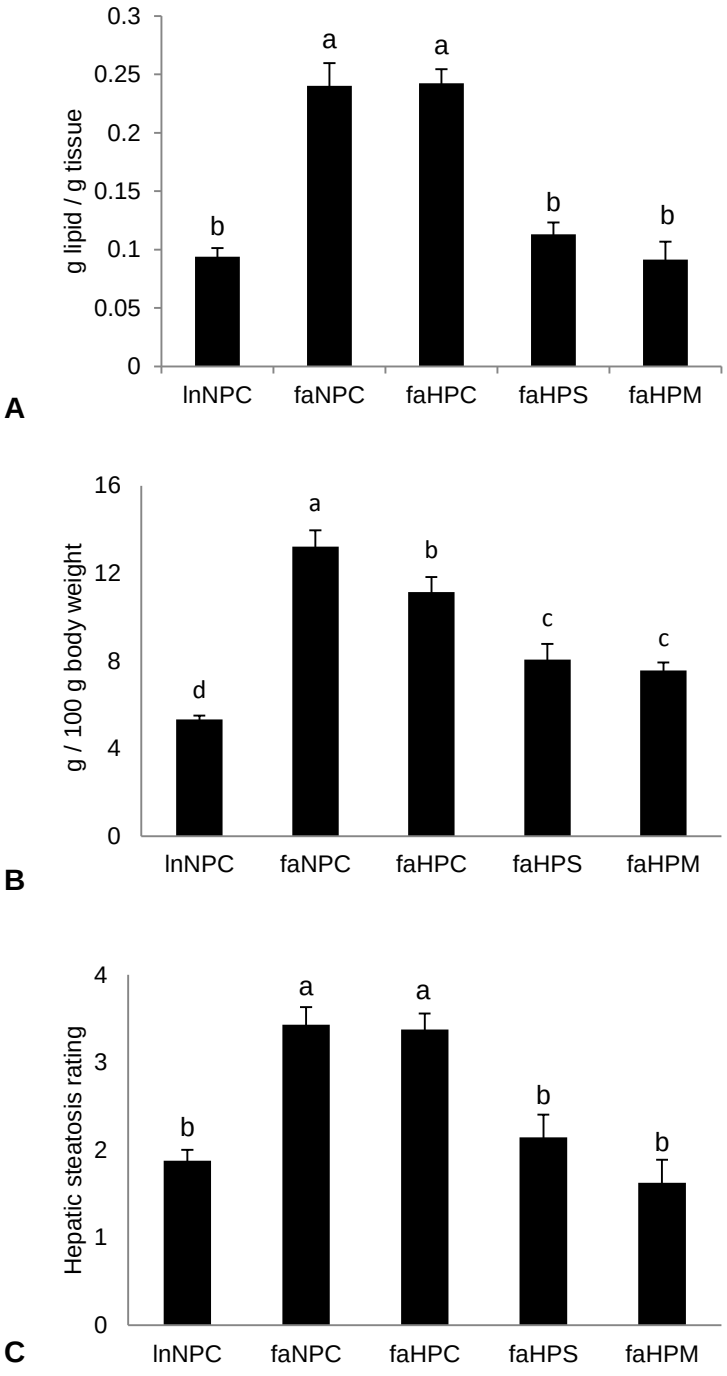


**FIGURE 5** Liver lipid concentration (A), liver weight per body weight (B) and hepatic steatosis rating (C) in lean and *fa/fa* Zucker rats given normal or high protein diets for 12 weeks. Data are presented as means ± SEM (n = 8 – 11 for liver weight; n = 4 per group for liver lipid concentration; n = 6 – 8 for hepatic steatosis rating). Values with different superscript letters are significantly different ($P < 0.05$) from each other.

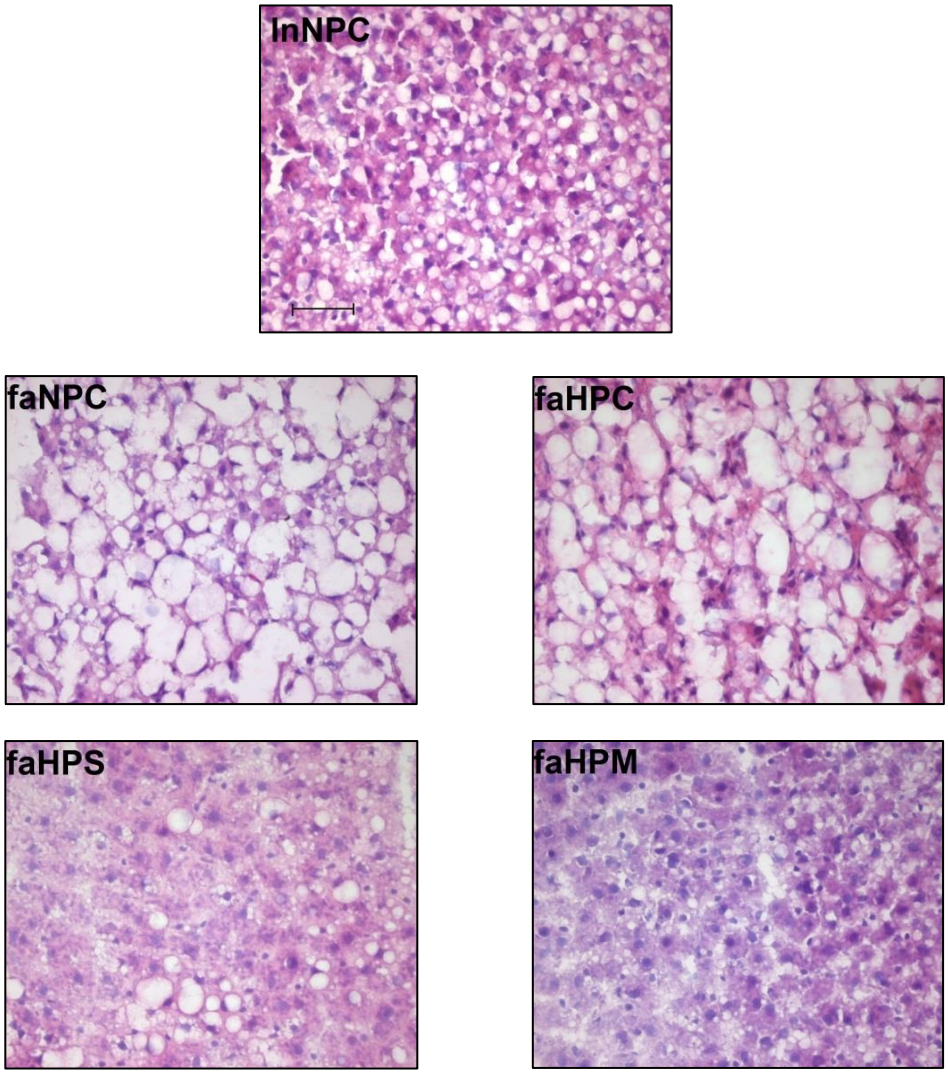


**FIGURE 6** Liver sections stained with hematoxylin and eosin from lean and *fa/fa* Zucker rats given normal or high protein diets for 12 weeks. Scale bar = 50 µm.

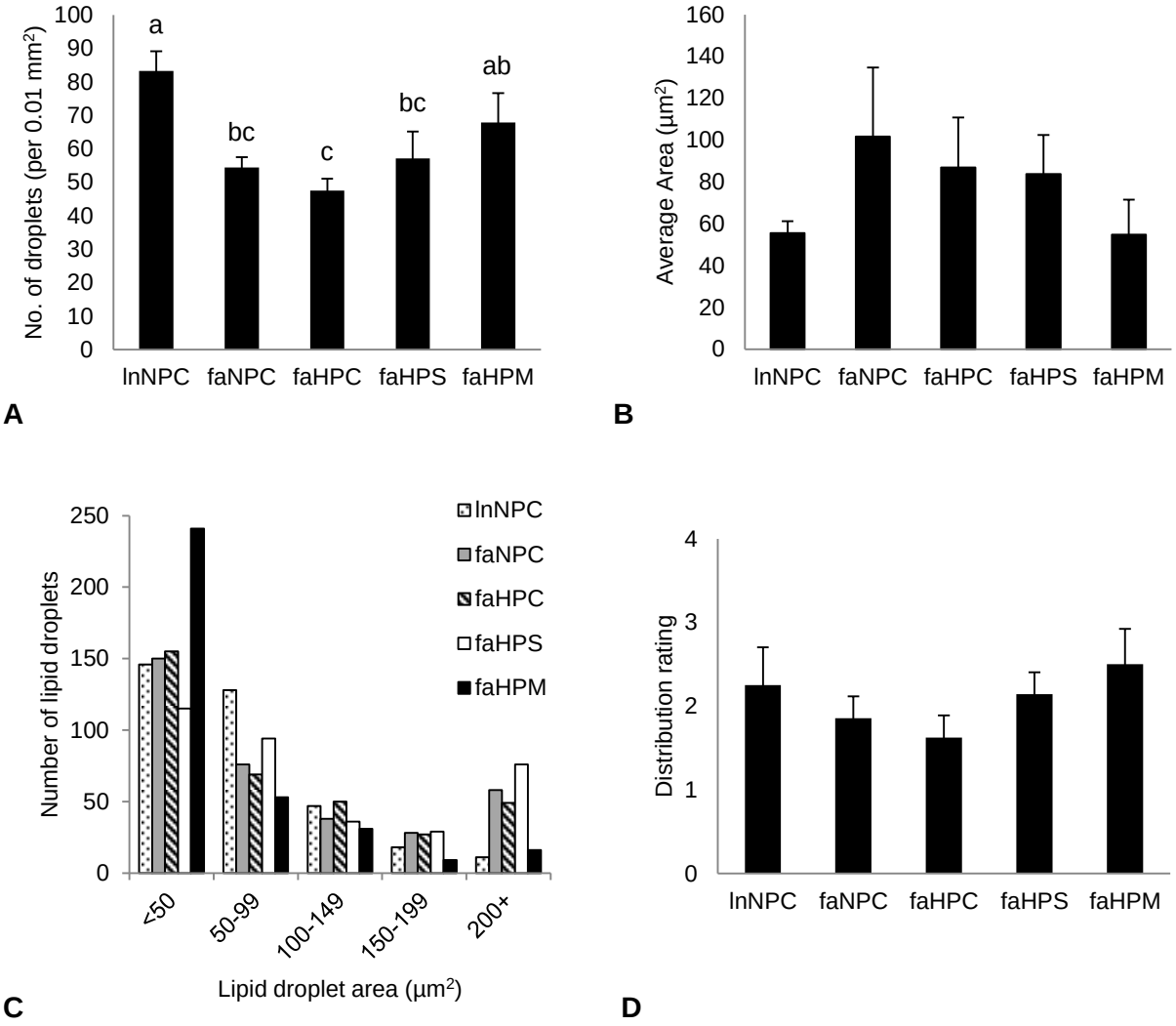


**FIGURE 7** Number of liver lipid droplets (A), size of liver lipid droplets (B), size distribution of liver lipid droplets (C) and liver lipid droplet distribution rating (D) in lean and *fa/fa* Zucker rats given normal or high protein diets for 12 weeks. Data are presented as means ± SEM (n = 6 – 8 per group). Values with different superscript letters are significantly different ($P < 0.05$) from each other. For (C), chi-squared testing indicated differences among the dietary groups for all size ranges except the 100 – 149 grouping.

altering the source of protein in a high protein diet had an effect on the number of liver lipid droplets with the faHPM group having 30% more liver lipid droplets compared to the faHPC group (Fig 7A). A higher number of lipid droplets in the faHPM group was associated with smaller lipid droplet area compared to the faHPS and faHPC groups (35% and 37% smaller, respectively); however, due to the large variation in data, there were no differences among groups for liver lipid droplet size (**Fig 7B**).

For size distribution of liver lipid droplets, chi-squared testing indicated differences among the dietary groups for all size ranges except the 100 – 149 grouping (**Fig 7C**). Upon visual examination, it appeared that the faHPM group had the largest proportion of small lipid droplets (< 50 grouping) and the smallest proportion of large lipid droplets (150 – 199 and 200+ groupings) compared to the faHPS and faHPC groups (Fig 7C). The faHPS group had a smaller proportion of lipid droplets that were < 50 $\mu m^2$ compared to the faHPC and faHPS groups; however they had a larger proportion of lipid droplets in the 50 – 99 grouping. Interestingly, the faHPS group also had the largest proportion of larger lipid droplets (200+ group) compared to the other high protein diet groups (Fig 7C). The faHPC and faNPC group showed a similar pattern for the size distribution data (Fig 7C). As expected, the lnNPC group had larger proportions of small lipid droplets (< 50 and 50 – 99 groupings) and the smallest proportion of large lipid droplets (200+ grouping; Fig 7C). The distribution rating was completed to measure the uniformity of lipid droplet size across a tissue section and to complement the lipid droplet size data; however, due to the large variation in data no significant differences among groups were observed (**Fig 7D**).

## 2.6 Discussion

The main finding of the present study was that different sources of protein within a high protein diet had varying effects on insulin resistance and hepatic steatosis in the *fa/fa* Zucker rat, but no effects on body weight, food intake and measures of adiposity. Specifically, a high protein diet containing a mixture of animal- and plant-based protein sources was the most effective at modulating reductions in insulin resistance and hepatic steatosis, followed by a high protein diet with soy protein isolate as the protein source. A high protein diet with casein as the protein source was the least effective among the high protein diet groups as it demonstrated no improvements on insulin resistance or hepatic steatosis. Furthermore, the high protein casein diet demonstrated similar effects to those observed in the normal protein casein diet, confirming that the source of protein within a high protein diet is critical.

The proposed benefit of high protein diets on weight loss has been the primary reason why high protein diets have been promoted as an effective tool in managing the metabolic syndrome. Indeed, several studies have demonstrated reductions in body weight and fat mass with the consumption of *ad libitum* high protein diets (Claessens, van Baak, Monsheimer, & Saris, 2009; Due, Toubro, Skov, & Astrup, 2004; McAuley et al., 2005; McAuley et al., 2006; Samaha et al., 2003; Skov, Toubro, Ronn, Holm, & Astrup, 1999). However, in the present study, *ad libitum* intake of high protein diets did not result in a reduction in body weight or measures of adiposity in *fa/fa* Zucker rats, regardless of the protein source.

Perhaps the reason reductions in body weight and adiposity were not observed was due to the fact that the high protein diets did not cause a reduction in food intake in the *fa/fa* groups. It has been proposed that under *ad libitum* conditions, subjects tend to have reduced energy intake when consuming a high protein diet compared to a high carbohydrate diet due to the satiating effect of foods high in protein. This reduced

energy intake leads to reductions in weight (Weigle et al., 2005). It is possible that the high protein diets provided to the animals were not more satiating compared to the normal protein diet. It could also be argued that because *fa/fa* Zucker rats have a mutation in the leptin receptor which leads to hyperphagia, perhaps any satiating effects from the high protein diets were simply overpowered by the genetic predisposition of *fa/fa* Zucker rats to overeat. Other studies that have compared high protein diets to normal protein diets under *ad libitum* conditions using different animal models have demonstrated both reductions and no differences in food intake. For example, in a recent study that used a model of diet-induced obesity (DIO), Wistar rats were given a high fat diet for nine weeks to induce obesity and then switched to either a low protein or high protein diet. The high protein diet resulted in reduced energy intake, as well as reduced visceral and subcutaneous fat compared to the low protein diet (Belobrajdic, McIntosh, & Owens, 2004). Another study using Wistar rats also demonstrated reduced energy intake in rats given a high protein diet compared to those given a normal protein diet over a six month period (Lacroix et al., 2004). Conversely, three studies using Sprague-Dawley rats (Baum et al., 2006; Devkota & Layman, 2011; Wakefield, House, Ogborn, Weiler, & Aukema, 2011) and one study using Wistar rats (Aparicio et al., 2013) all demonstrated no differences in food intake between the high protein and normal protein diet groups.

The proposed benefit of providing diets *ad libitum* is that *ad libitum* intake is more likely to detect unique properties of protein in facilitating weight loss as opposed to energy-restricted diets, since strict control of energy will achieve clinically important weight loss that will conceal any relevant metabolic effects of protein (Te Morenga & Mann, 2012). In fact, when examining energy-restricted weight loss diets that compare high protein diets to high carbohydrate diets, both diets result in similar reductions in body weight, and any improvements in metabolic syndrome parameters such as insulin

resistance or hyperlipidemia are attributed to the weight loss and not to the diet itself (Brinkworth et al., 2004; Layman, Clifton, Gannon, Krauss, & Nuttall, 2008; Noakes, Keogh, Foster, & Clifton, 2005)

Interestingly, despite no reductions in body weight or fat mass, *fa/fa* rats in the high protein diet groups had higher absolute LBM compared to the *fa/fa* rats in the normal protein casein group regardless of the protein source in the high protein diets. Studies have demonstrated that compared to high carbohydrate diets, high protein diets appear to preserve LBM during weight loss which is particularly relevant in regards to the metabolic syndrome as it can lead to enhanced insulin sensitivity (Baba et al., 1999; Claessens et al., 2009; Farnsworth et al., 2003; Piatti et al., 1994; Skov et al., 1999). Our results did demonstrate enhanced insulin sensitivity in the faHPM group. However, the faHPC and faHPS groups showed no improvements in insulin sensitivity. Furthermore, the increases in absolute LBM in the high protein diet groups were diminished once adjusted for body weight, and no further statistical differences were observed between the normal protein and high protein diet groups. As such, while the higher absolute LBM may have had a small role in enhancing insulin sensitivity in the faHPM group, it is much more likely that the enhanced insulin sensitivity in the faHPM group was due to other factors.

In assessing insulin sensitivity in the present study, the high protein mixed diet was the most effective at enhancing insulin sensitivity compared to both the high protein casein and high protein soy diets. Compared to the faHPC group, the faHPM group had lower $AUC_i$ during OGTT, and lower fasting serum insulin concentrations, HOMA-IR and insulin:glucose ratio at week 12. Compared to the faHPS group, the faHPM group had lower fasting serum insulin concentrations and HOMA-IR at week 12. It is important to note that plasma glucose and insulin concentrations under fasting conditions and during OGTT are commonly used to derive indexes of insulin sensitivity, such as HOMA-IR and

insulin:glucose ratio, and they correlate with insulin sensitivity measured with the gold-standard, hyperinsulinemic-euglycemic clamp (Abdul-Ghani, Tripathy, & DeFronzo, 2006).

In addition to the data presented above, examination of the pancreas further confirmed the beneficial effects being observed with the high protein mixed diet. Compared to the faHPC group, the faHPM group demonstrated pancreatic preservation as evidenced by lower pancreatic islet cell area (i.e. reduced β-cell hypertrophy). The smaller islet cells in the faHPM group resulted in reduced pancreatic insulin production which lead to lower fasting serum insulin at week 12 and during OGTT, which in turn was reflected in lower HOMA-IR, insulin:glucose ratio and $AUC_i$. Since there were no differences in fasting blood glucose concentrations among groups at week 12, it can be stated that due to increased insulin sensitivity in peripheral tissues, the pancreas had to secrete less insulin in the faHPM group to maintain blood glucose concentrations, and the lower HOMA-IR and insulin:glucose ratio were attributed to the reduced pancreatic insulin production and enhanced insulin sensitivity. The faHPM group also had a higher pancreas to body weight ratio which was similar to the pancreas to body weight ratio of the lnNPC group.

Conversely, the higher HOMA-IR and insulin:glucose ratio at week 12 and $AUC_i$ during OGTT in the faHPC group compared to the faHPM group were likely a result from an increase in pancreatic insulin production as indicated by larger islet size (i.e. increased β-cell hypertrophy) and higher fasting serum insulin concentrations at week 12 and during OGTT. Furthermore, the lower pancreas to body weight ratio in the faHPC group compared to both the faHPM and faHPS groups is also a reflection of β-cell destruction, an important etiological factor in the development and progression of T2DM (DeFronzo, 2004; Gastaldelli et al., 2004; Saad et al., 1989).

Given these findings, we were surprised to see that during OGTT, the faHPM group had higher $AUC_g$ compared to the faHPC group due to elevated serum glucose concentrations at 15, 30 and 60 minutes after ingestion of glucose. Despite these elevations in blood glucose concentrations, serum insulin concentrations remained relatively constant in the faHPM group and were the lowest concentrations among all *fa/fa* groups at all time points, although only significant differences were observed at t = 0, 60 and 120. The elevated blood glucose concentrations during these time points suggest that different sources of protein have varying post-prandial responses; however, the exact reasoning for this cannot be explained. Perhaps the varying post-prandial effects of different protein sources is related to the composition of amino acids and digestibility of the protein, which has been shown to have different effects on the concentrations of hormones involved in glucose and insulin metabolism such as glucagon-like-peptide-1 (GLP-1) (Te Morenga & Mann, 2012; Veldhorst et al., 2008). It is important to recognize though that after 120 minutes, blood glucose concentrations in the faHPM group returned to baseline levels and no differences among any of the high protein diet groups were observed. Furthermore, blood glucose concentrations were able to return to baseline without requiring the pancreas to secrete more insulin. Due to the higher $AUC_g$ in the faHPM group compared to the faHPC group, we also did not see a statistically lower $AUC_{index}$, which is another marker to measure insulin sensitivity, in the faHPM group compared to the faHPC group.

In assessing the effects of the high protein soy diet on insulin sensitivity, the faHPS group did not demonstrate any improvements except for having a pancreas to body weight ratio that was similar to that of the faHPM and lnNPC groups and higher than the faHPC group. This data suggests that soy protein alone is not sufficient to result in improvements in insulin sensitivity, but as part of a high protein mixed diet it is effective.

While the faHPC group demonstrated no improvements in measures of insulin resistance compared to the faHPS and faHPM groups, all data used to assess both insulin sensitivity and pancreatic function in the faHPC group were similar to the faNPC group. This has two important implications. Firstly, simply increasing the casein protein content of the diet is not enough to modulate reductions in insulin resistance. Secondly, the similar values between the faHPC and faNPC group signify that the high protein casein diet was not detrimental, rather it just did not have any positive effects. This is important because some studies have reported that consumption of high protein diets leads to negative effects on insulin resistance (Linn et al., 2000; Tremblay et al., 2007; Weickert et al., 2011).

It was somewhat surprising to see that the high protein soy diet had a lesser effect on insulin resistance compared to the high protein mixed diet as several studies have demonstrated positive effects of soy protein on insulin resistance. For example, male lean SHHF (+/cp) rats, a unique rat model that exhibits the early features of the metabolic syndrome, were given casein or soy protein for 36 weeks. Rats given soy protein had significantly lower body weight, liver weight, fasting blood glucose, and plasma insulin compared to rats given casein (Davis et al., 2005). In another study, consumption of a high-isoflavone soy protein diet improved glucose tolerance, insulin resistance, and hepatic cholesterol and triglyceride levels in obese Zucker rats (Mezei et al., 2003).

However, the high protein mixed diet contained complete milk protein, in addition to soy protein isolate, wheat gluten and egg white. Several studies have reported positive effects of dairy intake on insulin sensitivity. For instance, two recent large epidemiological studies reported dairy intake was positively linked to insulin sensitivity (Choi, Willett, Stampfer, Rimm, & Hu, 2005; Liu et al., 2006). In addition, the Coronary Artery Risk Developing in Young Adults study found a 21% reduction in risk of insulin

resistance in overweight individuals with each daily serving of low-fat dairy food (Pereira et al., 2002). In another study, complete dairy protein was shown to improve insulin sensitivity in a DIO rat model (Eller & Reimer, 2010). So perhaps the high protein mixed diet was the most effective diet at improving insulin resistance due to the addition of complete milk protein, or perhaps it was the synergistic effect of all four protein sources within the high protein mixed diet that resulted in the beneficial effects.

Despite the differences observed among the groups in regards to insulin resistance, we did not see any differences in the insulin signaling proteins IRS-1 and Akt in skeletal muscle among groups. The action of insulin is mediated through a tightly regulated signaling cascade that eventually results in glucose uptake into muscle cells. During insulin resistance, elevated plasma FFA and accumulation of triglycerides in muscle cells causes an increase in serine phosphorylation of IRS-1, the main docking protein in skeletal muscle, preventing the tyrosine phosphorylation of IRS-1 that is required to continue the insulin signaling cascade process. This disruption in the insulin signaling cascade process results in impaired Akt activation due to decreased phosphorylation of Ser473 and Thr308, and ultimately a decrease in glucose transport (Abdul-Ghani & DeFronzo, 2010). As such, examining the expression of the total and phosphorylated forms of IRS-1 and Akt are critical in understanding the effects of high protein diets at the cellular level.

The insulin signaling protein data in the present study reflects the response to chronic insulin exposure as Western immunoblotting was performed on gastrocnemius muscle tissue that was collected after a 12 hour fast. Since insulin levels are relatively low in the fasting state, insulin signaling proteins may not be active in the fasting state. As such, it may have been more appropriate to use a different method that ensures activation of insulin signaling proteins. For example, an acute insulin stimulation method, which involves injection of insulin prior to removal of tissue, has been used in

previous studies that have examined the effects of different protein sources on insulin signaling proteins, with differences being observed (Tremblay, Lavigne, Jacques, & Marette, 2003). Alternatively, other high protein studies have measured insulin signaling protein levels following consumption of a meal. For instance, in a recent study, total and phosphorylated forms of Akt, p70S6K and Erk1/2 were measured in muscle and adipose tissue of Sprague-Dawley rats prior to either a high carbohydrate or high protein meal (12 hour fasting) and 30 and 90 minutes following the meal. There were no differences in any of the insulin signaling proteins in either muscle or adipose tissue in the fasted state. However, differences were observed at 30 and 90 minutes following consumption of either a high carbohydrate or high protein meal (Devkota & Layman, 2011).

While the improvement in insulin sensitivity observed in the faHPM group would contribute to pancreatic β-cell preservation, it is also closely linked to the profound reduction in hepatic lipid accumulation observed in the faHPM group as evidenced by lower total liver lipid concentrations, lower liver to body weight ratio, and lower hepatic steatosis rating compared to the faHPC group. It is well established that reduction in hepatic fat contributes to improved glucose handling since hepatic insulin resistance secondary to hepatic lipid accumulation leads to increased rates of gluconeogenesis and glycogenolysis (Parekh & Anania, 2007). As such, the combined reduction in hepatic lipid accumulation and insulin resistance observed with the high protein mixed diet in *fa/fa* rats clearly results from simultaneous improvements in the function of several tissue systems, illustrating the importance of investigating multi-organ responses to high protein diets in explaining their effects.

The faHPS group also demonstrated profound reductions in hepatic lipid accumulation with the faHPM and faHPS groups both demonstrating lower liver to body weight ratios, lower hepatic lipid concentrations and lower hepatic steatosis ratings compared to the faHPC group. The total liver lipid concentration and hepatic steatosis

rating in the faHPM and faHPS groups were also similar to the values in the lnNPC group. Given the similar results between the faHPS and faHPM groups for hepatic steatosis, and the strong association between insulin resistance and hepatic steatosis, it was surprising that only the faHPM group demonstrated statistically significant improvements in insulin resistance as previously indicated. The high protein casein diet demonstrated no beneficial effects on hepatic steatosis in *fa/fa* rats except for reduced liver to body weight ratio compared to the faNPC group, suggesting that the source of protein within a high protein diet is critical at reducing hepatic steatosis.

While the study of high protein intake on NAFLD is relatively new, the majority of studies thus far have shown favorable effects of high protein diets on the liver. For instance, a high protein diet (35% energy from protein) prevented and reversed hepatic steatosis, the first stage of NAFLD, in male C57Bl/6 mice independently of fat and carbohydrate intake and more efficiently than a 20% reduction in energy intake. Interestingly, mice given the high protein diet gained less body weight and had smaller epididymal fat pads compared to mice given a low protein diet (11% energy from protein), but liver weight was not different between the groups (Garcia-Caraballo et al., 2013). Other studies have demonstrated that the source of protein appears to play a critical role in the effects of high protein on NAFLD. For instance, soy protein prevented the development of fatty liver in Wistar rats, while those given a casein diet developed fatty liver. Rats given the soy protein diet also demonstrated lower insulin concentrations compared to the casein-fed rats, but there was no difference in weight gain between the two groups (Ascencio et al., 2004).

Staining of liver sections allowed us to quantify lipid droplet number and size to further support hepatic lipid accumulation data and verify the attenuation of hepatic steatosis. The high protein mixed diet was associated with a greater number of hepatic lipid droplets compared to the high protein casein diet; however, the smaller size of

these droplets meant fewer lipids were present in the cells. This finding is consistent with the lower total liver lipid concentration in *fa/fa* Zucker rats given a high protein mixed diet. The faHPS group had the largest proportion of large lipid droplets but also the highest number of lipid droplets in the 50 – 99 grouping compared to the other high protein diet groups. The combination of large and small lipid droplets in the faHPS group suggests that total hepatic lipid measurements do not reflect number, size and size distribution of lipid droplets. The fact that we did not observe any differences between the faNPC and faHPC groups in lipid droplet number, size and size distribution data is consistent with other data measured in the present study that also failed to show differences between these two groups.

Interestingly, reductions in both insulin resistance and hepatic steatosis were observed despite no reductions in body weight. This is a unique finding considering the critical role of obesity in the development of both insulin resistance and NAFLD and the fact that the majority of studies that have demonstrated beneficial effects of high protein diets on metabolic syndrome parameters attribute these benefits solely to reductions in body weight (Brinkworth et al., 2004; Noakes et al., 2005; Soenen et al., 2013; Westerterp-Plantenga, Lemmens, & Westerterp, 2012). Only a limited number of studies have been able to demonstrate improvements due to the actual high protein diet and not attributed to any corresponding weight loss (Farnsworth et al., 2003). For instance, after only 3 weeks on either a high protein or high carbohydrate diet, obese women consuming a high protein diet showed improvements in insulin sensitivity despite no significant differences in weight loss between the two groups. These improvements were attributed to a greater retention of LBM in the high protein diet group (Piatti et al., 1994). Another study demonstrated that while high protein diets (32% of energy) were responsible for reducing energy intake and adiposity, whey protein (provided at either 8% or 32% of energy) was more effective than red meat at increasing insulin sensitivity

(Belobrajdic et al., 2004). Finally, in previous studies in our lab, *fa/fa* Zucker rats given a mixture of conjugated linoleic acid (CLA) isomers, a naturally occurring group of positional and geometric linoleic acid isomers present in ruminant meat and dairy products, had improved peripheral insulin resistance and oral glucose tolerance, and reduced hepatic steatosis despite an increase in visceral adipose mass (Noto, Zahradka, Ryz et al., 2007; Noto, Zahradka, Yurkova et al., 2007).

A confounding factor of the present study is we do not know whether the beneficial effects of high protein diets on insulin resistance and hepatic steatosis are due to an increase in protein or a reduction in carbohydrates. Several studies have reported that a high protein intake has favorable effects on insulin resistance due to a lower glycemic load because of reduced carbohydrate intake (Layman et al., 2003; Layman et al., 2003; Noakes et al., 2005). It is also well documented that dietary carbohydrates, particularly simple sugars, promote dyslipidemia (Grundy, 1997; Layman et al., 2008). In fact, high protein studies that have demonstrated improvements in blood lipids often have difficulty concluding whether it is an increase in protein or a reduction in carbohydrates that is responsible for the favorable effects on blood lipids.

The assessment of serum FFA concentrations can be used as a reflection of both insulin resistance and hepatic steatosis. Both obesity and insulin resistance cause an increase in circulating serum FFA concentrations (Grundy et al., 2004). The increased FFA also impacts hepatic lipid production by decreasing HDL cholesterol and increasing LDL cholesterol and triglycerides, which further enhances insulin resistance (Grundy et al., 2004; Potenza & Mechanick, 2009). In addition, increased serum FFA concentrations due to obesity play a critical role in the transition from hepatic steatosis to nonalcoholic steatohepatitis (NASH) (Basaranoglu et al., 2010). Due to the beneficial effects the high protein mixed diet had on both insulin resistance and hepatic steatosis, it was not surprising to see that the faHPM group had lower fasting serum FFA

concentrations at week 12 compared to the faHPS group. Although, it is interesting that the faHPS group and elevated fasting serum FFA concentrations compared to the faHPM group considering both groups demonstrated similar reductions in hepatic steatosis. Perhaps the increased serum FFA concentrations in the faHPS group is one of the reasons why the faHPS group did not demonstrate improved insulin sensitivity. Furthermore, reductions in serum triglycerides were not observed among the *fa/fa* groups which was again interesting considering the reductions in insulin resistance and hepatic steatosis observed in the faHPM group, and reductions in hepatic steatosis observed in the faHPS group. Perhaps this is due to the fact that there was no weight loss or reduction in fat mass in the *fa/fa* rats, as similar to other metabolic syndrome parameters, the majority of high protein studies that have demonstrated improvements in triglycerides in conjunction with weight loss have attributed the improvements to the reduction in body weight and not to the high protein diet itself (Azadbakht, Izadi, Surkan, & Esmaillzadeh, 2013; Layman et al., 2008).

In conclusion, we demonstrated that *ad libitum* high protein diets had varying beneficial effects on insulin resistance and hepatic steatosis dependent on the source of protein within the diet, but no effect on body weight, food intake or adiposity in *fa/fa* Zucker rats. Future studies are required to determine the biologically active components in the high protein mixed and high protein soy diets that are responsible for the beneficial effects observed. Furthermore, comparing the high protein mixed and high protein soy diets to normal protein mixed and normal protein soy diets will provide further clarity as whether the source of protein is solely responsible for improvements in metabolic syndrome parameters, or whether it is both the source and amount of protein provided in the diet that results in the beneficial outcomes.

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# CHAPTER 3 – OVERALL DISCUSSION

### 3.1 Summary

The metabolic syndrome, a collection of risk factors including obesity, insulin resistance, dyslipidemia, hypertension, inflammation and NAFLD, is a major health issue due to the growing obesity epidemic. Moderate weight loss is the first-line approach for managing the metabolic syndrome and preventing the progression to cardiovascular disease or T2DM. There has been increasing interest regarding the consumption of high protein diets as an effective tool in reducing body weight and improving metabolic syndrome parameters. However, results have been inconsistent making it difficult for researchers and health care professionals to make concrete conclusions regarding the use of high protein diets in the treatment and management of the metabolic syndrome.

In the present study, high protein diets did not reduce body weight, food intake or fat mass regardless of the protein source used in the high protein diet. However, different sources of protein within a high protein diet did have varying effects on insulin resistance and hepatic steatosis in the *fa/fa* Zucker rat, an animal model commonly used to study the metabolic syndrome. Specifically, a high protein diet containing a mixture of animal- and plant-based protein sources was the most effective at modulating reductions in insulin resistance and hepatic steatosis. This was followed by a high protein diet with soy protein isolate as the protein source, with mainly reductions in hepatic steatosis being observed in *fa/fa* rats consuming the high protein soy diet. Finally, a high protein diet with casein as the protein source was the least effective high protein diet, demonstrating no improvements in insulin resistance and hepatic steatosis.

The results of the study partially support the general hypothesis. The first part of the hypothesis was that a high protein intake would have positive effects on metabolic syndrome parameters compared to normal protein intake. This hypothesis was not supported, as the high protein casein diet and normal protein casein diet showed similar effects on nearly all metabolic syndrome parameters assessed except for a higher

absolute LBM and a lower liver to body weight ratio in the faHPC group compared to the faNPC group. Therefore, simply increasing the casein protein content of the diet was not sufficient to produce improvements in metabolic syndrome parameters assessed in *fa/fa* Zucker rats.

The second part of the hypothesis was that varying the source of protein in a high protein diet would have positive effects on metabolic syndrome parameters, with the high protein soy diet being the most beneficial, followed by the high protein mixed diet, and lastly the high protein casein diet. This hypothesis was partially supported as varying the source of protein in a high protein diet did indeed result in beneficial effects on metabolic syndrome parameters. However, the high protein mixed diet was the most beneficial diet as *fa/fa* rats consuming the high protein mixed diet demonstrated reductions in both insulin resistance and hepatic steatosis, whereas *fa/fa* rats consuming the high protein soy diet mainly demonstrated reductions in hepatic steatosis. As mentioned above, the high protein casein diet was indeed the least effective at improving metabolic syndrome parameters. It is important to mention though that there were no differences among the high protein diet groups for measures of obesity (i.e. body weight, food intake, fat mass), triglycerides, haptoglobin, insulin signaling protein levels, liver lipid droplet size and distribution rating. Therefore, even altering the source of protein in a high protein diet did not result in beneficial effects on all metabolic syndrome parameters assessed in the present study. At the same time, no detrimental effects were observed among the high protein diet groups on the metabolic syndrome parameters assessed.

Specifically, the high protein diets had the following improvements (statistically significant) on the metabolic syndrome parameters assessed in the present study:

*High protein mixed diet vs. high protein casein diet*

- Lower $AUC_i$ during OGTT
- Lower fasting serum insulin concentrations, HOMA-IR and insulin:glucose ratio at week 12
- Lower pancreatic islet cell area
- Higher pancreas to body weight ratio
- Lower liver to body weight ratio
- Lower hepatic lipid concentrations
- Lower hepatic steatosis rating
- Higher number of liver lipid droplets

*High protein mixed diet vs. high protein soy diet*

- Lower fasting serum insulin concentrations and HOMA-IR at week 12
- Lower fasting serum FFA concentrations at week 12

*High protein soy diet vs. high protein casein diet*

- Higher pancreas to body weight ratio
- Lower liver to body weight ratio
- Lower hepatic lipid concentrations
- Lower hepatic steatosis rating

*High protein casein diet vs. normal protein casein diet*

- Higher absolute LBM
- Reduced liver to body weight ratio

Table 5 further summarizes the effects of high protein diets on the metabolic syndrome parameters measured in this study. The first column compares the lnNPC and faNPC groups to determine if any genotype effects were observed. The second column compares the faNPC to faHPC groups to determine if there were differences between a normal protein casein diet and high protein casein diet on metabolic syndrome parameters, which was identified as the first objective of the study. Finally, the third column compares the faHPC, faHPS and faHPM groups to determine if different sources of protein in a high protein diet had varying effects on metabolic syndrome parameters, which was identified as the second objective of the study. In addition, the statistical results of the lnNPC group were used as a marker to determine if any of the high protein diet groups had similar values to the lnNPC group.

**TABLE 5** Summary of effects of high protein diets on metabolic syndrome parameters

| | Genotype Effect (lnNPC vs faNPC) | Objective 1 (faNPC vs faHPC) | Objective 2 (faHPC vs faHPS vs faHPM) |
|---|---|---|---|
| **OBESITY** | | | |
| Final body weight | faNPC had ↑ final body weight | No differences | No differences |
| Weight gain | No differences | No differences | No differences |
| Total food intake | faNPC had ↑ total food intake | No differences | No differences |
| Adiposity | faNPC had ↑ visceral adipose to body weight ratio<br><br>faNPC had ↑ perirenal to adipose body weight ratios<br><br>No differences in mesenteric to body weight ratio<br><br>No differences in epididymal adipose to body weight ratio | No differences | No differences |
| LBM | faNPC had ↓ absolute LBM and LBM to body weight ratio | faHPC had ↑ absolute LBM but no difference in LBM to body weight ratio | No differences |
| **INSULIN RESISTANCE** | | | |
| OGTT | faNPC had ↑ $AUC_i$ and $AUC_{index}$<br><br>No differences in $AUC_g$ | No differences | faHPM had ↑ $AUC_g$ and ↓ $AUC_i$ compared to faHPC<br><br>faHPM had similar $AUC_i$ and $AUC_{index}$ values to lnNPC |

**TABLE 5 (continued)**

| | Genotype Effect (lnNPC vs faNPC) | Objective 1 (faNPC vs faHPC) | Objective 2 (faHPC vs faHPS vs faHPM) |
|---|---|---|---|
| Fasting serum glucose and insulin – week 12 | faNPC had ↑ fasting insulin, I:G ratio, and HOMA-IR<br><br>No differences in fasting glucose | No differences | faHPM had ↓ fasting insulin and HOMA-IR compared to faHPS and faHPC; and ↓ I:G ratio compared to faHPC<br><br>faHPM had similar fasting insulin values to lnNPC<br><br>No differences in fasting glucose |
| Pancreas | faNPC had ↑ islet cell area<br><br>faNPC had ↓ pancreas to body weight ratio | No differences | faHPM had ↓ islet cell area compared to faHPC<br><br>faHPM had similar islet cell area to lnNPC<br><br>faHPM and faHPS had ↑ pancreas to body weight ratios; similar values to lnNPC |
| Insulin signaling protein levels | No differences | No differences | No differences |
| **DYSLIPIDEMIA** | | | |
| Fasting serum FFA – week 12 | No differences | No differences | faHPM had ↓ fasting FFA compared to faHPS |
| Fasting serum TG – week 12 | faNPC had ↑ fasting TG | No differences | No differences |
| **INFLAMMATION** | | | |
| Fasting serum haptoglobin – week 12 | faNPC had ↑ fasting haptoglobin | No differences | No differences |

**TABLE 5 (continued)**

| | Genotype Effect (lnNPC vs faNPC) | Objective 1 (faNPC vs faHPC) | Objective 2 (faHPC vs faHPS vs faHPM) |
|---|---|---|---|
| **NAFLD** | | | |
| Liver weight | faNPC had ↑ liver to body weight ratio | faHPC had ↓ liver to body weight ratio | faHPM and faHPS had ↓ liver to body weight ratio |
| Hepatic lipid concentra-tion | faNPC had ↑ hepatic lipids | No differences | faHPM and faHPS had ↓ hepatic lipids; similar values to lnNPC |
| Hepatic steatosis rating | faNPC had ↑ hepatic steatosis rating | No differences | faHPM and faHPS had ↓ hepatic steatosis rating; similar values to lnNPC |
| Number of lipid droplets | faNPC had ↓ number | No differences | faHPM had ↑ number compared to faHPC |
| Lipid droplet size | No differences | No differences | No differences |
| Distribution rating | No differences | No differences | No differences |

**KEY:** $AUC_g$ = area under the curve, glucose; $AUC_i$ = area under the curve, insulin; $AUC_{index}$ = area under the curve, glucose-insulin index; FFA = free fatty acids; HOMA-IR = homeostatic model assessment index for insulin resistance; I:G ratio = insulin to glucose ratio; LBM = lean body mass; NS = not significant; OGTT = oral glucose tolerance testing; TG = triglycerides

### 3.2 Conclusion

In conclusion, a high protein diet containing a mixture of animal- and plant-based protein sources was the most effective at improving certain metabolic syndrome parameters, particularly insulin resistance and hepatic steatosis, assessed in the *fa/fa* Zucker rat. The next most effective diet was a high protein diet with soy protein isolate as the protein source. The improvements observed in the high protein mixed and soy diet groups were likely attributed mainly to the effect of both pancreatic β-cell preservation and reduced hepatic lipid accumulation, while an increase in absolute LBM played a lesser role. Interestingly, the improvements in insulin resistance and hepatic steatosis observed in the high protein mixed and soy diet groups occurred despite no reductions in body weight or fat mass. A high protein diet with casein as the protein source was the least effective high protein diet as it demonstrated no benefits on improving metabolic syndrome parameters compared to the high protein mixed and soy diets, and showed similar effects to those observed with the normal protein casein diet. This confirms that the source of protein within a high protein diet is critical.

While the results of the present study provide promise to obese individuals with insulin resistance and hepatic steatosis who may struggle with weight loss, it still must be stressed that obesity is still one of the major underlying causes of the metabolic syndrome and is strongly linked to a number of health conditions including cardiovascular disease and T2DM. In addition, the fact that *ad libitum* intake of the high protein diets did not cause a reduction in body weight and fat mass may also explain the lack of improvement in dyslipidemia and inflammation. Therefore, while high protein diets may be a tool for the treatment of specific metabolic syndrome parameters, more research is required to determine the most effective protein sources within a high protein diet for reducing all metabolic syndrome parameters including obesity.

### 3.3 Strengths

- While prevention of obesity is key, the current obesity epidemic has amplified the need for effective treatment options to prevent the development of further debilitating health conditions such as cardiovascular disease and T2DM. Therefore, the fact that this study was a treatment design makes it more applicable to today's society as it can be used as an effective tool to manage obesity and the metabolic syndrome.
- The *fa/fa* Zucker rat was an appropriate rodent model to use as it is the most widely used animal model of genetic obesity and the most representative rat strain to study metabolic syndrome parameters.
- The use of the lean control group allowed us to determine if improvements of metabolic syndrome parameters were similar to lean control normal values.
- All metabolic syndrome parameters (obesity, insulin resistance, dyslipidemia, hypertension, inflammation and NAFLD) were examined in a variety of ways.
- The macronutrient distribution of all experimental diets was representative of the current AMDRs for carbohydrate, fat and protein. In addition, obesity development in the *fa/fa* Zucker rat is not dependent on a high fat diet which could have limited the diet design. For instance, if a DIO model were used, a high fat diet consisting of approximately 45 – 60% energy from fat, well above the current AMDR for fat, would be needed to develop obesity. Furthermore, maintaining the protein content of the high protein diets at 35% of energy would result in carbohydrate intake being between 5 – 20% of energy, which is well below its current AMDR.
- The use of *ad libitum* intakes increased the likelihood of detecting unique metabolic effects of protein since energy-restricted diets would have likely resulted in weight loss among all groups that would have masked any potential metabolic effects of protein.

- The use of an animal model that did not lose weight with high protein intake allows for the separation of weight loss and diet effects.
- The HPM diet was representative of the consumption of protein in the Canadian diet which is typically a mixture of both animal- and plant-based protein sources.
- Staining of liver sections allowed us to further support hepatic lipid accumulation data and verify the attenuation of hepatic steatosis.
- Quantification of pancreatic islet cell size provided histological data to support insulin resistance results.

### 3.4 Limitations

- While multiple tools were used to assess insulin resistance, the hyperinsulinemic euglycemic clamp still remains the gold standard for this assessment.
- Insulin sensitivity could have been assessed by an insulin tolerance test, a test whereby animals are injected with insulin and blood glucose is then measured at specific time intervals. Animals with larger reductions in blood glucose are more insulin sensitive compared to those with lower reductions.
- We cannot confirm whether improvements in insulin resistance and hepatic steatosis observed in the faHPM and faHPS groups were due entirely to protein content or whether a reduction in carbohydrate intake in these diets played a role.
- While a normal protein casein diet was used to compare the effects of a high protein casein diet, we did not have a normal protein soy diet or normal protein mixed diet in the *fa/fa* groups. Therefore, we do not know whether the reductions in insulin resistance and hepatic steatosis observed in faHPM and faHPS groups were due solely to the protein source, or whether they were attributed to the amount of protein.
- No baseline data was collected so we could not state whether data was “increased” or “decreased”.
- We do not know which of the four components in the high protein mixed diet (soy protein, wheat gluten, complete milk protein, egg white) was mainly responsible for the reductions observed in insulin resistance and hepatic steatosis and/or whether it was the synergistic effect of these four components acting together that resulted in the improvements.
- Muscle tissue used for Western immunoblotting of insulin signaling molecules was collected after a 12 hour fast, representing chronic basal insulin exposure under fasting conditions. It may be more appropriate to use an insulin stimulation method

just before tissue collection to determine insulin signaling protein activity in response to acute insulin stimulation, and/or collect tissue at certain time points following consumption of meal. However, this would have required more animals.

- Since the reduction in hepatic lipid accumulation contributed to the improvements in insulin sensitivity, specifically in the faHPM group, Western immunoblotting on liver tissue may have provided more insight regarding the mechanism behind the effects of high protein diets on insulin resistance and hepatic steatosis. For example, studies examining the effects of dietary interventions on hepatic steatosis have assessed levels of sterol-regulatory element binding protein-1 (SREBP-1) in the liver. This is due to evidence indicating that hepatic lipid accumulation in insulin-resistant states is caused by the activation of SREBP-1, which is increased in response to high insulin levels. As SREBP-1 activates genes involved in fatty acid synthesis, an increase in SREBP-1 increases the rate of lipogenesis in the liver (Tovar et al., 2005). Interestingly, one study demonstrated that compared to casein, soy protein prevented the development of hepatic steatosis in rats due to lower serum insulin concentrations and reduced expression of hepatic SREBP-1 (Ascencio et al., 2004).
- While serum was collected at week 6, we could not accurately compare the 6 week and 12 week serum values due to different blood collection procedures (jugular vs. trunk) and different lengths of fasting (5 hour vs. 12 hour).
- Parameters of kidney function and bone health, which could have been used to assess any adverse effects of high protein intake, were beyond the scope of this project.

### 3.5 Future Research

One of the main unanswered questions is whether the beneficial effects observed for insulin resistance and hepatic steatosis in *fa/fa* Zucker rats given high protein mixed and high protein soy diets were due to the high protein content (i.e. 35% of energy) or the source of protein. Future studies investigating normal protein mixed and normal protein soy diet groups (i.e. 15% energy as protein) are required to clarify the role the source of protein compared to the amount of protein for improving insulin resistance and hepatic steatosis. In addition, future studies are needed to determine whether the reduced carbohydrate intake in the high protein diets played a role on modulating reductions in insulin resistance and hepatic steatosis. Furthermore, while the high protein mixed and high protein soy diets proved to have favorable effects, we do not know the biologically active components in these diets that are responsible for these improvements. Research in this area has focused on a few possibilities, specifically branched-chain amino acids such as leucine and isoflavone content of soy protein, but more studies are required to elucidate this further.

### 3.6 Implications

Firstly, this study was representative of the current AMDRs for protein, carbohydrate and fat. This has several implications:

- It is reasonable to expect individuals to be able to follow and adhere to the diet used in the present study as it does not require extreme dietary changes, such as eliminating certain foods or entire food groups which is commonly seen in very high protein/very low carbohydrate diets.
- The fact that the most effective diet was one that contained a mixture of animal- and plant-based protein sources meant to represent the typical protein consumption in the Canadian diet also means minimum dietary changes would be required, plus individuals would be able to consume 35% of energy as protein from food sources and not supplements.
- It is reasonable to expect health care professionals to recommend this type of diet due to the fact that it does not require extreme dietary changes, plus because the diet is representative of the current AMDRs, this helps ensure individuals will meet their daily requirements for all nutrients.
- The majority of studies that have examined the potential adverse effects of high protein diets containing 35% of energy as protein have concluded that this amount of protein is generally safe for people to consume (Johnstone, 2012; Santesso et al., 2012; Schwingshackl & Hoffmann, 2013).

Secondly, the beneficial effects of the high protein mixed and soy diets were observed despite no reductions in body weight or fat mass. Therefore, individuals who

have insulin resistance or hepatic steatosis would benefit from consuming this type of diet even if they were struggling with weight loss.

Finally, because we saw improvements in some components of the metabolic syndrome and no improvements in others, it is possible that individuals with the metabolic syndrome who present with certain risk factors may respond better to a particular dietary approach than those who present with a different set of risk factors. As such, tailoring the dietary treatment based on the specific metabolic syndrome parameters present in an individual may be a more successful approach in treating and managing the risk factors (Muzio et al., 2007)(){{}}().

# CHAPTER 4 - APPENDICES

**Protocol 1 – Glucose Assay**

Six week serum (5 – 7 hour fast, jugular blood), termination serum (12 hour fast, cardiac puncture) and serum from OGTT (5 hour fast, saphenous vein) were collected and initially stored on ice until centrifuged at 2000 g for 20 minutes at 4$^{o}$C. Serum was aliquoted and stored at -80$^{o}$C until samples were analyzed for glucose using an enzymatic colorimetric assay kit provided by Genzyme Diagnostics (Charlottetown PE, Catalogue No. 220-32). The principle of the assay is that in the presence of glucose oxidase, β-D-glucose, $O_2$ and $H_2O$ are converted to D-gluconic acid and $H_2O_2$. Next, $H_2O_2$, hydroxybenzoate and 4-aminoantipyrine react with peroxidase to form quinoneimine dye and $H_2O$. The quinoneimine dye that is produced can be measured and is proportional to the amount of glucose in the sample.

*Reagents:*

- Glucose colour reagent: A solution containing a buffer (pH 7.25 at 25ºC), 0.25 mmol/L 4-aminoantipyrine, 20 mmol/L p-hydroxybenzoate, >40,000 U/L glucose oxidase (microbial), >200 U/L peroxidase (botanical), and preservatives after reconstitution with 100 mL ultrapure $H_2O$.
- Glucose calibrator (standard): A solution containing 5 mmol/L glucose and preservatives. This solution was serially diluted to produce 4 additional standards at concentrations of 2.5, 1.25, 0.625 and 0.3125 mmol/L.
- Samples: Samples were thawed on ice, vortexed and diluted with ultrapure $H_2O$.

  Dilution for 6 week serum – lean 1:4; obese 1:8

  Dilution for termination serum – lean 1:4; obese 1:8

  Dilution for OGTT – lean 1:10; obese 1:10 – 1:25.

Six week samples and termination samples were assayed separately; however, one termination sample from each group was assayed with the six week samples to validate results.

- Quality control: The quality control (DC-TROL Level 2, Genzyme Diagnostics, Charlottetown PE, Catalogue No. SM-056) was reconstituted with 5 mL of ultrapure $H_2O$ and then diluted 1:4.

*Procedure:*

Blank, standards, quality control and samples were vortexed and 5 µL of each were plated in triplicate on a 96-well plate (NUNC™, Roskilde Denmark, Catalogue No. 167008). Using a multi-channel pipette, 200 µL of the glucose colour reagent was then added to each well, pipetting up and down 3 times to mix contents. The plate was then incubated for 10 minutes at room temperature. After incubation, all bubbles were popped by gently blowing on the plate and/or by using a needle tip. The absorbance of the colour in each well was then measured at 505 nm (PowerWave™ XS/XS2, BioTek Instruments, Inc., Winooski VT). Using a computer software program (Gen5™, BioTek Instruments, Inc., Winooski VT), the absorbance of the blank was subtracted from standards and samples, and a standard curve was constructed and the sample concentrations were determined. If the glucose concentration of the quality control did not fall between 15.2 – 18.6 mmol/L, samples were re-assayed to ensure proper dilutions were used. Samples with coefficients of variation greater than 10% were re-assayed and the standard curve of all assays had a correlation coefficient of 0.9 or greater.

**Protocol 2 – Triglyceride Assay**

Six week serum (5 – 7 hour fast, jugular blood) and termination serum (12 hour fast, cardiac puncture) were collected and initially stored on ice until centrifuged at 2000 g for 20 minutes at 4$^{o}$C. Serum was aliquoted and stored at -80$^{o}$C until samples were analyzed for triglycerides with an enzymatic colorimetric assay kit from Genzyme Diagnostics (Charlottetown PE., Catalogue No. 236-60). The test principle for this assay is a four-step reaction. First, triglycerides are hydrolyzed with lipase to form glycerol and fatty acids. Second, in the presence of glycerol kinase and magnesium, glycerol and ATP are converted to glycerol-1-phosphate and ADP. Third, in the presence of glycerol phosphate oxidase, glycerol-1-phosphate and $O_2$ form $H_2O_2$ and dihydroxyacetone phosphate. Finally, in the presence of peroxidase, $H_2O_2$ and p-chlorophenol and 4-aminoantipyrine react to produce quinoneimine dye and $H_2O$. The amount of quinoneimine dye produced can be measured and is proportional to the amount of triglycerides in the sample.

*Reagents:*

- Triglyceride reagent: A buffered solution containing 0.4 mmol/L 4-aminoantipyrine, 2.6 mmol/L adenosine triphosphate, 3.0 mmol/L p-chlorophenol, >2400 U/L glycerol phosphate oxidase (microbial), >1000 U/L lipoprotein lipase (microbial), >540 U/L peroxidise (botanical), >400 U/L glycerol kinase (microbial), stabilizers and preservatives.
- Triglyceride calibrator (standard): A solution containing 2.03 mmol/L triglycerides (DC-CAL Calibrator, Genzyme Diagnostics, Catalogue No. SE-035). This solution was serially diluted to produce 4 additional standards at concentrations of 1.015, 0.5075, 0.25375 and 0.1269 mmol/L.
- Samples: Samples were thawed on ice, vortexed and diluted with ultrapure $H_2O$.

Dilution for 6 week serum – lean 1:2; obese 1:16

Dilution for termination serum – lean 1:4; obese 1:8 – 1:16, 2 samples diluted 1:40

Six week samples and termination samples were assayed separately; however, one termination sample from each group was assayed with the six week samples to validate results.

- Quality control: The quality control (DC-TROL Level 2, Genzyme Diagnostics, Charlottetown PE, Catalogue No. SM-056) was reconstituted with 5 mL of ultrapure $H_2O$.

*Procedure:*

Blank, standards, quality control and samples were vortexed and 10 µL of each were plated in triplicate onto a 96-well plate (NUNC™, Roskilde Denmark, Catalogue No. 167008). Using a multi-channel pipette, 200 µL of the triglyceride reagent was then added to each well, pipetting up and down 3 times to mix contents. The plate was then incubated at 37$^{o}$C for 18 minutes. After the incubation period, all bubbles were popped by gently blowing on the plate and/or by using a needle tip. The absorbance of each well was then measured at 520 nm (PowerWave™ XS/XS2, BioTek Instruments, Inc., Winooski VT). Using a computer software program (Gen5™, BioTek Instruments, Inc., Winooski VT), the absorbance of the blank was subtracted from standards and samples, and a standard curve was constructed and the sample concentrations were determined. If the triglyceride concentration of the quality control did not fall between 0.85-1.15 mmol/L, samples were re-assayed. Samples with coefficients of variation greater than 10% were re-assayed and the standard curve of all assays had a correlation coefficient of 0.9 or greater.

**Protocol 3 – Free Fatty Acid Assay**

Six week serum (5 – 7 hour fast, jugular blood) and termination serum (12 hour fast, cardiac puncture) were collected and initially stored on ice until centrifuged at 2000 g for 20 minutes at 4$^{o}$C. Serum was aliquoted and stored at -80$^{o}$C until samples were analyzed for FFA with an enzymatic colorimetric assay kit from Roche Diagnostics (Mannheim Germany, Catalogue No. 11 383 175 001). The kit was adapted for a microplate reader by Dr. Carla Taylor's lab. The test principle is a three step reaction. First, acyl-coA synthetase converts FFA, CoA and ATP to acyl-CoA, AMP and phyrophosphate. Second, acyl-CoA oxidase converts acyl-CoA and $O_2$ to enoyl-CoA and $H_2O_2$. Finally, peroxidase converts $H_2O_2$, 4-aminoantipyrine and 2,4,6-tribromo-3-benzoic acid to HBr, $2H_2O$ and red dye. The amount of red dye produced can be measured and is proportional to the amount of FFA in the sample.

*Reagents:*

- Reaction mixture A: 1 tablet (ATP, CoA, acyl-CoA synthetase, peroxidise, ascobate oxidase, 4-aminoantipyrine and stabilizers, bottle 2)) dissolved in 11 mL potassium phosphate buffer (pH 7.8, bottle 1). This was prepared at room temperature and tablets were allowed to completely dissolve for at least 10 minutes.
- Reaction mixture B: 1 tablet (acyl-CoA oxidase and stabilizers, bottle 5) dissolved in 0.6 mL acyl-CoA oxidase dilution solution and stabilizers (bottle 4). This was prepared at room temperature and tablets were allowed to completely dissolve for at least 10 minutes.
- Solution C: N-ethyl-maleinimide solution with stabilizers (bottle 3).
- Samples: Samples were thawed on ice and vortexed. Six week samples and termination samples were assayed separately; however, one termination sample from each group was assayed with the six week samples to validate results.

*Procedure:*

Using a multichannel pipette, 200 µL of reaction mixture A was added to each well of a 96-well plate (NUNC™, Roskilde Denmark, Catalogue No. 167008). Pipette tips were changed between each use to avoid excessive bubble build up. The blank and samples were then vortexed and 10 µL of each were plated in triplicate. Pipette tips were changed between each use. The plate was then shaken by hand (keeping the plate on countertop and moving it in a circular motion) for 30 seconds, covered and incubated for 10 minutes at room temperature.

After the incubation period, 10 µL of solution C was added to each well using a multichannel pipette and all bubbles were popped by blowing gently on the plate and/or using a needle tip. The plate was mixed for 30 seconds using the shaking feature on the microplate reader and the absorbance of each well was measured at 546 nm (PowerWave™ XS/XS2, BioTek Instruments, Inc., Winooski Vermont). This absorbance was labelled $A_1$. The plate was then removed from the microplate reader and 10 µL of reaction mixture B was added to each well using a multichannel pipette. The plate was again shaken, uncovered, for 20 seconds by hand, then covered, and allowed to incubate at room temperature for 20 minutes. After the incubation period, all bubbles on the plate were popped, and the plate was shaken in the plate reader for 30 seconds and the absorbance was measured at 546 nm (PowerWave™ XS/XS2, BioTek Instruments, Inc., Winooski VT). This absorbance was labelled $A_2$.

Serum FFA concentrations were calculated using the following formula:

$$C\ (mmol/L) = \frac{V}{\varepsilon \times d \times v} \times \Delta A$$

Where:

C = concentration of FFA in the sample

V = total well volume in mL (0.230 mL)

v = sample volume in mL (0.010 mL)

d = light path in cm (0.53326 cm)

ε = absorption coefficient at 546 nm [19.3 x (1 x $mmol^{-1}$ x $cm^{-1}$)$^3$]

ΔA = change in absorbance of the sample minus the change in absorbance of the blank;

$(A_2\text{-}A_1)_{sample} - (A_2\text{-}A_1)_{blank}$

**Protocol 4 – Haptoglobin Assay**

Six week serum (5 – 7 hour fast, jugular blood) and termination serum (12 hour fast, cardiac puncture) were collected and initially stored on ice until centrifuged at 2000 g for 20 minutes at 4$^{o}$C. Serum was aliquoted and stored at -80$^{o}$C until samples were analyzed for haptoglobin with an enzymatic colorimetric assay kit from Tridelta Development Ltd. (Maynooth Ireland, Catalogue No. TP801). Free haemoglobin exhibits peroxidase activity, which is inhibited at a low pH. Haptoglobin present in serum combines with haemoglobin and at a low pH preserves the peroxidase activity of the bound haemoglobin. Preservation of the peroxidase activity of haemoglobin is directly proportional to the amount of haptoglobin present in the specimen.

*Reagents:*

- Reagent 1: Haemoglobin
- Reagent 2: Chromogen
- Sample/calibrator diluent: Phosphate buffered saline (PBS)
- Calibrator: A solution containing 2.5 mg/mL haptoglobin. This solution was serially diluted to produce 4 additional standards at concentrations of 1.25, 0.625, 0.312 and 0.156 mg/mL.
- Samples: Samples were thawed on ice, vortexed and diluted with sample/calibrator diluent.

  Dilutions for 6 week serum – lean, no dilution; obese, 1:4

  Dilutions for termination serum – lean, no dilution; obese no dilution, 1:2 or 1:4

Six week samples and termination samples were assayed separately; however, one termination sample from each group was assayed with the six week samples to validate results.

*Procedure:*

Standards and samples were vortexed and 7.5 µL of each were plated in duplicate onto a 96-well plate (NUNC™, Roskilde Denmark, Catalogue No. 167008). Using a multi-channel pipette, 100 µL of reagent 1 was added to each well, pipetting up and down 3 times to mix contents. Using a multi-channel pipette, 140 µL of reagent 2 was added to each well, pipetting up and down 3 times to mix contents. The plate was then incubated for 5 minutes at room temperature. After the incubation period, all bubbles were popped by gently blowing on the plate and/or by using a needle tip. The absorbance of each well was then measured at 630 nm. Samples with coefficients of variation greater than 10% were re-assayed and the standard curve of all assays had a correlation coefficient of 0.9 or greater.

**Protocol 5 – Insulin Assay**

Six week serum (5 – 7 hour fast, jugular blood), termination serum (12 hour fast, cardiac puncture) and serum from OGTT (5 hour saphenous vein) were collected and initially stored on ice until centrifuged at 2000 g for 20 minutes at 4$^{o}$C. Serum was aliquoted and stored at -80$^{o}$C until samples were analyzed for insulin using an ELISA kit provided by ALPCO diagnostics (Salem NH, Catalogue No. 80-INSRT-E10). Monoclonal antibodies specific for insulin are immobilized to the 96-well microplate as the solid phase. Standards, controls and samples are added to the appropriate wells with a horseradish peroxidase enzyme labeled monoclonal antibody (Conjugate), resulting in insulin molecules being sandwiched between the solid phase and the Conjugate. After incubation, wells are washed with wash buffer to remove unbound Conjugate. 3,3',5,5' – Tetramethylbenzidine (TMB) substrate is added to each well, resulting in a blue color as the TMB substrate reacts with the bound Conjugate. Stop solution is added to stop the reaction and changes the color from blue to yellow. The intensity of the color is directly proportional to the amount of insulin in the sample.

*Reagents:*

- Insulin microplate (coated with mouse monoclonal anti-insulin)
- Conjugate buffer
- Conjugate stock (HRP labeled monoclonal anti-insulin antibody)
- Mammalian insulin high and low controls
- Wash buffer concentrate
- Insulin standards (0.0, 0.15, 0.4, 1.0, 3.0, 5.5 ng/mL)
- TMB substrate
- Stop solution

- Samples: Samples were thawed on ice and all reagents were brought to room temperature prior to use. Samples requiring dilution were diluted with ultrapure $H_2O$. Samples below the range of the standard curve were re-assayed with the addition of lower standards (0.0375 and 0.075 ng/mL) to the plate.

    Dilutions for 6 week serum – lean, no dilutions; obese 1:10

    Dilutions for termination serum – lean, no dilutions; obese 1:4 – 1:10

    Dilutions for OGTT serum – lean 1:4 – 1:8; obese 1:8 – 1:10

Six week samples and termination samples were assayed separately; however, one termination sample from each group was assayed with the six week samples to validate results.

*Procedure:*

The mammalian insulin controls were reconstituted with 0.6 mL of ultrapure $H_2O$ and allowed to stand for 30 minutes. Thirty-five microlitres of the low and high controls were aliquoted into labeled microcentrifuge tubes and stored at -20$^{o}$C for future assays. Ten microlitres of standards, mammalian insulin controls, and samples were then plated in triplicate onto the 96-well plate (NUNC™, Roskilde Denmark, Catalogue No. 167008). Pipette tips were changed between every single well to ensure the antibody did not get transferred from the microplate to the original sample. Using a multi-channel pipette, 75 µL of working strength conjugate was added to each well. The plate was then covered and incubated for 2 hours at room temperature while being shaken at 700 – 900 rpm.

After incubation, the plate was washed 6 times with working strength wash buffer. For each wash, 200 µL of wash buffer was added to each well using a multi-channel pipette. The plate was then quickly flipped upside down and patted on a paper towel. After the final wash, the plate was patted on a paper towel to ensure no liquid remained in the wells after washing was complete.

Next, 100 µL of TMB substrate was added to each well and the plate was incubated for 15 minutes at room temperature while being shaken at 700 – 900 rpm. After the incubation period, 100 µL of stop solution was added to each well and the absorbance of each well was immediately measured at 450 nm (PowerWave™ XS/XS2, BioTek Instruments, Inc., Winooski VT). Using a computer software program (Gen5™, BioTek Instruments, Inc., Winooski VT), a standard curve was constructed (cubic spline fit, log scale) and the sample concentrations were determined. If the insulin concentration of the mammalian insulin controls did not fall between 0.45 – 0.88 ng/mL (low) and 2.7 – 4.79 ng/mL (high), samples were re-assayed. Samples with coefficients of variation greater than 10% were re-assayed.

*Insulin Sensitivity Calculations:*

1. HOMA-IR was calculated as follows (Matthews et al., 1985):

$$\frac{\text{fasting serum insulin (uU/mL) x fasting serum glucose (mmol/L)}}{22.5}$$

Fasting serum insulin was first converted from ng/mL to pmol/L (using the molecular weight of insulin 5800 ng/nmol), then from pmol/L to uU/mL (using the conversion factor 1 uU/mL = 6.945 pmol/L )

2. An insulin to glucose ratio was also calculated as a simple screening test for insulin resistance:

$$\text{Insulin:glucose ratio} = \frac{\text{fasting serum insulin (pmol/L)}}{\text{fasting serum glucose (mmol/L)}}$$

3. AUC was calculated for both glucose and insulin from serum collected during OGTT using the following equation (Brouns et al., 2005):

$$AUC = \left[\frac{(t15 + t0)}{2} \times 15(min)\right] + \left[\frac{(t30 + t15)}{2} \times 15(min)\right] + \left[\frac{(t60 + t30)}{2} \times 30(min)\right] + \left[\frac{t120 + t60)}{2} + 60(min)\right]$$

$AUC_{index}$ = AUC glucose (min*mmol/L) x AUC insulin (min*pmol/L)

**Protocol 6 – Quantification of Pancreatic Islet Cell Size**

*a) Insulin immunostaining*

*Reagents:*

- Old and new xylene – The “45 slide” rule is used to determine “old” xylene versus “new” xylene. New xylene is xylene that has been used for less than 45 slides. After 45 slides have been immersed in it, it is considered old xylene.
- 100%, 95%, 70% ethanol
- 3% $H_2O_2$: 225 mL deionized $H_2O$ + 25 mL of 30% $H_2O_2$ (made fresh every day; Cat # H323, Fisher).
- PBS: Dissolve 1 packet of PBS pH 7.4 (Cat # P5368, Sigma) in 1 L deionized $H_2O$ (made fresh every day).
- 3% Hematoxylin: Dissolve 50 g aluminum potassium sulfate in 1000 mL deionized $H_2O$. When completely dissolved, add 1 g hematoxylin. Next, add 0.2 g sodium iodate and 20 mL glacial acetic acid. Bring to a boil and cool. Filter if necessary.
- PAP pen/liquid blocker pen (Cedarlane, Burlington ON, Catalogue No. MU22)
- STAT-Q peroxidase-DAB staining system (Innovex Biosciences, Richmond CA, Catalogue No. 314KLD), includes DAB, DAB substrate buffer, secondary antibody (antibody is HRP-labeled Strepavidin) and tertiary antibody (antibody is Mouse Link Rat Absorbed) antibodies
- Primary antibody**:** Mouse Monoclonal Antibody to Insulin (Innovex Biosciences, Richmond CA, Catalogue No. MAB391P)
- Cyto Q background buster (Innovex Biosciences, Richmond CA, Catalogue No. NB306)
- Permount: to adhere cover slips to slides (Fisher Scientific, Ottawa ON, Catalogue No. SP15)

- Fisherfinest premium cover glass: 22 X 50 (Fisher Scientific, Ottawa ON, Catalogue No.12-548-5E)

*Procedure:*

In the fume hood, tissue sections were deparafinnized by placing them in a slide holder and into old xylene for 15 minutes and then new xylene for another 15 minutes. Tissue sections were then rehydrated by placing slides in 100% ethanol for 3 minutes, 100% ethanol for 3 minutes, 95% ethanol for 3 minutes, 70% ethanol for 3 minutes, and deionized water for 5 minutes. Endogenous peroxidase activity of tissues was then blocked by placing slides in fresh 3% $H_2O_2$ for 10 minutes followed by PBS for 5 minutes.

Slides were then dried and a PAP pen was used to draw a circle around each tissue section. Background buster (250 µL) was then pipetted onto each tissue section and slides were incubated in an incubation chamber (a container with a lid that had moist paper towels added for humidity) for 20 minutes. After the incubation period, the background buster was poured off and a paper towel was used to blot underneath each slide and around each tissue.

To identify insulin, 250 µL of primary antibody (1:75 dilution; anti-insulin) was pipetted onto one tissue section on each slide (the tissue section furthest from the slide label). Next, 250 µL of PBS was pipetted on the tissue section closest to the slide label (control section). Slides were incubated in the incubation chamber for 20 minutes.

Following incubation, slides were rinsed with PBS from a wash bottle and then placed in a PBS bath for 5 minutes. Next, 250 µL of the secondary antibody was pipetted onto each tissue section and allowed to incubate for 10 minutes. This was followed by another rinse with PBS and placement in a fresh PBS bath for 5 minutes. Next, 250 µL of the tertiary antibody was then added onto each tissue section and

allowed to incubate for 10 minutes. This was followed by another rinse with PBS and placement in a fresh PBS bath for 5 minutes.

Slides were dried and placed in a dark fume hood. Prepared DAB solution was then pipetted onto each tissue section (250 µL) and incubated in the fume hood for 5 minutes. The unreacted DAB was then washed off the slides, followed by submersion of the slides in 3% hematoxylin for 1 minute. Slides were then washed in a beaker with tap water. Tissue sections were then dehydrated by placing slides in compartments with 70% ethanol for 1 minute, 95% ethanol for 1 minute, 100% ethanol for 1 minute, and 100% ethanol for 1 minute. Slides were then immersed in new xylene for 3 minutes, tapped on a paper towel to remove excess xylene, and then placed flat in the fume hood. Cover slips were then mounted on each slide with a drop of Permount and allowed to dry overnight.

*b) Quantification of Islet Cell Size*

Sections were visualized with Zeiss Axioskop 2 Mot Plus microscope and pictures of islet cells were taken with Zeiss Axiocam digital camera, Axio Version 4.6 software (Carl Zeiss Canada Ltd, Toronto ON). Next, ImageJ Software (NIH, Bethesda MA) was used to measure islet cell area. An average islet cell area was calculated for each sample.

**Protocol 7 – Western Immunoblotting**

*a) Extraction of Protein from Gastrocnemius Muscle*

To quantify the amount of protein in gastrocnemius muscle, 40 mg of gastrocnemius muscle was placed in a mortar and 30 µL/mg (1200 µL) of 3X sample buffer was added. The tissue was ground to a fine powder with a pestle. After all samples were ground, samples were allowed to sit for 15 minutes at room temperature to permit lysis of the muscle cells. Next, the mixture was transferred to a microcentrifuge tube and centrifuged for 20 minutes at 13 000 g. The supernatant containing the protein was removed and sonicated (Sonic dismembrator, Model 100, Thermo Fisher Scientific Inc, Ottawa ON) for 10 seconds. After sonication, samples were allocated to another microcentrifuge tube and stored at -80$^{o}$C until used.

*b) Protein Assay*

The Pierce Bicinchoninic Acid (BCA) assay kit (Thermo Fisher Scientific, Ottawa ON, Catalogue No. 23225) was used to determine the concentration of protein in the protein extracts of gastrocnemius muscle tissue. These concentrations were needed in order to load the same amount of protein per lane into gels for Western immunoblotting. The principle of the protein assay involves the reduction of $Cu^{2+}$ to $Cu^{1+}$ by protein in an alkaline medium followed by the highly sensitive and selective colorimetric detection of the cuprous cation ($Cu^{1+}$) by BCA which produces a purple complex. The intensity of the colored end product can be measured by spectrometric analysis at 550 nm and is directly proportional to the amount of protein in the sample.

*Reagents:*

- Reagent A: Bicinchonic acid and tartrate in an alkaline carbonate buffer
- Reagent B: 4% copper sulfate pentahydrate solution

- BSA standard: A solution containing 2.0 mg/mL BSA (Thermo Fisher Scientific, Ottawa ON, Catalogue No. 23225). This solution was serially diluted to produce 5 additional standards at concentrations of 0.2, 0.4, 0.6, 0.8, and 1.0.
- Samples: Samples were thawed on ice, vortexed and diluted with ultrapure $H_2O$

  Dilutions: 1:4 (10 µL sample + 30 µL ultrapure $H_2O$)

*Procedure:*

Standards and samples were vortexed and 10 µL of each were plated in triplicate onto a 96-well plate (NUNC™, Roskilde Denmark, Catalogue No. 167008). Using a multi-channel pipette, 200 µL of reagent mixture (50 parts Reagent A, 1 part Reagent B) was added to each well, pipetting up and down 3 times to mix contents. The plate was then incubated for 30 minutes at 37$^{o}$C temperature. After the incubation period, all bubbles were popped by gently blowing on the plate and/or by using a needle tip. The absorbance of each well was then measured at 550 nm (PowerWave™ XS/XS2, BioTek Instruments, Inc., Winooski VT). Using a computer software program (Gen5™, BioTek Instruments, Inc., Winooski VT), a standard curve was constructed and the sample concentrations were determined. Samples with coefficients of variation greater than 10% were re-assayed and the standard curve of all assays had a correlation coefficient of 0.9 or greater.

*c) Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)*

After quantification of the protein sample, SDS-PAGE was used to separate proteins based on their molecular weights. Briefly, protein samples were heated in SDS-containing buffer and then loaded onto a polyacrylamide gel. The gel was exposed to an electric current which draws the proteins through the porous gel, with higher molecular weight proteins traveling slower through the gel than lower molecular weight proteins.

*Reagents:*

- 6 – 7.5% separating gel: 20% acrylamide, 10% SDS, 1.5 M Tris HCl pH 8.8, 10% ammonium persulfate, N, N, N', N' –Tetramethylethylenediamine (TEMED) and dd$H_2O$
- 5% stacking gel: 20% acrylamide, 10% SDS, 0.5 M Tris HCl pH 6.8, 10% ammonium persulfate, TEMED and dd$H_2O$
- $H_2O$-saturated butanol
- 10% bromophenol blue
- β-mercaptoethanol
- SDS-PAGE electrode buffer: 0.125 M Tris, 0.96 M glycine, 0.5% SDS

*Procedure:*

All components of the apparatus were cleaned by rinsing with dd$H_2O$, followed by wiping the large and small glass plates with ethanol using a Kimwipe. The glass plates (spaced apart with a 1.0 mm spacer) were placed into the sandwich clamp assembly and then placed into a casting stand. Components for the separating gel were mixed and poured between the glass plates. A 6% gel was made for the higher molecular weight antibodies [IRS-1 and pIRS-1(Ser636/639)], while a 7.5% gel was made for the lower molecular weight antibodies [Akt, pAkt(Ser473) and pAkt(Thr308)]. $H_2O$-saturated butanol was pipetted over top of the separating gel to ensure that the top of the gel was even. After the gel polymerized (approximately 45 minutes), the $H_2O$-saturated butanol was poured off and the gel was rinsed with dd$H_2O$. Next, the stacking gel was mixed and poured over the separating gel. A 15-well comb was quickly inserted into the stacking gel, ensuring no bubbles were trapped below the comb, and left to polymerize

for 15 minutes. After the gel polymerized, the comb was removed and the wells were rinsed with $ddH_2O$.

From the protein assay, the volumes of all protein samples were calculated (volume of protein sample needed [µL] = concentration of protein to load into gel [7.5 – 10 µg based on dose response curves] / protein concentration from protein assay [µg/µL]) and added to microcentrifuge tubes. Next, 10 µL of 3X sample buffer was added to the sample and approximately 10% of blue dye mixture (1:1 ratio of β-mercaptoethanol and bromophenol blue). In a microwave, $ddH_2O$ was brought to a boil. Samples were then placed in a holding container and placed in the $ddH_2O$ and heated in the microwave for another 5 minutes to denature the proteins.

The sandwich clamp assembly was then transferred to the electrophoresis apparatus and placed in a transparent buffer tank. The SDS-PAGE electrode buffer was poured over the middle of the electrophoresis apparatus and then into the buffer tank. A molecular mass marker, samples, and a reference sample were loaded into the gel using a glass syringe. The reference sample, which was created by pooling 10 µL of homogenate from one sample per dietary group and then calculating the protein content using the BCA assay, was loaded on all gels to control for different intensities on different gels. The electrophoresis apparatus was then connected to a power supply and electrophoresis was conducted at 20 mA of current per gel for 1 hour, or until the blue dye was near the bottom of the gel.

*d) Gel Transfer*

After proteins were separated according to molecular weight, they were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane.

*Reagents:*

- Transfer buffer: 20% methanol, 0.25 mM Tris, 130 mM glycine and dd$H_2O$ (for 6% gel, add 50 mL methanol, 160 mL 5X Transfer Buffer and bring up to 1 L with dd$H_2O$ in a large container. For 7.5% gel, add 200 mL methanol, 160 mL 5X Transfer Buffer and bring up to 1 L with dd$H_2O$)
- 5X Tris-buffered saline in Tween-20 (TBST): 0.1 M Tris HCl pH 7.4, 0.25% Tween-20
- 1X TBST: 1 part 5X TBST and 4 parts dd$H_2O$
- Methanol

*Procedure:*

The glass plates from the sandwich assembly were separated and the stacking gel was cut away and discarded. The gel was removed from the glass plate by adhering to a piece of blotting paper. A PVDF membrane, which was soaked in methanol and then equilibrated in transfer buffer for 5 minutes, was placed on top of the gel. The membrane was covered with another piece of blotting paper and both sides were covered with a fiber pad. All components were completely submerged in transfer buffer. At this point, the fiber pads were gently pressed to remove any trapped air bubbles from between the membrane and gel and then placed inside a transfer cassette. The transfer cassette was placed inside the electrode module with the negative side of the cassette facing the negative side of the module. An ice pack and a stir bar were placed inside the transfer tank with the cassettes, and the transfer buffer was poured over the entire apparatus. The apparatus was placed on a magnetic stirrer (set at medium pace), and the protein transfer was achieved by electrophoresis at 100 volts for 75 minutes for 6% gels, and 100 volts for 60 minutes for 7.5% gels. Following electrophoresis, membranes

were removed, placed in a container and covered with 1X TBST and stored at 4$^{o}$C until used, or blocked as described below.

*e) Identification of Proteins*

Western immunoblotting uses antibodies to detect a protein of interest. Firstly, a primary antibody specific to the protein of interest is applied, followed by a secondary antibody conjugated to horseradish peroxidase (HRP). The HRP catalyzes a reaction that results in the emission of light due to the production of a chemiluminescent agent. The intensity of the light produced is proportional to the amount of protein of interest that is present in the sample.

*Reagents:*

- 3% bovine serum albumin (BSA) in TBST
- 1% BSA in TBST
- 1X TBST
- Primary antibodies
- Horseradish peroxidase-linked secondary antibody
- Chemiluminescent reagent: Chemiluminescent Peroxidase Substrate-1 (Sigma-Aldrich, St. Louis MO)

*Procedure:*

The membrane was placed in 12 mL of 3% BSA in TBST and agitated for 1 hour on an orbital shaker. After blocking, primary antibodies were then added at specific dilutions (**Table S1**) to the 3% BSA in TBST. Parafilm was stretched around the container to prevent air from entering. The container was placed on a shaker in the 4$^{o}$C

**Table S1** Antibodies used in Western blotting analysis

| Primary Antibody | Source | Primary Dilution | Secondary Antibody | Secondary Dilution | Molecular Weight (kDa) | % Separating gel |
|---|---|---|---|---|---|---|
| pAkt(Ser473)[1] | Cell Signaling Cat # 9271 | 1:1000 | anti-rabbit horseradish peroxidase | 1:10 000 | 60 | 7.5 |
| pAkt(Thr308)[2] | Cell Signaling Cat # 9275 | 1:1000 | anti-rabbit horseradish peroxidase | 1:10 000 | 60 | 7.5 |
| Akt | Cell Signaling Cat # 9272 | 1:1000 | anti-rabbit horseradish peroxidase | 1:10 000 | 60 | 7.5 |
| MAPK p44/42 (loading control)[3] | Cell Signaling Cat # 9102 | 1:2000 | anti-rabbit horseradish peroxidase | 1:10 000 | 42, 44 | 7.5 |
| pIRS-1(Ser636/639)[4] | Cell Signaling Cat # 2388 | 1:1000 | anti-rabbit horseradish peroxidase | 1:10 000 | 180 | 6 |
| IRS-1[5] | Cell Signaling Cat # 2382 | 1:1000 | anti-rabbit horseradish peroxidase | 1:10 000 | 180 | 6 |
| eEF2 (loading control)[6] | Cell Signaling Cat # 2332 | 1:1000 | anti-rabbit horseradish peroxidase | 1:10 000 | 95 | 6 |

[1]Phosphorylated Akt at serine 473
[2]Phosphorylated Akt at threonine 308
[3]Mitogen-activated protein kinase p44/42
[4]Phosphorylated insulin receptor substrate-1 at serine 636/639
[5]Insulin receptor substrate-1
[6]Eukaryotic elongation factor 2

walk-in fridge and agitated gently overnight. The following day, the primary antibodies were removed and the membrane was rinsed 4 times with 1X TBST for 5 minutes each. Horseradish peroxidase-linked secondary antibodies, specific to the animal source of the primary antibody, were added to 1% BSA in TBST at specific dilutions (Table S1) and incubated for 1 hour on an orbital shaker. Following the incubation, the secondary antibody was removed and the membranes were again rinsed 4 times with 1X TBST for 5 minutes each. A chemiluminescent reagent was applied to the membranes and detected using a FluorChem®Q gel scanning system with a charge-coupled device camera (Proteinsimple) and AlphaView®Software (version 1.3.0.6; Alpha Innotech Corporation). Because several different membranes were probed for the same protein, all bands had to be standardized to account for differences in exposure intensity. To do this, the trace quantity of each protein of interest was divided by the trace quantity of the reference sample on the same membrane. Then, to determine the degree of protein phosphorylation, the trace quantities of the phosphorylated protein bands [pAkt(Ser473), pAkt(Thr308), pIRS-1(Ser636/639)] were divided by the trace quantities of the respective non-phosphorylated protein bands (Akt and IRS-1). To determine total protein levels, the trace quantities of the non-phosphorylated protein bands (Akt and IRS-1) were divided by the trace quantities of the respective loading control bands (MAPK p44/42 and eEF2).

*f) Stripping of Membrane Blots*

Membranes were stripped after Western blotting to allow the membrane to be probed with additional antibodies. To strip the membranes, the membranes were placed in a container and covered with 25 mL of stripping buffer (10% SDS, 0.5 M Tris HCl pH 6.8 and dd$H_2O$) plus 200 µL of β-mercaptoethanol. The container was closed and placed into another larger container to help contain the strong odour of β-

mercaptoethanol, and then placed on the orbital shaker for a minimum of 3 hours or overnight. After membranes have been stripped, the stripping buffer was poured down the sink in the fume hood and the membranes were placed in a new container and covered with 1X TBST. The membranes were washed with 1X TBST 5 – 6 times for 5 minutes each, or until no odour was detectable. After the final wash, the membranes were either kept in a clean container with 1X TBST in 4$^{o}$C until needed, or blocked with primary antibody as per protocol.

**Protocol – 8 Total Liver Lipid Concentration**

Total liver lipid was determined by a modified Folch method (Folch, Lees, & Sloane Stanley, 1957). First, 1.0 g of liver was weighed and homogenized in 22 mL of 2:1 chloroform:methanol for 20 seconds with a Polytron PT-MR 1600 (Kinematica, Lucerne Switzerland). The homogenate (22 mL) was then filtered through a #1 Whatman filter paper into a 25 mL graduated cylinder. Next, 0.73% NaCl was added at a volume of 20% of the volume of the homogenate in the graduated cylinder. The cylinder was stoppered and the mixture was inverted 5 times and left to separate overnight at room temperature in the fume hood. Additionally, 20 mL glass scintillation vials were placed in a dessicator for use the next day.

The following day, the volume of the lower chloroform/lipid layer was recorded and 10 mL of this layer was placed in dried and weighed 20 mL glass scintillation vials. The chloroform was evaporated under nitrogen at 30$^{o}$C with a N-EVAP III Nitrogen Evaporator (Organomation Associates, Inc., Berlin MA). Next, the 20 mL glass scintillation vials containing the lipid were dried in the dessicator overnight.

The next day, the vials were weighed and the lipid content was calculated as follows:

$$\text{g lipid/g tissue} = \frac{(\text{dry weight vial + lipid}) - (\text{dry weight vial})}{10 \text{ mL chloforom used}} \times \text{volume of chloroform layer}$$

**Protocol 9 – Liver Section Staining**

*a) Preparation of Slides*

*Reagents:*

- 4% paraformaldehyde solution
- Acidified ethanol: 400 mL 70% ethanol, 1 mL concentrated HCl
- Hematoxylin: 50 g aluminum potassium sulfate, 1000 mL dd$H_2O$, 1 g hematoxylin, 0.2 g sodium iodate, 20 mL glacial acetic acid
- Eosin Y: 5 g eosin Y (Fisher Scientific, Ottawa ON, Catalogue No. #E511), 500 mL dd$H_2O$

*Procedure:*

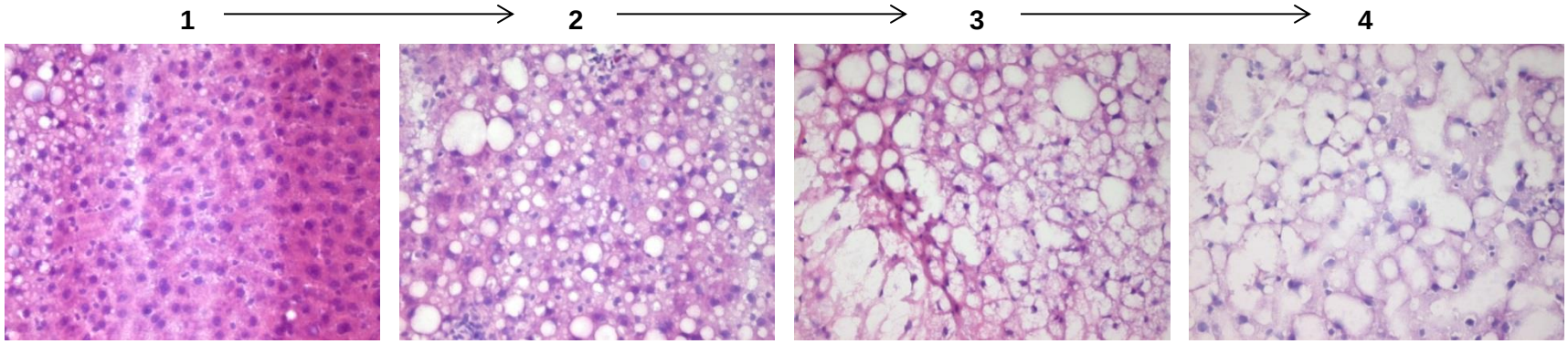
Slides were submerged in 4% paraformaldehyde solution for 8 – 10 minutes, washed with 1X PBS for 5 minutes, then dd$H_2O$ for 15 minutes. Slides were then stained with hematoxylin for 5 minutes and then rinsed with dd$H_2O$. Next, the slides were rinsed in acidified ethanol, rinsed with tap water and then washed in dd$H_2O$ for 2 minutes. Slides were then stained with eosin Y for 8 minutes. This was followed by a 5 minute wash with 5% ethanol, and then two, 5 minute washes with 100% ethanol. Slides were then soaked in xylene for 15 minutes and then allowed to dry for 1 hour.

*b) Quantification of Lipid Droplet Size and Number*

Sections were visualized with Zeiss Axioskop 2 Mot Plus microscope and images were taken with Zeiss Axiocam digital camera, Axio Version 4.6 software (Carl Zeiss Canada Ltd, Toronto ON). Quantification of lipid droplet number and size was carried out using ImageJ software (NIH, Bethesda MA). For lipid droplet size, 2 lipid droplets were randomly selected from 25 different sections of liver tissue for a total of 50 lipid droplets for each rat. An average was then taken for each rat. For lipid droplet number,

liver sections were divided into 0.01 $mm^2$ and all lipid droplets within this area were counted.

*c) Qualitative Assessment Scale for Hepatic Steatosis Rating*




Hepatic steatosis rating = a progressive rating scale used to assess degree of hepatic steatosis. All slides were rated in a blinded manner.

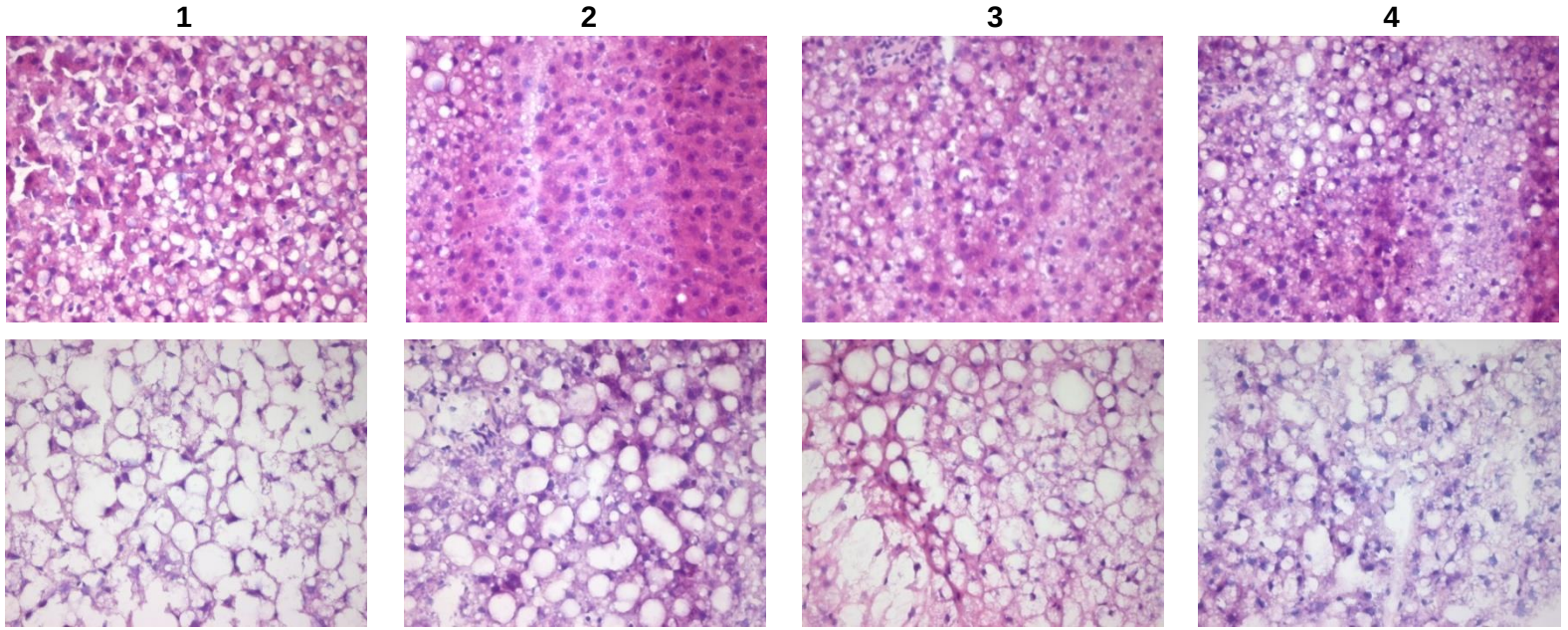
1. Low hepatic steatosis
    - Tissue quite smooth, almost no noticeable lipid droplets.

2. Low – medium hepatic steatosis
    - Noticeable droplets but relatively small, may have some large droplets.

3. Medium – high hepatic steatosis
    - Relatively large lipid droplets, possibly with some smaller droplets present as well. Liver tissue matrix somewhat intact, but large gaps are present.

4. High hepatic steatosis
    - Predominantly large lipid droplets, not much liver tissue present.

*d) Qualitative Assessment Scale for Distribution Rating*




Distribution rating = used to assess how even lipid droplet size is across the tissue section. All slides were rated in a blinded manner.

1. Lipid droplets are mostly uniform, all similar size.
2. Small degree of unevenness in lipid droplet size.
3. Moderate amount of unevenness in lipid droplet size (approximately 50/50).
4. High degree of unevenness in lipid droplet size.

**Protocol 10 – Blood pressure**

At weeks 3, 7 and 10, blood pressure was assessed by the tail cuff method using a CODA™ multi-channel, computerized, non-invasive blood pressure system for mice and rats (Kent Scientific, Torrington CT). Prior to the week 3 blood pressure readings, rats were pre-exposed to the procedure to reduce the stress response during the actual testing period. The rats were restrained in a holder containing a nose cone at the front to limit their view and to reduce the level of stress, as well as a holder at the back which allowed their tails to be free. The procedure room was kept at 26$^{o}$C and a heating platform and heat lamp were used to maintain the rats' surface temperature between 30 – 33$^{o}$C. An occlusion cuff was placed at the base of the tail followed by a volume pressure recording (VPR) sensor. Once the rats' body temperatures were between 30 – 33$^{o}$C, nine acclimation blood pressure readings (9 cycles) were taken to help the rats get accustomed to the procedure. Next, 15 blood pressure readings (15 cycles) were taken and used to calculate an average blood pressure reading. During one cycle, the occlusion cuff inflated to block blood flow to the tail, then the occlusion cuff deflated and the VPR sensor measured the tail swelling as a result of the arterial pulsations from the blood flow. The CODA™ Software automatically determined systolic blood pressure (when the tail first begins to swell) and diastolic blood pressure (when the increased rate of swelling in the tail ceases).

**TABLE S2** Weekly body weights and total weight gain[1]

| | lnNPC | faNPC | faNPC-wtm[3] | faHPC | faHPS | faHPM |
|---|---|---|---|---|---|---|
| Week 0 (initial) | 321 ± 9[b] | 497 ± 10[a] | 504 ± 14[a] | 499 ± 17[a] | 498 ± 16[a] | 502 ± 11[a] |
| Week 1 | 365 ± 10[b] | 537 ± 12[a] | 536 ±15[a] | 542 ± 18[a] | 542 ± 20[a] | 537 ± 12[a] |
| Week 2 | 400 ± 10[b] | 571 ± 14[a] | 573 ± 13[a] | 574 ± 21[a] | 585 ± 23[a] | 570 ± 15[a] |
| Week 3 | 422 ± 13[b] | 586 ± 13[a] | 588 ± 14[a] | 599 ± 21[a] | 616 ± 23[a] | 601 ± 16[a] |
| Week 4 | 444 ± 12[b] | 596 ± 11[a] | 599 ± 14[a] | 621 ± 20[a] | 638 ± 24[a] | 629 ± 18[a] |
| Week 5 | 466 ± 14[b] | 605 ± 10[a] | 613 ±14[a] | 632 ± 21[a] | 659 ± 25[a] | 656 ± 19[a] |
| Week 6 | 481 ± 14[c] | 610 ± 10[b] | 618 ± 15[b] | 641 ± 22[ab] | 678 ± 28[a] | 675 ± 20[a] |
| Week 7 | 499 ± 15[c] | 627 ± 9[b] | 631 ± 14[b] | 657 ± 22[ab] | 694 ± 30[a] | 691 ± 22[a] |
| Week 8 | 515 ± 15[c] | 639 ± 9[b] | 639 ± 14[b] | 676 ± 23[ab] | 714 ± 32[a] | 708 ± 23[a] |
| Week 9 | 532 ± 15[c] | 654 ± 11[b] | 652 ± 15[b] | 692 ± 23[ab] | 732 ± 34[a] | 727 ± 25[a] |
| Week 10 | 552 ± 15[c] | 671 ± 11[b] | 670 ± 16[b] | 712 ± 23[ab] | 758 ± 37[a] | 746 ± 27[a] |
| Week 11 | 557 ± 15[c] | 678 ± 11[b] | 676 ± 16[b] | 719 ± 24[ab] | 771 ± 36[a] | 753 ± 28[a] |
| Week 12 (final) | 558 ± 14[c] | 682 ± 12[b] | 678 ± 15[b] | 726 ±24[ab] | 777 ± 37[a] | 755 ± 31[ab] |
| Total weight gain[2] | 237 ± 10 | 184 ± 12 | 173 ± 7 | 220 ± 14 | 278 ± 29 | 253 ± 23 |

[1]Data are presented as means ± SEM (grams; n = 8 – 11 per group). Values in the same row with different superscript letters are significantly different ($P < 0.05$) from each other. An absence of letters indicates that means are not statistically different.
[2]Total weight gain = week 12 (final) – week 0 (initial).
[3]The faNPC-wtm group and the high protein group with the lowest average weekly body weights were weighed an additional 1-2 times per week to ensure weights were not statistically different. Throughout the study, the faHPC group had the lowest body weights in the high protein diet groups and there were no significant differences between the faNPC-wtm and the faHPC groups for the entire 12 weeks. In addition, the faNPC-wtm group and faNPC group had no significant differences between their weights. Therefore, the faNPC-wtm group was dropped from any further analysis; faNPC-wtm = *fa/*fa Zucker rats weight matched group given normal protein casein diet.

**TABLE S3** Average daily food intake[1]

| | lnNPC | faNPC | faHPC | faHPS | faHPM |
|---|---|---|---|---|---|
| Week 1 | 23.6 ± 1.0$^{c}$ | 34.7 ± 1.3$^{a}$ | 32.9 ± 1.4$^{a}$ | 32.2 ± 1.5$^{a}$ | 28.0 ± 1.5$^{b}$ |
| Week 2 | 21.3 ± 1.2$^{b}$ | 28.9 ± 1.5$^{a}$ | 27.7 ± 1.6$^{a}$ | 30.6 ± 1.7$^{a}$ | 26.4 ± 1.1$^{a}$ |
| Week 3 | 22.2 ± 0.9$^{a}$ | 26.6 ± 1.9$^{a}$ | 24.6 ± 1.6$^{a}$ | 27.1 ± 1.2$^{a}$ | 25.6 ± 1.6$^{a}$ |
| Week 4 | 21.6 ± 1.5$^{b}$ | 25.5 ± 1.6$^{ab}$ | 26.2 ± 1.6$^{ab}$ | 28.1 ± 1.4$^{a}$ | 28.6 ± 1.8$^{a}$ |
| Week 5 | 23.1 ± 1.3$^{a}$ | 27.1 ± 1.8$^{a}$ | 22.7 ± 1.9$^{a}$ | 26.8 ± 2.6$^{a}$ | 27.4 ± 2.0$^{a}$ |
| Week 6 | 19.2 ± 0.8$^{b}$ | 28.3 ± 2.8$^{a}$ | 24.2 ± 1.0$^{ab}$ | 26.6 ± 2.4$^{a}$ | 28.2 ± 3.0$^{a}$ |
| Week 7 | 19.1 ± 1.8$^{b}$ | 27.8 ± 1.9$^{a}$ | 23.9 ± 0.8$^{ab}$ | 29.0 ± 3.2$^{a}$ | 29.7 ± 1.5$^{a}$ |
| Week 8 | 23.9 ± 1.1$^{b}$ | 30.8 ± 2.0$^{a}$ | 25.2 ± 1.0$^{ab}$ | 29.2 ± 2.6$^{ab}$ | 30.5 ± 2.0$^{a}$ |
| Week 9 | 20.3 ± 0.7$^{c}$ | 27.8 ± 1.5$^{ab}$ | 26.7 ± 0.8$^{b}$ | 30.4 ± 2.3$^{ab}$ | 31.5 ± 1.7$^{a}$ |
| Week 10 | 20.7 ± 15.1$^{c}$ | 27.2 ± 1.4$^{ab}$ | 24.7 ± 0.8$^{bc}$ | 30.1 ± 2.8$^{ab}$ | 29.6 ± 2.0$^{a}$ |
| Week 11 | 19.5 ± 0.7$^{c}$ | 28.0 ± 1.7$^{ab}$ | 25.4 ± 0.9$^{b}$ | 28.5 ± 2.3$^{ab}$ | 30.3 ± 1.5$^{a}$ |
| Week 12 | 20.3 ± 2.0$^{b}$ | 22.2 ± 1.6$^{ab}$ | 24.5 ± 2.0$^{ab}$ | 24.3 ± 2.0$^{ab}$ | 27.1 ± 2.0$^{a}$ |
| Average daily food intake | 21.2 ± 0.5$^{b}$ | 27.9 ± 0.9$^{a}$ | 25.7 ± 0.8$^{a}$ | 28.6 ± 0.6$^{a}$ | 28.6 ± 0.5$^{a}$ |

[1]Data are presented as means ± SEM (grams; n = 8 – 11 per group). Values in the same row with different superscript letters are significantly different ($P < 0.05$) from each other.

**TABLE S4** Absolute weights for adipose tissue data[1]

| | lnNPC | faNPC | faHPC | faHPS | faHPM |
|---|---|---|---|---|---|
| Total adipose tissue (g)[2] | 124 ± 10[b] | 352 ± 13[a] | 362 ± 17[a] | 409 ± 27[a] | 394 ± 24[a] |
| Visceral adipose tissue (g)[3] | 38.8 ± 2.4[b] | 79.6 ± 3.51[a] | 79.2 ± 4.5[a] | 82.1 ± 5.4[a] | 91.2 ± 5.8[a] |
| Epididymal adipose (g) | 15.1 ± 0.8[b] | 17.1 ± 1.14[b] | 16.2 ± 1.0[b] | 21.4 ± 1.4[a] | 21.7 ± 1.4[a] |
| Perirenal adipose (g) | 14.5 ± 0.1[b] | 47.8 ± 2.64[a] | 49.7 ± 3.9[a] | 47.3 ± 4.1[a] | 53.9 ± 4.6[a] |
| Mesenteric adipose (g) | 9.20 ± 0.8[b] | 14.8 ± 1.16[a] | 13.3 ± 1.4[a] | 13.5 ± 0.7[a] | 15.7 ± 1.6[a] |
| Subcutaneous adipose tissue (g)[4] | 85.4 ± 7.5[c] | 272 ± 11[b] | 281 ± 16[ab] | 327 ± 23[a] | 302 ± 19[ab] |

[1]Data are presented as means ± SEM (n = 8 – 11 per group). Values in the same row with different superscript letters are significantly different ($P < 0.05$) from each other.
[2]Total adipose = visceral + subcutaneous adipose tissue.
[3]Visceral adipose = epididymal + perirenal + mesenteric adipose tissue.
[4]Subcutaneous adipose tissue weight obtained from dual-energy x-ray absorptiometry.

**TABLE S5** Gastrocnemius muscle data[1]

| | lnNPC | faNPC | faHPC | faHPS | faHPM |
|---|---|---|---|---|---|
| Gastrocnemius muscle (g) | 4.70 ± 0.08[a] | 3.29 ± 0.07[c] | 3.42 ± 0.10[bc] | 3.52 ± 0.11[bc] | 3.69 ± 0.10[b] |
| Gastrocnemius muscle (g/100 g bwt) | 0.859 ± 0.02[a] | 0.490 ± 0.01[b] | 0.482 ± 0.02[b] | 0.471 ± 0.03[b] | 0.501 ± 0.02[b] |

[1]Data are presented as means ± SEM (n = 8 – 11 per group). Values in the same row with different superscript letters are significantly different ($P < 0.05$) from each other.

**TABLE S6** Oral glucose tolerance testing and area under the curve data[1]

| | lnNPC | faNPC | faHPC | faHPS | faHPM |
|---|---|---|---|---|---|
| Glucose (mmol/L) | | | | | |
| t = 0 | 9.68 ± 0.42$^{c}$ | 13.1 ± 0.6$^{a}$ | 10.9 ± 0.4$^{bc}$ | 12.4 ± 1.1$^{ab}$ | 11.5 ± 0.6$^{ab}$ |
| t = 15 | 14.4 ± 0.6$^{b}$ | 16.6 ± 0.9$^{ab}$ | 14.5 ± 0.7$^{b}$ | 15.7 ± 0.4$^{ab}$ | 19.3 ± 1.3$^{a}$ |
| t = 30 | 16.5 ± 0.7$^{c}$ | 18.2 ± 1.0$^{bc}$ | 17.9 ± 0.9$^{bc}$ | 20.5 ± 0.9$^{ab}$ | 23.7 ± 1.5$^{a}$ |
| t = 60 | 18.6 ± 0.8$^{bc}$ | 17.0 ± 1.1$^{c}$ | 19.1 ± 1.0$^{bc}$ | 22.2 ± 1.1$^{ab}$ | 24.1 ± 1.5$^{a}$ |
| t = 120 | 9.63 ± 0.6$^{b}$ | 11.3 ± 1.0$^{ab}$ | 10.5 ± 0.5$^{ab}$ | 12.8 ± 1.1$^{a}$ | 12.0 ± 0.8$^{a}$ |
| $AUC_g$ (min*mmol/L)[2] | 1784 ± 58$^{b}$ | 1860 ± 110$^{b}$ | 1875 ± 82$^{b}$ | 2169 ± 83$^{ab}$ | 2311 ± 118$^{a}$ |
| Insulin (pmol/L) | | | | | |
| t = 0 | 641 ± 45$^{d}$ | 3260 ± 390$^{a}$ | 2853 ± 688$^{ab}$ | 1945 ± 148$^{bc}$ | 1384 ± 89$^{c}$ |
| t = 15 | 1096 ± 107$^{c}$ | 3722 ± 627$^{a}$ | 3062 ± 539$^{ab}$ | 2036 ± 248$^{b}$ | 1986 ± 302$^{b}$ |
| t = 30 | 794 ± 83$^{c}$ | 3706 ± 516$^{a}$ | 3197 ± 576$^{ab}$ | 2296 ± 239$^{b}$ | 1966 ± 151$^{b}$ |
| t = 60 | 769 ± 98$^{d}$ | 3700 ± 545$^{a}$ | 3522 ± 845$^{ab}$ | 2179 ± 177$^{bc}$ | 1883 ± 169$^{c}$ |
| t = 120 | 1206 ± 231$^{c}$ | 4342 ± 707$^{a}$ | 3720 ± 575$^{a}$ | 2790 ± 360$^{ab}$ | 1921 ± 206$^{b}$ |
| $AUC_i$ (min*pmol/L x $10^3$)[3] | 110 ± 10$^{d}$ | 460 ± 65$^{a}$ | 409 ± 79$^{ab}$ | 279 ± 19$^{bc}$ | 227 ± 14$^{cd}$ |
| $AUC_{index}$ (mmol*pmol*$min^2$ x $10^8$)[4] | 1.96 ± 0.20$^{c}$ | 8.96 ± 1.75$^{a}$ | 7.58 ± 1.42$^{ab}$ | 6.02 ± 0.44$^{ab}$ | 5.23 ± 0.43$^{bc}$ |

[1]Data are presented as means ± SEM (n = 7-10 per group). Values in the same row with different superscript letters are significantly different ($P < 0.05$) from each other.
[2]Area under the curve for glucose
[3]Area under the curve for insulin
[4]Glucose-insulin index

**TABLE S7** Fasting serum glucose and insulin at 6 weeks, 11 weeks and 12 weeks[1]

| | lnNPC | faNPC | faHPC | faHPS | faHPM |
|---|---|---|---|---|---|
| Glucose (mmol/L) | | | | | |
| 6 weeks[2] | 9.52 ± 0.80 | 11.5 ± 0.9 | 10.4 ± 0.9 | 11.1 ± 1.0 | 11.5 ± 0.6 |
| 11 weeks[3] | 9.68 ± 0.42$^{c}$ | 13.1 ± 0.6$^{a}$ | 10.9 ± 0.4$^{bc}$ | 12.4 ± 1.1$^{ab}$ | 11.5 ± 0.6$^{ab}$ |
| 12 weeks[4] | 13.1 ± 0.6 | 16.2 ± 0.7 | 15.7 ± 1.6 | 18.8 ± 2.3 | 14.1 ± 0.6 |
| Insulin (pmol/L) | | | | | |
| 6 weeks[2] | 273 ± 25$^{c}$ | 3723 ± 445$^{a}$ | 4060 ± 403$^{a}$ | 3230 ± 378$^{a}$ | 1924 ± 228$^{b}$ |
| 11 weeks[3] | 641 ± 45$^{d}$ | 3260 ± 390$^{a}$ | 2853 ± 688$^{ab}$ | 1945 ± 148$^{bc}$ | 1384 ± 89$^{c}$ |
| 12 weeks[4] | 75 ± 16$^{c}$ | 663 ± 75$^{a}$ | 597 ± 68$^{ab}$ | 440 ± 99$^{b}$ | 246 ± 25$^{c}$ |
| Insulin:glucose ratio (pmol/mmol) | | | | | |
| 6 weeks[2] | 29.3 ± 3.1$^{c}$ | 338 ± 41$^{a}$ | 414 ± 60$^{a}$ | 302 ± 43$^{a}$ | 174 ± 24$^{b}$ |
| 11 weeks[3] | 68.4 ± 6.9$^{c}$ | 251 ± 29$^{ab}$ | 258 ± 60$^{a}$ | 161 ± 16$^{bc}$ | 124 ± 10$^{c}$ |
| 12 weeks[4] | 5.23 ± 1.25$^{c}$ | 43.0 ± 5.4$^{a}$ | 42.3 ± 4.7$^{a}$ | 29.4 ± 9.6$^{ab}$ | 16.9 ± 2.1$^{b}$ |
| HOMA-IR (mmol/L*µU/mL)[5] | | | | | |
| 6 weeks[2] | 17.0 ± 2.0$^{c}$ | 270 ± 35$^{a}$ | 278 ± 45$^{a}$ | 230 ± 35$^{a}$ | 140 ± 17$^{b}$ |
| 11 weeks[3] | 39.0 ± 2.23$^{d}$ | 276 ± 38$^{a}$ | 203 ± 51$^{ab}$ | 155 ± 18$^{bc}$ | 101 ± 6.4$^{cd}$ |
| 12 weeks[4] | 6.89 ± 1.58$^{d}$ | 66.4 ± 8.0$^{a}$ | 56.7 ± 9.3$^{ab}$ | 44.7 ± 8.9$^{b}$ | 23.6 ± 2.3$^{c}$ |

[1]Data are presented as means ± SEM (n = 7 – 10 per group). Values in the same row with different superscript letters are significantly different ($P < 0.05$) from each other. An absence of letters indicates that means are not statistically different.
[2]Blood collected via jugular vein after 5 – 7 hour fast.
[3]Oral glucose tolerance testing blood sample at t = 0; blood collected via saphenous vein after 5 hour fast.
[4]Termination blood sample; blood collected via cardiac puncture after 12 hour fast.
[5]Homeostatic model assessment index for insulin resistance

**TABLE S8** Fasting serum free fatty acids, triglycerides and haptoglobin at 6 weeks and 12 weeks[1]

| | lnNPC | faNPC | faHPC | faHPS | faHPM |
|---|---|---|---|---|---|
| Free fatty acids (mmol/L) | | | | | |
| 6 weeks[2] | 0.494 ± 0.05$^{b}$ | 0.664 ± 0.03$^{a}$ | 0.631 ± 0.06$^{a}$ | 0.705 ± 0.04$^{a}$ | 0.675 ± 0.04$^{a}$ |
| 12 weeks[3] | 0.273 ± 0.03$^{ab}$ | 0.313 ± 0.04$^{a}$ | 0.282 ± 0.03$^{ab}$ | 0.395 ± 0.07$^{a}$ | 0.167 ± 0.04$^{b}$ |
| Triglycerides (mmol/L) | | | | | |
| 6 weeks[2] | 1.67 ± 0.17$^{d}$ | 3.75 ± 0.44$^{bc}$ | 3.05 ± 0.37$^{c}$ | 4.45 ± 0.45$^{ab}$ | 6.59 ± 0.96$^{a}$ |
| 12 weeks[3] | 1.31 ± 0.20$^{c}$ | 6.90 ± 0.94$^{b}$ | 11.12 ± 2.45$^{ab}$ | 10.20 ± 1.84$^{ab}$ | 12.94 ± 2.06$^{a}$ |
| Haptoglobin (mg/mL) | | | | | |
| 6 weeks[2] | 1.24 ± 0.12$^{c}$ | 3.42 ± 0.27$^{a}$ | 2.65 ± 0.37$^{b}$ | 2.12 ± 0.29$^{b}$ | 2.24 ± 0.16$^{b}$ |
| 12 weeks[3] | 1.13 ± 0.12$^{b}$ | 2.84 ± 0.30$^{a}$ | 2.17 ± 0.28$^{a}$ | 2.12 ± 0.35$^{a}$ | 2.46 ± 0.21$^{a}$ |

[1]Data are presented as means ± SEM (n = 8 – 11 per group). Values in the same row with different superscript letters are significantly different ($P < 0.05$) from each other.
[2]Blood collected via jugular vein after 5 – 7 hour fast.
[3]Termination blood sample; blood collected via cardiac puncture after 12 hour fast.

**TABLE S9** Pancreatic islet size and pancreas weight[1]

| | lnNPC | faNPC | faHPC | faHPS | faHPM |
|---|---|---|---|---|---|
| Islet cell area (µm x $10^2$) | $1.01 \pm 0.25^{c}$ | $4.55 \pm 0.84^{a}$ | $5.17 \pm 1.47^{a}$ | $3.17 \pm 0.79^{ab}$ | $2.02 \pm 0.63^{bc}$ |
| Pancreas (g) | $0.78 \pm 0.03^{b}$ | $0.66 \pm 0.04^{b}$ | $0.78 \pm 0.05^{b}$ | $1.03 \pm 0.04^{a}$ | $1.12 \pm 0.05^{a}$ |
| Pancreas (g/100 g bwt) | $0.14 \pm 0.01^{a}$ | $0.10 \pm 0.01^{b}$ | $0.11 \pm 0.01^{b}$ | $0.14 \pm 0.01^{a}$ | $0.15 \pm 0.01^{a}$ |

[1]Data are presented as means ± SEM (n = 7-10 per group for islet cell area; n = 8 – 11 per group for pancreas weight). Values in the same row with different superscript letters are significantly different ($P < 0.05$) from each other.

**TABLE S10** Western blotting data[1]

| | lnNPC | faNPC | faHPC | faHPS | faHPM |
|---|---|---|---|---|---|
| pAkt(Ser473)[2]:Akt | 0.92 ± 0.14 | 0.92 ± 0.19 | 0.89 ± 0.10 | 0.94 ± 0.16 | 0.71 ± 0.06 |
| pAkt(Thr308)[3]:Akt | 0.87 ± 0.19 | 0.86 ± 0.14 | 0.79 ± 0.07 | 1.02 ± 0.14 | 0.92 ± 0.16 |
| Akt:MAPK p44/42[4] | 1.19 ± 0.10 | 1.34 ± 0.12 | 1.14 ± 0.15 | 1.08 ± 0.15 | 1.09 ± 0.10 |
| pIRS-1(Ser636/639)[5]:IRS-1[6] | 0.86 ± 0.13 | 1.09 ± 0.13 | 1.20 ± 0.17 | 1.24 ± 0.26 | 1.19 ± 0.25 |
| IRS-1:eEF2[7] | 1.23 ± 0.10 | 0.99 ± 0.14 | 1.31 ± 0.27 | 0.98 ± 0.20 | 1.09 ± 0.20 |

[1]Data are presented as means ± SEM (n = 8 per group). An absence of letters indicates that means are not statistically different. All values given as arbitrary units.
[2]Phosphorylated Akt at serine 473
[3]Phosphorylated Akt at threonine 308
[4]Mitogen-activated protein kinase p44/42
[5]Phosphorylated insulin receptor substrate-1 at serine 636/639
[6]Insulin receptor substrate-1
[7]Eukaryotic elongation factor 2

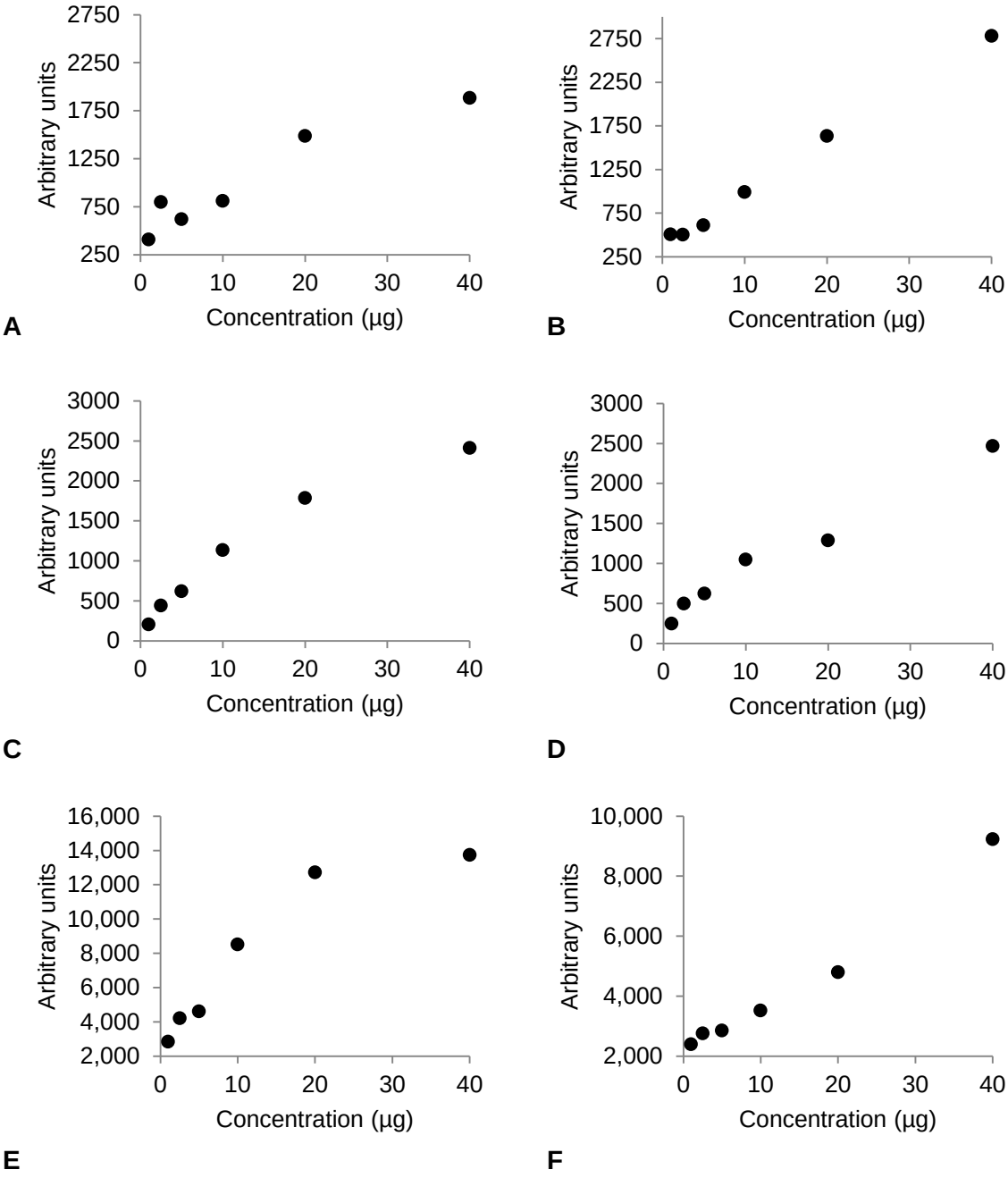



**FIGURE S1** Dose response analysis for Western blots: pAkt(Ser473) – lean (A), pAkt(Ser473) – *fa/fa* (B), pAkt(Thr308) – lean (C), pAkt(Thr308) – *fa/fa* (D), Akt – lean (E), Akt – *fa/fa* (F). For subsequent experimental samples, 7.5 µg was loaded for all proteins listed.

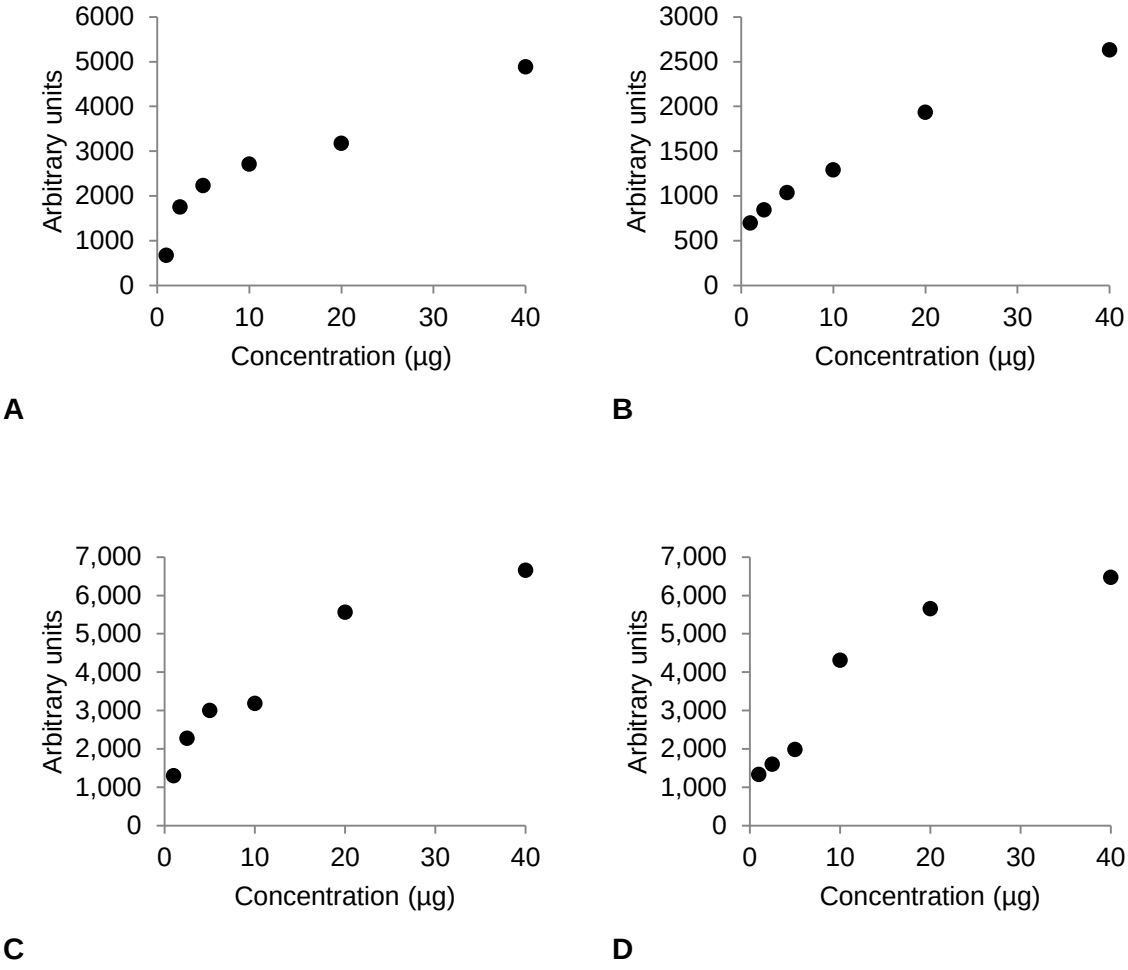



**FIGURE S2** Dose response analysis for Western blots: pIRS-1(Ser636/639) – lean (A), pIRS-1(Ser636/639) – *fa/fa* (B), IRS-1 – lean (C), IRS-1 – *fa/fa* (D). For subsequent experimental samples, 10 µg was loaded for all proteins listed.

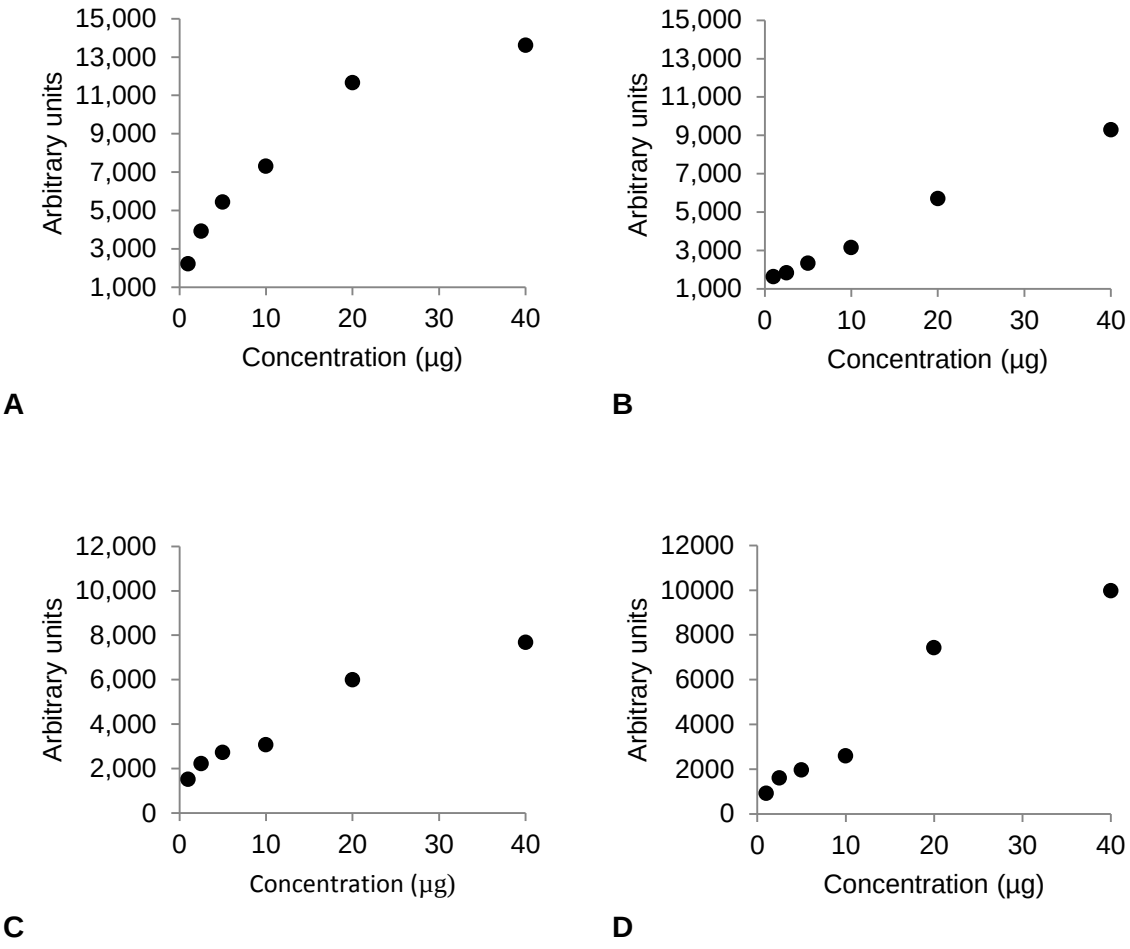



**FIGURE S3** Dose response analysis for Western blot loading controls: MAPK p44/42 – lean (A), MAPK p44/42 – *fa/fa* (B), eEF2 – lean (C), eEF2 – *fa/fa* (D). For subsequent experimental samples, 7.5 µg was loaded for MAPK p44/42 and 10 µg was loaded for eEF2.

**TABLE S11** Liver weight, hepatic steatosis and intrahepatocellular lipid accumulation data[1]

| | lnNPC | faNPC | faHPC | faHPS | faHPM |
|---|---|---|---|---|---|
| Liver (g) | 14.7 ± 0.9$^{c}$ | 44.2 ± 2.2$^{a}$ | 39.5 ± 2.1$^{a}$ | 30.4 ± 2.5$^{b}$ | 28.2 ± 2.0$^{b}$ |
| Liver (g/100 g bwt) | 5.33 ± 0.18$^{d}$ | 13.2 ± 0.8$^{a}$ | 11.1 ± 0.7$^{b}$ | 8.07 ± 0.71$^{c}$ | 7.57 ± 0.37$^{c}$ |
| Total liver lipid concentration (g lipid/g tissue) | 0.094 ± 0.01$^{b}$ | 0.240 ± 0.02$^{a}$ | 0.242 ± 0.01$^{a}$ | 0.113 ± 0.01$^{b}$ | 0.091 ± 0.02$^{b}$ |
| Hepatic steatosis rating (1 = low degree, 4 = high degree) | 1.88 ± 0.13$^{b}$ | 3.43 ± 0.20$^{a}$ | 3.38 ± 0.18$^{a}$ | 2.14 ± 0.26$^{b}$ | 1.63 ± 0.26$^{b}$ |
| Number of liver lipid droplets (per 0.01 $mm^2$) | 83.3 ± 5.9$^{a}$ | 54.4 ± 3.1$^{bc}$ | 47.5 ± 3.6$^{c}$ | 57.1 ± 8.0$^{bc}$ | 67.9 ± 8.8$^{ab}$ |
| Liver lipid droplet size ($\mu m^2$) | 55.6 ± 5.7 | 102 ± 33 | 86.9 ± 23.9 | 83.8 ± 18.6 | 54.8 ± 16.7 |
| Liver lipid droplet distribution rating (1 = uniform fat droplets, 4 = very varied fat droplets) | 2.25 ± 0.45 | 1.86 ± 0.26 | 1.63 ± 0.26 | 2.14 ± 0.26 | 2.50 ± 0.42 |

[1]Data are presented as means ± SEM (n = 8 – 11 per group for liver weight; n = 4 per group for liver lipid concentration; n = 6-8 per group for all other results). Values in the same row with different superscript letters are significantly different ($P < 0.05$) from each other. An absence of letters indicates that means are not statistically different.

**TABLE S12** Non-lipid and lipid liver content[1,2]

| | **lnNPC** | **faNPC** | **faHPC** | **faHPS** | **faHPM** |
|---|---|---|---|---|---|
| Non-lipid weight (%) | 90.6 ± 0.7[a] | 76.0 ± 2.0[b] | 75.8 ± 1.20[b] | 88.7 ± 1.0[a] | 90.9 ± 1.5[a] |
| Lipid weight (%) | 9.40 ± 0.71[a] | 24.0 ± 2.0[b] | 24.2 ± 1.2[b] | 11.3 ± 1.0[a] | 9.14 ± 1.53[a] |

[1]Data are presented as means ± SEM (n = 4 per group). Values in the same row with different superscript letters are significantly different ($P < 0.05$) from each other.
[2]Data calculated using total hepatic lipid concentration data.

**TALBE S13** Blood pressure data

| | lnNPC | faNPC | faHPC | faHPS | faHPM |
|---|---|---|---|---|---|
| Systolic pressure (mmHg) | | | | | |
| Week 3 | 140 ± 3[ab] | 145 ± 3[ab] | 148 ± 4[a] | 135 ± 6[b] | 151 ± 3[a] |
| Week 7 | 154 ± 3[a] | 151 ± 3[a] | 149 ± 7[ab] | 137 ± 4[b] | 158 ± 3[a] |
| Week 11 | 148 ± 4[a] | 162 ± 5[a] | 150 ± 6[a] | 149 ± 6[a] | 155 ± 3[a] |
| Diastolic pressure (mmHg) | | | | | |
| Week 3 | 101 ± 2[a] | 102 ± 4[a] | 101 ± 4[a] | 95.8 ± 5.0[a] | 106 ± 4[a] |
| Week 7 | 107 ± 3[a] | 104 ± 3[ab] | 106 ± 7[a] | 93.1 ± 3.2[b] | 114 ± 2[a] |
| Week 11 | 107 ± 3[a] | 113 ± 5[a] | 105 ± 6[a] | 104 ± 5.4[a] | 108 ± 4[a] |
| Mean arterial pressure (mmHg) | | | | | |
| Week 3 | 115 ± 7[ab] | 116 ± 11[ab] | 115 ± 11[ab] | 109 ± 15[b] | 121 ± 11[a] |
| Week 7 | 124 ± 9[a] | 120 ± 10[a] | 120 ± 21[a] | 108 ± 10[b] | 129 ± 8[a] |
| Week 11 | 121 ± 9[a] | 129 ± 15[a] | 120 ± 17[a] | 118 ± 17[a] | 124 ± 11[a] |

[1]Data are presented as means ± SEM (n = 8 – 11 per group). Values in the same row with different superscript letters are significantly different ($P < 0.05$) from each other. The faHPS group had delayed progression to hypertension as evidenced by lower systolic pressure at week 3 compared to both the faHPC and faHPM groups and at week 7 compared to the faHPM group, lower diastolic pressure at week 7 compared to the faHPM group, and lower mean arterial pressure at week 3 compared to the faHPM group and week 7 compared to both the faHPC and faHPM groups.

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