INSULIN RESISTANCE IN OBESITY: TARGETING THE MOLECULAR MECHANISMS OF METABOLIC DISEASE

A dissertation submitted
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by

Ciarán E. Fealy

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Abbreviations

\( \Delta \Psi_m \) Membrane Potential

13C-MRS Carbon-13 Magnetic Resonance Spectroscopy

1H-MRS Proton Magnetic Resonance Spectroscopy

31P-MRS Phosphorus-31 Magnetic Resonance Spectroscopy

ACSM American College of Sports Medicine

ADA American Diabetes Association

ADP Adenosine Diphosphate

AKT Protein Kinase B

ALT Alanine Aminotransferase

AMP Adenosine Monophosphate

AMPK AMP-activated protein kinase

AMRAP As Many Repetitions as Possible

ANT Adenine Nucleotide Translocator

AS160 Akt Substrate of 160 kDa

AST Aspartate Aminotransferase

ATP Adenosine Triphosphate

AUC Area under the Curve

BAX BCL-2-Associated X Protein

BCL-2 Beta Cell Lymphoma 2

BMI Body Mass Index
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<td>Creatine Kinase</td>
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<td>CK18</td>
<td>Cytokeratin 18</td>
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<td>CKD</td>
<td>Chronic Kidney Disease</td>
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<td>Coenzyme A</td>
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<td>Diacylglycerol</td>
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<td>Diastolic Blood Pressure</td>
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<td>Ethylenediaminetetraacetic acid</td>
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<td>FOX</td>
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<td>Glycated Hemoglobin</td>
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<td>HIIT</td>
<td>High Intensity Interval Training</td>
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<tr>
<td>HIPT</td>
<td>High Intensity Power Training</td>
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<td>HMW</td>
<td>High Molecular Weight</td>
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<td>HOMA-IR</td>
<td>Homeostatic Model Assessment of Insulin Resistance</td>
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<td>Intramyocellular Lipids</td>
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<td>IRS</td>
<td>Insulin Receptor Substrate</td>
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<td>KB</td>
<td>Kettlebell</td>
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<td>LDL</td>
<td>Low Density Lipoproteins</td>
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<td>MAP</td>
<td>Mean Arterial Pressure</td>
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<td>Mitochondrial Division Inhibitor-1</td>
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<td>MEF</td>
<td>Mouse Embryonic Fibroblast</td>
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<tr>
<td>Mff</td>
<td>Mitochondrial Fission Factor</td>
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<td>Mfn1</td>
<td>Mitofusin Protein 1</td>
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<td>Mfn2</td>
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<tr>
<td>mPTP</td>
<td>Mitochondrial Permeability Transition Pore</td>
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<td>mTOR</td>
<td>Mammalian Target of Rapamycin</td>
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<td>NADH</td>
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<td>NASH</td>
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<td>NFκB</td>
<td>Nuclear Factor Kappa-light-chain-enhancer of Activated β Cells</td>
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<td>NIDDM</td>
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<td>Non-leptin Producing Obese Mutant Mouse</td>
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<td>Oral Glucose Tolerance Test</td>
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<td>PGC1α</td>
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<td>Ribonucleic Acid</td>
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<td>VO2max</td>
<td>Maximal Oxygen Consumption</td>
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<tr>
<td>WOD</td>
<td>Workout of the Day</td>
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DEDICATION

To my parents James and Dolores Fealy; thank you for the love and support over the years. Your unstinting belief in me helped carry me through the hardest days.
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Abstract

The prevalence of obesity has been increasing rapidly over the past 30 years such that obesity-associated metabolic diseases including non-alcoholic fatty liver disease (NAFLD) and type 2 diabetes (T2D) are now among the leading causes of morbidity and mortality. This makes the discovery of novel, practical therapies for prevention and treatment, along with better understanding of the mechanisms that link obesity to metabolic disease, the most significant challenge for biomedical research in the 21st century. Insulin resistance is a common pathology underlying the development of obesity associated metabolic disease and is a target for disease progression. Exercise has long been recognized as an effective tool for managing glycemic control and its use in research provides insight into the mechanisms that contribute to insulin resistance. We used an exercise approach to investigate the effect of exercise training, independent of weight loss, on markers of hepatocyte apoptosis in individuals with NAFLD. Our data indicate that hepatocyte apoptosis is reduced following 7 days of exercise training. We also examined the efficacy of a novel high intensity exercise program for improving insulin sensitivity and reducing cardiometabolic risk in T2D. We concluded that CrossFit exercise reduces insulin resistance and cardiovascular risk in T2D. We also used an exercise approach to investigate a novel target that may link mitochondrial dysfunction to insulin resistance. We demonstrated that the activation of the mitochondrial fission mediator dynamin-related protein-1 was reduced by exercise in insulin resistant muscle and subsequent cell culture experiments provide evidence that this may be linked to improvements in insulin signalling and mitochondrial coupling efficiency.
CHAPTER 1.

Introduction

1.1 Background

Overweight, defined as a body mass index (BMI) of \( \geq 25 \text{ kg/m}^2 \), and obesity (BMI \( \geq 30 \text{ kg/m}^2 \)) are recognized as major contributing factors in the development of hypertension (1), heart disease (2, 3), type 2 diabetes (T2D) (4), stroke (5), non-alcoholic fatty liver disease (NAFLD) (6), pulmonary and respiratory disease (7), chronic kidney disease (CKD) (8), along with several variants of cancer (9) and neurological conditions such as Alzheimer’s (10) and Parkinson’s disease (11). In fact, overweight and obesity contribute to 7 of the 10 leading causes of death in the US (12) and contributed to an annual medical cost of $147 billion in 2008, with the medical cost for an obese individual amounting to an average of $1429 more than that of a normal weight individual (13).

Despite this knowledge, overweight and obesity continue to rise in the general population. In the period between 1960 and 2012, the relative proportion of the United States population categorized as overweight/obese has risen from \(~42\%\) to almost 75\% (14). These figures include a doubling of obesity rates (15.8±0.6 to 36.6±1.6 \%) and a greater than 6-fold increase in extreme obesity (BMI \( \geq 40\text{ kg/m}^2 \); 1.4±0.2 to 8.6±0.7\%). Even more troubling is the rise in early childhood obesity (15) and the associated appearance of metabolic diseases such as NAFLD (16) and T2D in children. Recent reports indicate that 8–45\% of children with newly diagnosed diabetes have non-
immune-mediated diabetes, with up to 85% of affected children either overweight or obese at diagnosis (17). Given that these trends appear to be a global phenomenon (18), it is reasonable to suggest that treating the obesity epidemic is poised to be one of the most significant public health challenges of the 21st century. Thus, the discovery of new therapeutic targets to prevent the progression of obesity associated diseases, along with the development of practical treatment modalities for overweight and obesity, are urgently required.

1.2 Obesity and Metabolic Disease

As the writings of both Galen and Hippocrates illustrate, the relationship between obesity and diminished health has been recognized for millennia. However, it is only in the past century that we have begun to understand the mechanisms that link obesity to metabolic diseases such as NAFLD and T2D. In the following sections I will examine the relationship between obesity and metabolic disease, specifically NAFLD and T2D. Furthermore, I will make the case that insulin resistance is the common underlying pathology that links metabolic disease with obesity and review some of the possible mechanisms that contribute to insulin resistance with a particular focus on skeletal muscle and liver insulin resistance. Finally, I will examine the use of exercise as a therapeutic and research tool in insulin resistance.

1.2.1 Obesity and NAFLD

Non-alcoholic fatty liver disease (NAFLD) is among the most common forms of chronic liver disease today. Current estimates suggest that approximately 20-30% of the
population are affected by the condition (19). NAFLD is strongly linked to obesity, with a reported prevalence as high as 80% in obese patients and only 16% in individuals with a normal BMI and without metabolic risk factors (20, 21).

NAFLD represents a spectrum of disorders ranging from benign steatosis, to more severe forms of the disease including non-alcoholic steatohepatitis (NASH) and cirrhosis (Figure 1-1). NAFLD is defined clinically as steatosis, or fatty infiltration, affecting >5% of hepatocytes in the absence of excessive significant alcohol consumption, other liver disease or the consumption of steatogenic drugs (22), while progression to NASH is characterized by the development of liver fibrosis, ballooning necrosis, and inflammation (23). As such, diagnosis of NASH requires a liver biopsy. Whilst only 10-20% of individuals with simple steatosis will progress to NASH (19), up to 25% of those with NASH develop cirrhosis, of which 30–40% succumb to liver related death within 10 years (24).

![Figure 1-1. The Pathogenesis of NAFLD](image)
According to the most widespread and prevailing model, the "two-hit hypothesis", the pathogenesis of NAFLD is marked by excessive accumulation of intrahepatic lipid moieties - the first-hit - resulting from obesity induced insulin resistance (25, 26). The "first-hit" increases the vulnerability of the liver to many factors that constitute the "second-hit" and promote hepatic injury, inflammation and fibrosis (25, 26). Recently it has become apparent that hepatocyte apoptosis is a major contributor to this profibrogenic state (27). Apoptosis, a form of programmed cell death, is an active ATP-dependent process that under normal physiological conditions contributes to the maintenance of normal tissue homeostasis. However, in certain pathophysiological conditions, such as NAFLD, apoptosis is upregulated, overwhelming the normal phagocytic engulfment of apoptotic cells, triggering a proinflammatory and profibrogenic response from hepatocytes (28). There are several molecular pathways involved in the induction of apoptosis. In Chapter 2 we will examine exercise induced responses in one of these pathways – The Fas pathway.

1.2.2 Obesity and Type 2 Diabetes

Overweight and obesity are significant risk factors for the development of T2D (29). Recent data have shown that 27.6 million Americans (9.3%) currently have T2D (30). As of 2010 it was estimated that 285 million adults had diabetes worldwide with the further prediction that 439 million people would have the disease by 2030 (30). Moreover, poorly controlled T2D has been shown to lead to several significant comorbidities including cardiovascular disease, stroke, blindness, kidney disease, and
amputations (31-34). These conditions have contributed to an elevated cost of healthcare for diabetic individuals with a total cost of diagnosed diabetes care exceeding 245 billion dollars in 2012 (30).

The Pathophysiology of Type 2 Diabetes

T2D is a chronic metabolic disease characterized by an abnormal increase in plasma glucose. The diagnosis criteria for diabetes outlined by the American Diabetes Association includes the following; 1. Fasting plasma glucose greater than 126 mg/dL, 2. a random plasma glucose higher than 200 mg/dL in association with symptoms of diabetes, and/or 3. a persistent elevation in plasma glucose following an oral glucose load (greater than 200mg/dL two hours after glucose ingestion) (35). Figure 1.2 illustrates the natural progression of T2D.

![Figure 1-2 The Natural History of Type 2 Diabetes](image-url)
Similar to NAFLD, the “first-hit” in T2D is also associated with the onset of insulin resistance. The presence of insulin resistance in peripheral tissues, most notably the skeletal muscle, adipose tissue, and liver, results in a compensatory rise in pancreatic insulin secretion. During this phase, hyperinsulinemia maintains blood glucose at normal levels. Eventually however, the degree of insulin resistance may overwhelm the ability of the pancreas to compensate, and blood glucose levels begin to rise. This is sometimes associated with failure of the pancreatic β-cells, a corresponding reduction in insulin secretion, leading to uncontrolled plasma glucose increases. The long term effects of such a loss of glycemic control and elevated insulin secretion include cardiovascular disease, hypertension and stroke, nephropathy, neuropathy and retinopathy. In addition, insulin resistance has been implicated in cancers such as breast, prostate, pancreatic, colorectal, and liver, while also increasing risk of neurodegenerative conditions such as Parkinson’s and Alzheimer’s disease. Thus, insulin resistance links overweight and obesity to the development of metabolic disease. Yet the underlying causes of insulin resistance remain poorly understood and, as a result, pharmacological interventions have had limited success in reducing the burden of the overweight/obesity epidemic. One of the key goals of the studies contained herein is to explore novel treatment strategies that target insulin resistance and the progression of associated comorbidities, and in doing so to develop a better understanding of the mechanisms that drive insulin resistance and metabolic disease.
1.3 The Insulin Signaling Pathway

Insulin is a peptide hormone secreted from the β-cells of the pancreas in response to an increase in plasma glucose. Insulin travels through the portal system to the liver and subsequently to peripheral tissues where it propagates its signal. Insulin binds to the insulin receptor in target tissues resulting in autophosphorylation, and subsequent recruitment of insulin receptor substrate (IRS). IRS is phosphorylated at multiple tyrosine residues, activating the docking of the phosphatidylinositol 3-kinase (PI3K) regulatory subunit, p85, to IRS. Once docked, PI3K catalyzes the production of phosphatidylinositol-3,4,5-triphosphate (PIP3) at the plasma membrane. PIP3 recruits PIP3-dependent kinase (PDK1) and Akt (protein kinase B). PDK1 subsequently

Figure 1-3. The Insulin Signaling Pathway
activates Akt via phosphorylation at threonine 308. Akt then phosphorylates numerous substrates including AS160. This ultimately results in translocation of GLUT4 vesicles to the plasma membrane and the internalization of glucose (36). Moreover, insulin has numerous additional physiological effects at target tissues including glycogen synthesis, regulation of protein synthesis and cell growth, and gene expression (Figure 1-3) (37).

1.4 The Physiological Effects of Insulin

1.4.1 Skeletal Muscle

Skeletal muscle is the major target tissue for insulin–stimulated glucose disposal. In healthy people subjected to a euglycemic, hyperinsulinemic clamp ~80% of the glucose is taken up by skeletal muscle. Insulin stimulates skeletal muscle glucose uptake through an increase of GLUT4 translocation to the plasma membrane and transverse tubules (38, 39). Additionally, insulin stimulates glycogen synthesis in muscle by activation of glycogen synthase (GS) (40, 41). Insulin activates glycogen synthase by dephosphorylation via Akt-mediated phosphorylation of GSK3 (41-43). Phosphorylation of GSK3 decreases kinase activity which will decrease phosphorylation of GS and increase glycogens synthase fractional activity (41, 44, 45). In addition to glycogen synthesis, insulin may also stimulate anabolic processes in muscle by activation of the mammalian target of rapamycin (mTOR) pathway (46).

1.4.2 Liver

Under normal physiological conditions, insulin action at the hepatocyte results in suppression of gluconeogenesis and glycogenolysis and increases glycogen synthesis,
resulting in decreased glucose output from the liver (47). This effect is both a direct inhibition through phosphoenolpyruvate carboxykinase (PEPCK) and also indirectly through inhibition of lipolysis in adipose tissue and the consequent reduction in glycerol as a substrate for gluconeogenesis (48). Insulin also increases fatty acid, predominantly palmitic acid (49), and cholesterol biosynthesis by activation of SREBP-c (50). Interestingly, this pathway does not appear to be effected to the same degree as Akt signaling in insulin resistant states (51). Thus, hepatocyte insulin resistance results in increased glucose output and dyslipidemia.

Figure 1-4. The Physiological Effects of Insulin
1.4.3 Adipose Tissue

In adipose tissue, insulin promotes glucose uptake via a GLUT4 dependent mechanism (52). Adipose tissue utilizes glycolysis for energy purposes. In addition, insulin promotes re-esterification of fatty acids into triglycerides while also increasing the pool of glucose-3-phosphate required for esterification (53). It is worth noting, however, that insulin resistance reduces the ability of insulin to promote esterification (54).

1.5 Mechanisms of Insulin Resistance

One of the earliest references to a defect of insulin action in diabetes was by Himsworth in 1936 (55). Here he demonstrated that a large number of patients with diabetes were insulin insensitive and advocated for the subdivision of diabetes into two categories – insulin sensitive and insulin insensitive. Notwithstanding the lack of appropriate diagnostic tools, the concept of insulin resistance and its association with obesity would become well established in the literature in the coming years (56, 57), until eventually the development of measures with a high degree of accuracy, in particular the radioimmunoassay for insulin and c-peptide, allowed confirmation of this finding (58) and prompted the eventual institutionalization of the subdivision of diabetes (59).

Initial research into the etiology of insulin resistance suggested that defects in carbohydrate metabolism may be a factor (56). This was supported by observations in first degree relatives of non-insulin dependent diabetics (NIDDM) who displayed reduced non-oxidative glucose metabolism. The test subjects were not obese and had basal glucose concentrations identical with those of an age-, weight-, and sex-matched control group with no family history of NIDDM. The authors went on to show that the
underlying cause of the problem was diminished muscle glycogen deposition and that this in turn stemmed from a lower than normal insulin activation of glycogen synthase (60).

Yet, even as these discoveries were being made, others were making the case for lipid involvement in development of insulin resistance. Reaven et al. (61) demonstrated that in non-obese normal, mildly diabetic, and severely diabetic patients, plasma free fatty acids (FFA) were positively associated with the degree of hyperglycemia, while inhibition of lipolysis, by Acipimox, and lipid oxidation, by etomoxir reduced plasma glucose concentration, decreased hepatic glucose production, and increased the rate of glucose disposal, in both streptozotocin induced diabetes in animals (61), and in patients with NIDDM (62, 63).

The development of accurate molecular imaging techniques such as $^1$H-MRS further supported the idea of lipid induced insulin resistance. Several studies in the late 1990s utilizing this technique showed a strong negative correlation between intramyocellular lipids (IMCL) and the rate peripheral glucose disposal, irrespective of BMI (64-66), while infusion of lipid/heparin in healthy individuals resulted in a reduction of insulin stimulated glucose disposal (67-70). These data illustrated the similarities between the onset of NAFLD and T2D and underscored the importance of ectopic lipid deposition in the etiology of insulin resistance and metabolic disease. However, as we have already observed in NAFLD, the presence of simple ectopic lipid per se does not appear to be sufficient to result in progression of metabolic disease. Similarly, in skeletal muscle, the discovery what became known as the athletes’ paradox suggested that the presence of IMCL in skeletal muscle is not sufficient to induce insulin resistance,
particularly in individuals with a high oxidative capacity (71). These data showed that endurance trained athletes, who are highly insulin-sensitive, have increased IMCL compared with lean sedentary controls, with levels even exceeding those of diabetics (71). In addition, 12–16 weeks of exercise training in obese subjects improved insulin sensitivity in conjunction with increased IMCL levels (72). As a result, several hypotheses have been suggested to explain the mechanisms responsible for the induction of insulin resistance by excess fatty acids.

### 1.5.1 The Glucose-Free Fatty Acid Cycle

One of the first proposed mechanisms to explain the effect of lipids on glucose metabolism was published by Randle and colleagues in 1963 (73). The central idea behind this thesis was glucose sparing during fatty acid availability. Randle and colleagues proposed that increased fatty acid delivery and oxidation would result in elevated production of citrate, a component of the TCA cycle. Elevated citrate levels result in allosteric regulation of phosphofructokinase-1 (PFK-1) resulting in inactivation of the enzyme and subsequent downregulation of glycolytic flux. This would necessitate a buildup of the precursor molecule glucose-6-phosphate with resultant saturation of glycogen stores. Although there is some evidence that in a healthy physiological state, glycogen saturation results in a feedback mechanism to reduce glucose uptake in muscle (74), individuals with insulin resistance and T2D have lower glycogen levels and reduced non-oxidative glucose disposal (75). Furthermore, $^{13}$C and $^{31}$P MRS studies found that intramyocellular concentrations of glucose-6-phosphate decreased during a lipid infusion,
demonstrating that fatty acids reduce insulin-stimulated glucose utilization in muscle by restricting glucose transport into the cell, not through inhibition of glycolysis (76).

1.5.2 The importance of lipid sub-species

Imaging studies of muscle and liver lipids reflect the presence of triglycerides which are generally considered to be metabolically inert, meaning that they have limited signaling properties. However, with the increasing availability of mass spectrometry techniques, it has become possible to detect intracellular concentrations of metabolically active lipid subspecies, which may regulate directly or indirectly regulate components of the insulin signaling pathway.

Diacylglycerol

Diacylglycerol (DAG) is a lipid molecule consisting of two fatty acid chains covalently bonded to a glycerol molecule through ester linkages. DAG is an intermediate in TAG synthesis and breakdown and liver and skeletal muscle DAGs have been found to be elevated in several models of insulin resistance (77, 78) and are elevated by lipid infusion in some (79, 80) but not all (81) human studies. The effect of DAG on insulin signaling in humans appears to be mediated by increased protein kinase-C (PKC) activation. Activation of PKCθ by lipid infusion in humans was associated with increased serine phosphorylation, and an associated inhibition of tyrosine phosphorylation, of IRS1 and subsequently reduced AKT phosphorylation in skeletal muscle (79, 80). Moreover, increased PKCδ following lipid infusion in human skeletal muscle was associated with increases in DAGs along with activation of pro-inflammatory
pathways (79). In NAFLD, hepatic DAG content is the best predictor of hepatic insulin resistance (82). In hepatocytes, DAG activation of PKCε is associated with reduced tyrosine phosphorylation of IRS1 (83).

Ceramides

Ceramides are members of the sphingolipid family and are integral to the structure of the lipid bilayer that makes up cell membranes (84). However, ceramides are pro-apoptotic (85) and also have cell-signaling properties, and their accumulation in the liver and muscle during periods of increased free fatty acids (FFA) may contribute to insulin resistance (86-88). Ceramides may inhibit insulin signaling by blocking Akt activation through two independent pathways. Ceramides catalyze the dephosphorylation of Akt by activating protein phosphatase 2A (89-91) and may also block Akt translocation to the plasma membrane (90). PKCζ may mediate this ceramide effect by phosphorylating Akt on an inhibitory residue (90). Thus, DAG and ceramides may mediate insulin resistance. However, DAGs and ceramides are also affected by the degree of saturation of fatty acids.

Saturated vs Unsaturated Fatty Acids

Data from nutritional interventions as well as cell culture studies have demonstrated that saturated fatty acids, such as palmitate, induce insulin resistance whereas unsaturated fatty acids, such as oleate, exert a protective effect. This effect is likely due to differential effects on DAG and ceramide accumulation. In a study by Coll et al. (92), exposure to palmitate increased DAG levels and activated the PKC0-NFκB
pathway, resulting in enhanced secretion of IL-6 and down-regulation of PPARγ coactivator 1α (PGC-1α) and acyl-coenzyme A:diacylglycerol acyltransferase 2 (DGAT2), the enzyme that controls the rate of triglyceride (TG) synthesis from DAG. In contrast, co-incubation of palmitate-exposed cells with oleate reversed these changes by promoting TG accumulation and mitochondrial β-oxidation, thus preventing DAG synthesis and activation of the PKCθ-NFκB pathway. Intriguingly, recent evidence also suggests that it is not total DAG *per se* but rather specific DAG-subspecies that may be important. Several studies have demonstrated that the degree of saturation is the most important factor in determining the link between DAG and insulin resistance in skeletal muscle. In a study by Dube et al (93) exercise reduced DAG species to a greater degree than diet-induced weight loss. In the diet group only the content of DAG C14:0 species were reduced, whereas in the exercise group C16:0/18:0, C16:0/18:1, C16:1/18:1, di-C16:0 and di-C18:1 DAG species were reduced. Moreover, it was demonstrated that athletes have a lower saturation of the DAG-fraction compared to sedentary controls (94), while males with metabolic syndrome had total DAG and TG content similar to controls, insulin resistant men had a higher degree of saturation of skeletal muscle DAG (95). This higher degree of saturation was explained by higher palmitic acid and lower oleic acid (95).

Long chain saturated FAs like palmitate are also precursors for ceramide synthesis and rates of ceramide synthesis are dependent on the availability of those FAs. As such, incubation of C2C12 myotubes with palmitate increases ceramide accumulation (89) and diets high in saturated fats induce intramyocellular ceramide accumulation (96).
Indeed, ceramide was identified as an essential intermediate linking saturated FAs to insulin resistance (97).

**Fatty Acyl-CoAs and Acylcarnitines**

Fatty acyl-CoAs and acylcarnitines are intermediate steps in β-oxidation of fatty acids. Upon entering a cell, fatty acids are sequestered by the addition of a Coenzyme A (CoA) group by acyl-CoA synthetase. In order to progress to oxidation acyl-CoA is transferred to the hydroxyl group of carnitine by carnitine acyltransferase I (CAT1), located on the cytosolic faces of the outer and inner mitochondrial membranes. Acyl-carnitine is shuttled inside by carnitine-acylcarnitine translocase, as a carnitine is shuttled outside. Acyl-carnitine is converted back to acyl-CoA by carnitine acyltransferase II (CAT2), located on the interior face of the inner mitochondrial membrane. The liberated carnitine is shuttled back to the cytosol, as an acylcarnitine is shuttled into the matrix (Figure 1-5). In circumstances where supply exceeds the demand or capacity for lipid synthesis or β-oxidation, FA-CoA and acylcarnitines will accumulate in the cell, either in the cytosol or in mitochondria. Whether acyl-CoAs and acylcarnitines contribute to insulin resistance is currently a subject of debate (98), however, similar to DAGs and ceramides, long chain acyl-CoAs and acylcarnitines are elevated in insulin resistance (99, 100) and some evidence suggests involvement in activation of PKC (101). Interestingly, Novgorodov et al. (102) recently demonstrated that ceramides are also produced in mitochondria by the coupled activities of mitochondrial thioesterase, which hydrolyses palmitoyl-CoA to CoA and palmitate, and neutral ceramidase, utilizing sphingosine and released palmitate in a reverse reaction to produce ceramide. Thus,
increased FA-CoA in or near the mitochondria might result in increased ceramide production, thus impairing mitochondrial function and eventually inducing apoptosis.

Acyl-CoA esters have been shown to be potent inhibitors of the adenine nucleotide translocase (ANT), which catalyses the exchange of ADP and ATP across the mitochondrial inner membrane, is generally accepted to be the overall rate limiting step in energy metabolism (103). Consequently, ATP synthesis can be expected to decrease under conditions where acyl-CoA buildup is present. In addition, it has been shown that acylcarnitines can disrupt membrane barriers (104) and thus acyl-CoAs and acylcarnitines may contribute to mitochondrial dysfunction and/or signaling properties at lipid membranes.

![Figure 1-5. The Carnitine Shuttle](image-url)
1.5.3 Mitochondrial Dysfunction and Insulin Resistance

Mitochondria are double membrane-bound organelles found in most eukaryotic cells and are the site of oxidative phosphorylation. The products of glycolysis and β-oxidation are catabolized to generate high energy substrates NADH and FADH$_2$ which are supplied to the electron transport system, a collection of protein complexes on the inner mitochondrial membrane. NADH and FADH$_2$ provide molecular hydrogen to the protein complexes at different points in the chain. Electrons are shuttled between complexes down a voltage gradient. The energy difference is used to pump the H$^+$ protons from the inner mitochondrial matrix to the intermembrane space, thereby generating an electrochemical gradient across the mitochondrial membrane in an elegant process first described by Peter Mitchell in 1961 (105). The membrane potential is then utilized by ATP synthase to add a phosphate group to ADP generating ATP (Figure 1-6).

![Figure 1-6. The Electron Transport System](image)

Figure 1-6. The Electron Transport System
Increased intramyocellular lipids (106, 107) and lipid intermediates, including diacylglycerols (DAGs) and ceramides (108), combined with impaired fat oxidation (109) led, in the later part of the 20th century, to an increase in research into the possibility of the mitochondrial dysfunction in the pathogenesis of insulin resistance. Kelley and colleagues, in a series of studies (110-113), measured the activity of several mitochondrial marker enzymes in skeletal muscle in insulin-resistant individuals and patients with T2D including citrate synthase (110, 112), cytochrome oxidase (111, 112), NADH oxidoreductase (110, 112), carnitine palmitoyl transferase (111), and succinate dehydrogenase (113). The activities of these enzymes were 20–40% lower in the diabetic patients than in normal control subjects. The mitochondria in diabetic muscle were also smaller than normal (110). This phenomenon was reversed by exercise training (72) and suggested that a defect in the mitochondria may be induced by sedentary behavior and that this “mitochondrial dysfunction” results in the buildup of IMCL. However, subsequent studies also found that mitochondrial content in insulin resistant muscle was reduced and when the data were corrected mitochondrial enzyme content and activity, the differences between insulin resistance and controls disappeared (114-116). In addition, reversal of T2D by weight loss does not result in normalization of muscle mitochondrial content despite improvements in insulin sensitivity (117). Moreover, transgenic knockout of mitochondrial transcription factor A (Tfam), a transcription factor that mediates transcription of genes encoded in the mitochondrial genome, in otherwise healthy mice resulted in an increase in insulin action rather than an expected decrease (118). Several reports also indicate that fat oxidation in may be increased in insulin
resistant states (109, 115), though data from indirect calorimetry studies suggest otherwise (119-121). Despite these data, interest in a mitochondrial mechanism for insulin persists. This is influenced, in part at least, by the mechanism by which adipokines such as adiponectin impart their insulin sensitizing effect (122). An alternative view posits that nutrient overload causes a build-up of intermediary metabolites, products of incomplete oxidation such as acyl-CoAs and acylcarnitines, in the mitochondria (123) and these moieties inhibit insulin signaling directly (98), or indirectly by forming lipid species (124) that activate inflammation (125, 126), oxidative stress (127) and stress kinases such as the PKCs (128, 129). This view has gained support from the work of Taddeo et al. (130) who found that oxidative stress led to opening the mitochondrial permeability transition pore (mPTP), a protein pore in the mitochondrial membrane activated by cellular stress and induction of apoptosis. This may permit release of these molecules into the cytosol where they might disrupt insulin signaling. Further, inhibition of the mPTP restored insulin sensitivity. One aspect of mitochondrial physiology that has heretofore received comparatively less attention is the potential role of altered mitochondrial dynamics in the pathogenesis of insulin resistance. Interestingly, data suggests that opening of the mPTP is just one step in the cascade that links nutrient oversupply and mitochondrial dynamics to insulin resistance in skeletal muscle (131).

1.5.4 Mitochondrial Dynamics

Emerging data show that mitochondria are highly dynamic organelles that continually fuse and divide. In healthy skeletal muscle, mitochondria form as a highly
reticular branched network (132, 133) and it is the balance between the processes of mitochondrial dynamics - fusion, fission, and mitochondrial autophagy (mitophagy) - that define the reticular nature of the network (134, 135). Mitochondrial fission is regulated by activation of Drp1 (phosphorylation at Ser616 or de-phosphorylation at Ser637) (136, 137), in coordination with mitochondrial fission protein-1 (Fis1) (138, 139), and/or mitochondrial fission factor (Mff) (140). Fusion is mediated by Optic Atrophy-1 (OPA1), and the mitofusin proteins (Mfn1 and 2) (141). Crucially, Drp1-mediated mitochondrial fission (Figure 1-7 (1)) results in depolarization of membrane potential ($\Delta\Psi_m$) in the excised mitochondrial network fragment (Figure 1-7 (2)) (142). The excised mitochondria may recover membrane potential (Figure 1-7 (3)) and, where fusion activity is high, rejoin the mitochondrial network (Figure 1-7 (4)) (143). Alternatively, the depolarized mitochondria be tagged for autophagy by PTEN-induced putative kinase-1 (PINK1) (Figure 1-7 (5)), which is stabilized on the outer mitochondrial membrane by membrane depolarization. It is hypothesized that mitochondrial networking facilitates the sharing of nutrients, proteins and DNA across the network with the goal of maintaining mitochondrial quality control (142).

1.5.5 Mitochondrial Dynamics and Energy Metabolism

Mitochondrial morphology and size varies widely between cell types according to the energy demands of the cell and OXPHOS activity. For example, mitochondrial size differs in skeletal muscle cells depending on fiber type, such that fast twitch glycolytic fibers have a more fragmented mitochondrial network while oxidative slow twitch fibers have more elongated mitochondria (144). While it is still unclear how the various
mitochondrial morphologies are mechanistically linked to mitochondrial function, it has been suggested that elongation of the mitochondrial network facilitates increased bioenergetic efficiency (145). In support of this theory, when MEF cells are starved of nutrients their mitochondria fuse in association with increased membrane potential (146). In contrast, muscle (147) and pancreatic beta cells (148) treated with excess nutrients are fragmented in association with a loss of membrane potential an effect likely mediated by increased production of ROS (147, 149).

Figure 1-7. Mitochondrial Dynamics

Evidence for Altered Mitochondrial Dynamics in the Pathophysiology of Insulin Resistance and T2D

Dysregulation of mitochondrial dynamics has been implicated in an increasing number of disease states (134, 150-152), including T2D (153, 154). However, data linking alterations in mitochondrial dynamics and skeletal muscle insulin resistance are limited. Muscle mitochondria from patients with type 2 diabetes, observed by electron
microscopy, are smaller in size when compared to non-diabetic controls (110), a morphological characteristic that is partially rescued by exercise training (155), while protein expression studies indicate a pro-fragmentary environment in muscle from obese individuals (156) and in T2D (157). Furthermore, Bach et al. (156) reported a 25% reduction in the mitochondrial network in skeletal muscle of obese Zucker rats, compared to wild type, in the absence of differences in mitochondrial mass. In the same study, fibroblasts treated with an Mfn2 antisense sequence exhibited decreased glucose oxidation and oxygen consumption. Mfn2 repression is also associated with decreased rates of pyruvate and palmitate oxidation in L6E9 muscle cells (158). A recent report by Jheng et al. (147) provides evidence that Drp1-mediated mitochondrial fission results in mitochondrial fragmentation, loss of mitochondrial membrane potential, increased oxidative stress, decreased ATP content, and insulin mediated glucose uptake in C2C12 cells pre-incubated with palmitic acid. Moreover, Drp1 knockdown experiments reversed these alterations. These findings were duplicated in ob/ob mice. In this case, mice treated with Mdivi-1, a Drp1 inhibitor, recovered tubular mitochondrial percentage and membrane potential, decreased oxidative stress to baseline levels, and increased insulin mediated glucose uptake.

Collectively, these data provide strong evidence implicating Drp1-mediated mitochondrial fission as a likely contributing factor to insulin resistance. As an adaptive mechanism the hypothesis that disrupted mitochondrial fission may contribute to induction of insulin resistance appears to have some merit. Given that cellular ATP levels are tightly regulated to the prevailing energy demand, nutrient oversupply to the
mitochondria in the context of obesity can be viewed as a build-up of forward pressure within the system. In this context, continuous delivery of substrate to the electron transport system leads to hyperpolarization of the mitochondrial membrane (159) and increased localized ROS production and oxidative stress (160-162). Based on these observations we have proposed this transient increase in ROS activates Drp1, which translocates to the mitochondrial membrane, initiating mitochondrial fission. Fission contributes to loss of $\Delta \Psi_m$ and recruitment of the mPTP, which then facilitates the release of intermediary metabolites and ROS into the cytosol leading to down-regulation of the insulin signalling pathway and nutrient uptake by the cell. Since depolarized mitochondria do not completely oxidize substrate, even transiently depolarized mitochondria would promote substrate catabolism without effecting ATP content, and the production of additional intermediary metabolites would serve to further inhibit insulin signalling (163). Thus, we propose that mitochondrial fragmentation acts as a mitochondrial pressure valve that releases forward pressure on the system, concomitantly shuts down nutrient uptake, which leads to nutrient storage in the form of fat, allowing the system to regain equilibrium. However, in obesity chronic elevations in fatty acids promote hyperglycemia and insulin resistance. The relationship between mitochondrial fission and insulin resistance is examined further in Chapters 4 and 5.

1.6 Exercise, Insulin Resistance and Metabolic Disease

The importance of exercise for the maintenance of glycemic control was recognized as far back as the early Roman Empire when Celcus wrote of how vigorous exercise should be prescribed for the treatment of thick urine (164). Unfortunately, this
knowledge appears to have been lost to the world from the Galenic period through to the 18th Century when John Brown “conceived life as motion. Diabetes, as a disease of weakness, should be treated by exercise, which should be neither too slight nor too severe” (164). That diabetes was widely regarded as a disease of the kidneys at the time likely hampered the translation of this insight into standard medical therapy. This began to change when Apolonaire Bouchardat, considered by many as the father of diabetology, read to the Paris Academy of Sciences 1838 (164) and described a strict diet and exercise regime for diabetes, noting that a patient requesting bread shall “earn your bread by the sweat of your brow” (164). There is evidence of clinical trials examining the effects of exercise for glycemic control as far back as Catani, a practicing physician in Rome in the mid to late 1800s. Allen (164) reported that Catani notes “the value of vigorous muscular exercise was recognized, and it was proved by clinical tests that glycosuria was thus diminished or abolished without change in the diet.” Moreover, a contemporary of Catani, Rudolph Eduard Kiilz reported:

“exercise is beneficial in strong patients with mild diabetes; in severe diabetes, where sugar is excreted on carbohydrate-free diet, exercise may diminish glycosuria, sometimes only transitorily, or it may have no effect; and in weak individuals with severe diabetes, there was no benefit from exercise.’ (164).

Ever since these pioneering works, physicians have prescribed exercise for the maintenance of glycemic control. Additionally, researchers have exploited this characteristic to probe the mechanisms that contribute to metabolic disease. Remarkably,
the first report (165) of increased insulin action by exercise was published just 5 years after Banting and Best discovered the hormone (166). In the intervening years, several mechanisms have been proposed explain how exercise imparts its effects on glycemic control and metabolic disease. These effects are due, in the short term, to both insulin dependent, and insulin independent mechanisms, and may, in the longer term, involve hormone secretion from adipose tissue, the liver, the gut, and the muscle.

1.6.1 Regulation of glucose uptake by exercise

Insulin independent mechanisms

The main insulin independent regulator of glucose uptake is the fuel-sensing enzyme AMP-activated protein kinase (AMPK). AMPK was first described as an enzyme activated by changes in the AMP/ATP ratio that could both increase cellular ATP generation (e.g., fatty acid oxidation) and diminish ATP use for less critical processes (e.g., fatty acid, triglyceride, and protein synthesis) (167). Activation of AMPK in peripheral tissues results in glucose transport, lipid and protein synthesis, and fuel metabolism (168). AMPK also regulates a wide array of other physiological events, including cellular growth and proliferation, mitochondrial function and biogenesis, and factors that have been linked to insulin resistance (IR), including inflammation, oxidative and ER stress, and autophagy (169).

AMPK is a heterotrimer consisting of a catalytic subunit (α) and 2 regulatory subunits (β and γ). Isoforms of each subunit have been identified (2 α and β isoforms, and 3 γ isoforms). AMPK is upregulated by metabolic stressors, yet paradoxically
AMPK protein expression and activity is diminished in obesity and type 2 diabetes (170-174). AMPK activation is increased following exercise in healthy adults (175), however, in obese individuals higher intensity exercise may be required to induce the same effects (176). It is important to note, however, that increased AMPK activation following exercise is transient in nature and downstream targets of AMPK activation such as PGC1α are increased in obesity and T2D following exercise (176). Thus increased AMPK activation may contribute to improved glycemic control following exercise.

**Insulin-dependent mechanisms**

In a study of fourteen obese patients with type 2 diabetes, seven days of exercise training was sufficient to increase insulin stimulated glucose disposal by ~45% while simultaneously suppressing hepatic glucose production in both basal and insulin stimulated conditions (177). Importantly, these effects were achieved independently of weight loss. The effects of exercise training on insulin stimulated glucose uptake may be mediated by increased GLUT4 expression in skeletal muscle (178-180). The increase in skeletal muscle GLUT4 occurs rapidly in response to an exercise stimulus (181-183) and is lost rapidly following cessation of training (184). The exact mechanisms by which exercise regulates GLUT4 expression is unclear, however GLUT4 expression is related to the oxidative capacity (185), and activation of AMPK and CaMKII may play a role in mediating the training effects of GLUT4 expression (186).

Exercise training also reduces DAG (187), ceramides (188), and acylcarnitine (189) profiles in obesity and T2D, while we have also shown a shift towards a more favorable lipid saturation index following 7-days of endurance exercise training in
NAFLD (190). These improvements are associated with increased oxidative capacity (121) and mitochondrial density (191) in obese humans. Again, these data are consistent with a possible link between increased insulin dependent glucose disposal, metabolic disease progression, and improved mitochondrial function, though the direction of causality remains to be determined.

Exercise and improved insulin sensitivity is also associated with altered hormonal signals from liver and adipose tissue, and reductions in inflammatory signaling. We have shown that reductions in the liver derived hormone Fetuin-A may be involved in regulating both hepatic and muscle insulin sensitivity in NAFLD (192, 193), while consistent changes in the insulin sensitizing, adipose derived hormone high molecular weight (HMW) adiponectin have been observed following exercise in obesity (194), NAFLD (190), and T2D (195). Interestingly, adiponectin appears to confer its insulin sensitizing properties by increasing PGC1α mediated mitochondrial biogenesis via the SIRT1/AMPK pathway (122), and also via the ceramidase activity of the adiponectin receptor (196). Additional benefits of exercise on insulin mediated glucose disposal may be mediated through decreased expression of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL6 (197) and adipokines such as resistin (198), though it is likely that these effects are secondary to improved energy balance and reduced lipotoxicity. In addition, there may be a role for gut derived factors in exercise mediated improvements in insulin sensitivity (199, 200), while recently described myokines such as Irisin (201) may also be involved.
1.6.2 Exercise Mode and Intensity

The vast majority of studies examining the effect of exercise on glycemic control, insulin resistance and metabolic disease focus on moderate intensity aerobic exercise. This is due, in part, to the consistent efficacy of moderate intensity exercise for the maintenance of glycemic control and improvement in fat oxidation and oxidative capacity, along with the comparative safety and accessibility of interventions. Recently, however, the importance of resistance training as an adjunct to aerobic exercise, and as a stand-alone therapy has been recognized (202). Additionally, the perceived barriers to exercise cited by those who engage in sedentary behavior, such as a lack of time, has prompted an increase in interest in high intensity training programs for the improvement in glycemic control. Despite this there are only a few studies which have compared exercise intensities vs. duration in the context of overweight and obesity. Houmard et al. (203), assessed the hypothesis that exercise training consisting of vigorous-intensity activity would enhance insulin sensitivity more substantially than moderate-intensity activity in 154 sedentary, overweight/obese individuals. Although all exercise groups showed an improvement in glycemic control, this study suggested that longer duration exercise was more effective at improving insulin sensitivity compared to shorter duration/high intensity exercise. That moderate intensity exercise is at least as effective at improving insulin sensitivity as high intensity exercise is supported by data from Braun et al (204). However, these studies were relatively short in duration and a longer study in older overweight women suggested that high intensity exercise outperforms moderate intensity after 9 months training (205). Moreover, the effects of training cessation on
insulin sensitivity and mitochondrial protein expression appear to persist for longer following high intensity training relative to moderate intensity exercise (206), while exercise at high intensity, when total energy expenditure is matched appears to convey greater cardioprotective benefits than exercise of a moderate intensity (207).

Increasing recognition of the role played by resistance training in improvements in glycemic control (208-210) prompted the American Diabetes Association and the American College of Sports Medicine to update exercise recommendations for prevention and treatment of T2D (202). In addition to aerobic exercise, the ADA/ACSM recommends resistance training 2-3 times per week, recognizing greater benefits from this combined training than either aerobic or resistance training alone (202). However, given the lack of adherence to current recommendations, the increased burden of the new recommendations, appear unlikely to increase participation in physical activity. Thus new, practical approaches may be required to target the progression of insulin resistance and metabolic disease. CrossFit Inc., a commercial entity which was founded in 2000, promote a form of high intensity power training (HIPT), defined by constantly varied, high intensity, functional movements including weightlifting, gymnastics or body weight exercises, and monostructural or endurance exercises. The combination of resistance and aerobic exercise performed at high intensity may confer the same benefits as including resistance and aerobic training independently, however, there is currently no data regarding its safety or efficacy in overweight/obese individuals with T2D. A study to determine the safety and efficacy of CrossFit HIPT for insulin sensitivity and cardiometabolic risk in this subject population is presented in Chapter 3.
1.6.3 Exercise and NAFLD progression

We have seen earlier that the “first-hit” in the progression of NAFLD is elevated lipid accumulation in hepatocytes in the presence of insulin resistance. While the effects of exercise on insulin resistance are now well established, it was unclear until relatively recently whether exercise training that improved insulin resistance might be able to limit or reverse the progression of NAFLD. Progression of NAFLD from steatosis to NASH and cirrhosis requires additional insults to the hepatocyte including elevated endoplasmic reticulum (ER) stress, increased oxidative stress, activation of pro-inflammatory pathways and hepatocyte apoptosis (211).

Under stressful conditions, in which the demand of protein synthesis is increased (e.g. NASH), an imbalance between the load of needed protein-folding and the response-related capability of the ER can lead to the accumulation of immature and/or misfolded proteins within the ER lumen (212, 213). This results in compromised functionality of this ER and decreased mitochondrial function, ultimately leading to liver dysfunction (212). Under both chronic ER and mitochondrial dysfunctions, restoration of ER homeostasis fails and the activation of the apoptotic signaling occurs (214). ER stress has been suggested to be a key mediator of hepatic inflammation in the context of NASH. The unfolded protein response pathway contributes to the inflammatory response associated with NASH, through activation of NF-κB (215). Additionally activation of ER stress pathways may contribute to increase reactive oxygen species (ROS) induced cell injury by downregulating expression of antioxidants (216). This effect is also
associated with increased mitochondrial depolarization (217). Endurance exercise may result in favorable alterations in ER stress in the liver in obesity (218).

Research from our own lab has demonstrated that exercise, independent of changes in body weight, is effective at increasing insulin sensitivity, HMW adiponectin, and maximal oxygen consumption (\(\text{VO}_2^{\text{max}}\)), while decreasing mononuclear cell ROS production in NAFLD (190). In Chapter 2 we also show that short term exercise, in the absence of body weight changes reduce plasma biomarkers of hepatocyte apoptosis and liver function enzymes. Interestingly, recent data from Concalves et al. (219) suggest that the anti-apoptotic effect of exercise training may be a result of reduced susceptibility of mPTP opening, which may be modulated by changes to mitochondrial dynamics. In addition, we also show that these improvements in liver health are associated with improvements in whole body fat oxidation.

1.6.4 Purpose of the study

The studies presented herein attempt to better understand the mechanisms by which insulin resistance and exercise contribute to metabolic disease progression.

1.7 Significance of Study

Epidemiological evidence indicates that type 2 diabetes is an independent risk factor for cardiovascular disease and microvascular complications, and suggests that the rate of cardiovascular disease is approximately 2-fold higher in people with diabetes than without (220, 221). In addition, the overweight and obesity epidemic sweeping the globe today is driving the rates of T2D ever higher while and affecting people at an
increasingly young age. Thus, the discovery of new therapeutic targets to prevent the progression of obesity associated diseases, along with the development of practical treatment modalities for overweight and obesity represents the most significant public health challenges of the 21st century. We hope that these data will provide novel insights into exercise regulation of insulin resistance along with identifying novel molecular targets for insulin resistance and consequently for prevention of obesity associated metabolic diseases such as NAFLD and T2D.

1.8 Specific Aims and Hypotheses

Aim 1. To investigate the effect of exercise training, independent of weight loss, on markers of hepatocyte apoptosis in individuals with NAFLD. We hypothesized that exercise training would provide an anti-apoptotic stimulus resulting in reductions in Cytokeratin 18 (CK18) fragments and that this would be mediated via reductions in the Fas signalling pathway.

Aim 2. To examine the safety and efficacy of a novel CrossFit high intensity power training program on insulin sensitivity and cardiometabolic risk in individuals with T2D. We hypothesized that, given the high intensity and combined aerobic and resistance components, CrossFit exercise would reduce body fat while maintaining lean tissue mass, and ameliorate insulin resistance and cardio-metabolic risk in type 2 diabetes.

Aim 3. To determine the relationship between Drp1-mediated mitochondrial fission and insulin sensitivity. We hypothesized that endurance exercise training would reduce Drp1 Ser616 phosphorylation in association with improved insulin sensitivity and
substrate metabolism. Moreover, we hypothesized that elevated saturated fat would induce mitochondrial fission in a Drp1 dependent manner and that Drp1 activity is required for lipid induced insulin resistance in skeletal muscle cells.
CHAPTER 2.

SHORT-TERM EXERCISE REDUCES MARKERS OF HEPATOCYTE
APOPTOSIS IN NON-ALCOHOLIC FATTY LIVER DISEASE

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2.1 Introduction

Non Alcoholic Fatty Liver Disease (NAFLD) is among the most common forms of chronic liver disease today. Current estimates suggest that approximately 20-30% of the population are affected by the condition (19). The pathogenesis of NAFLD is marked by excessive accumulation of intrahepatic lipid, increases in insulin resistance and an elevation in profibrogenic activities such as inflammation, oxidative stress (25) and activation of hepatic stellate cells (222). Recently it has been shown that hepatocyte apoptosis contributes to this profibrogenic state (27). Apoptosis, a form of programmed cell death, is an active ATP-dependent process that under normal physiological conditions contributes to the maintenance of tissue homeostasis. However, in certain pathophysiological conditions, such as NAFLD, apoptosis is upregulated, overwhelming the normal phagocytic engulfment of apoptotic cells triggering a proinflammatory and profibrogenic response from hepatocytes (28).

Activation of apoptosis in hepatocytes initiates the caspase cleavage of the intermediate filament cytokeratin 18 (CK18) at two sites, Asp238 and Asp326 (223, 224). This cleavage is highly specific to apoptosis and fragments (M30) can be detected by
antibody specific ELISA (225). In a recent study, Wieckowska et al. (226) demonstrated that M30 fragments were elevated in NAFLD patients compared to controls and that the levels of these fragments were correlated with the presence of liver fibrosis. This finding has been confirmed in subsequent studies (227, 228) and it is thought that caspase cleaved CK18 fragments may be a highly sensitive, non-invasive biomarker for determining the severity of NAFLD (229). Current evidence suggests that activation of the Fas apoptotic pathway may be involved in initiating hepatocyte apoptosis in NAFLD. Fas is a glycosylated protein that is widely expressed in the liver (230). It is activated by the binding of Fas ligand (FasL) leading to receptor trimerization and formation of the death-inducing signalling complex (DISC) (231). Data from Feldstein et al. (232) demonstrate elevations in Fas protein expression in liver samples from NASH patients. Additionally, Fas expression is upregulated in human Hep G2 cells exposed to FFA, and in a mouse model of NAFLD, resulting in increased sensitivity to Fas-mediated apoptosis (233).

Given its central role in the pathogenesis of NAFLD, identifying therapeutic treatments that minimize apoptosis in these patients may result in better outcomes for individuals with NAFLD. Due to its association with obesity, lifestyle interventions are recommended to patients presenting with NAFLD. As early as 1970, Drenick (234) showed that diet induced weight loss over 5 months reduced hepatic steatosis, and when weight loss was maintained for an average of 17 months, liver histology was either completely or almost completely normalized. More recently, Palmer and Schaffer (235) observed an improvement in ALT and AST, surrogate markers of liver damage,
following 10% weight loss over an average of 16 months. Lifestyle interventions utilising a combination of diet and exercise has also been shown to result in reductions in serum levels of ALT and AST, both in the presence and absence of weight loss (236). Furthermore, data from animal studies indicate that exercise training prevents the development of steatosis (237), even in the presence of a high fat diet (238). However, no study to date has investigated the effect of exercise training, independent of weight loss, on markers of apoptosis in individuals with NAFLD. We therefore examined the effect of a seven-day exercise program on plasma CK18 fragments in obese individuals with NAFLD. We hypothesized that exercise training would provide an anti-apoptotic stimulus resulting in reductions in CK18 fragments and that this would be mediated via reductions in the Fas signalling pathway.

2.2 Methods

2.2.1 Participants

13 obese, previously sedentary (individuals exercising for 20 minutes or more at least two times per week were excluded) adults (age 58±3 years; BMI 35.2±1.2 kg·m⁻²; mean±SEM) with NAFLD (>5% intrahepatic lipid (IHL) assessed by ¹H MR spectroscopy) were recruited from the local population to undergo a seven-day exercise training intervention. All volunteers underwent a medical history, physical exam, oral glucose tolerance test (OGTT), and complete blood profile (lipid profile, and hepatic/renal/hematological function tests). Medical screening excluded individuals with any disease, and/or taking medications known to affect our outcome variables. In
addition, individuals consuming more than 5 units of alcohol per week were excluded. Screening also excluded those with any contraindications to physical activity that was detected during a resting 12-lead electrocardiogram and a sub-maximal exercise stress test. Female subjects were either, postmenopausal and not using any hormone replacement therapy, or premenopausal and in the follicular phase of the menstrual cycle during the study period. The study was approved by the Institutional Review Board and all subjects provided signed informed consent in accordance with our guidelines for the protection of human subjects.

2.2.2 Aerobic Fitness

Each participant performed an incremental-graded treadmill exercise test to determine his or her maximal oxygen consumption ($\text{VO}_2\text{max}$), as previously described (239). Expired air was continuously sampled online with the use of an automated system (Jaeger Oxycon Pro; Viasys, Yorba Linda, CA). $\text{VO}_2\text{max}$ testing was conducted on the pre- and post-testing days following an oral glucose tolerance test.

2.2.3 Intervention.

All participants undertook 60 minutes of supervised aerobic exercise at ~80-85% of maximum heart rate each day for 7 consecutive days. Compliance to exercise intensity was monitored using a heart rate monitor (Polar Electro Inc., Woodbury, NY). Participants were instructed to consume their normal diet and not make any dietary changes during the 7-day period of the intervention. Furthermore, subjects were instructed to avoid caffeine consumption for 12- and alcohol for 48- hours prior to testing.
and to consume the same diet containing 250 g of carbohydrate on the day prior to the pre- and post-study testing days. Post-testing occurred the morning of the day following the final exercise bout.

2.2.4 Body Composition

Height and body weight were measured by standard techniques. Whole body adiposity was measured by dual-energy X-ray absorptiometry (model iDXA; Lunar, Madison, WI).

2.2.5 Intrahepatic Lipid

Hepatic triglyceride content was measured by $^{1}$H MR spectroscopy using a 3T MR system (Siemens Sonata, Erlangen, Germany). Subjects arrived at the laboratory at 06:00 am following an overnight (8 h) fast. Briefly, a body array MRI coil was affixed to each subject’s back with velcro straps. The center of the body array coil was aligned with the subject’s spine and shoulders for accurate repositioning during longitudinal studies. Each subject was positioned face down and head first in the Siemens Verio 3T MRI scanner on a memory foam mattress to further minimize respiratory motion. After localizer scans for positioning, an 8 cm³ voxel was positioned within the right posterior lobe of the subject’s liver with guidance from the high-resolution localization images. Manual shimming was performed to a line width of ~40 Hz to ensure high quality spectra required to delineate water and the various lipid species. MR spectra with and without water suppression were acquired with a single-voxel PRESS acquisition with a long repetition time (TR=5000ms), and a short echo time (TE=30ms) to limit the effects of
magnetic relaxation (240). The acquisition was acquired with 32 averages to obtain sufficient signal to accurately assess lipid components. The data was Fourier-Transformed, filtered, baseline corrected, and phased. All NAFLD patients were confirmed to have greater than 5% Intrahepatic lipid (IHL), which is the diagnostic criteria for hepatic steatosis (241).

2.2.6 Insulin Sensitivity and Substrate Metabolism

Subjects arrived at the Clinical Research Unit following an overnight fast and lay supine in bed for 30 minutes followed by assessment of whole body fat oxidation (FOX) by indirect calorimetry, as previously described (242). Subsequently, a 75 gram OGTT was administered. Baseline blood draws were obtained from an antecubital vein prior to ingestion of the glucose drink. Blood samples were drawn in EDTA tubes containing aprotonin at 30, 60, 90, 120 and 180 minutes after ingestion. Plasma glucose was determined using the YSI 2300 STAT Plus analyzer (Yellow Springs, OH), and plasma insulin was determined via radioimmunoassay (Millipore, Billerica, MA). Insulin sensitivity during the OGTT (ISI_{OGTT}) was calculated using the Matsuda Index (243).

2.2.7 Plasma Analyses

Plasma analyses were obtained from plasma which was stored at -80°C immediately following post-draw processing. Plasma caspase-3 generated CK18 fragments were quantified by M30 apoptosense ELISA (PEVIVA; Alexis, Grunwald, Germany). The Human Fas/TNFRSF6 Quantikine ELISA Kit and the Human Fas Ligand/TNFSF6 Quantikine ELISA Kit (R&D systems, Minneapolis, MN) were used for
quantitative measurement of soluble Fas (sFas) and soluble Fas ligand (sFasL), respectively. ALT and AST were assessed using the Cobra Integra Alanine Aminotransferase (ALTL) test, test ID 0-495, and the Aspartate Aminotransferase (ASTL) test, test ID 0-494 (Roche Diagnostics, Indianapolis, IN), respectively.

2.2.8 Statistical Analyses

Values were tested for normality using the D’Agostino & Pearson omnibus normality test on GraphPad Prism 4.0 (Graphpad Software Inc., San Diego CA). Pre- to post- intervention changes were assessed using a repeated measures-analysis of variance for normally distributed samples. Pre- to post- changes that were not normally distributed were log transformed (area under the curve [AUC] insulin). Data that were not normalized by log transformation (fasting plasma insulin [FPI] & ISI_{OGTT}) were assessed using a Wilcoxon signed rank test. Linear regression analysis was used to determine associations between normally distributed data. In addition, Spearman’s rank correlation analyses were used to identify relationships between variables that failed the test for normality (Δ FOX and Δ sFasL). Statistical significance was accepted when $P<0.05$. These analyses were carried out using StatView for Windows 5.0.1 (SAS Institute, NC), and all data are expressed as mean ± SEM.

2.3 Results

2.3.1 Participant Characteristics

Anthropometric data for the group are summarized in Table 1. Seven of the participants presented at screening with elevated ALT and AST values. Seven days of
exercise did not alter body weight or body composition, however, aerobic fitness, as measured by VO$_{2\text{max}}$, did increase following exercise training. In addition, total IHL assessed by 1H MR Spectroscopy remained unchanged following the intervention (Table 1).

Table 2-1 Summary of subject characteristics before and after the exercise training.

<table>
<thead>
<tr>
<th>Variable (unit)</th>
<th>Pre</th>
<th>Post</th>
<th>P- Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>58±3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body Weight (kg)</td>
<td>100.3±3.8</td>
<td>100.4±3.7</td>
<td>0.727</td>
</tr>
<tr>
<td>BMI (kg·m⁻²)</td>
<td>35.2±1.2</td>
<td>35.3±1.1</td>
<td>0.532</td>
</tr>
<tr>
<td>FM (kg)</td>
<td>45.8±2.4</td>
<td>45.6±2.3</td>
<td>0.298</td>
</tr>
<tr>
<td>FFM (kg)</td>
<td>54.4±3.1</td>
<td>54.9±3.1</td>
<td>0.065</td>
</tr>
<tr>
<td>IHL (%)</td>
<td>18.2±2.5</td>
<td>17.5±2.1</td>
<td>0.285</td>
</tr>
<tr>
<td>VO2max (ml·kg·min⁻¹)</td>
<td>22.0±1.4</td>
<td>23.6±1.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Fasting Glucose (mg·dL⁻¹)</td>
<td>112.9±5.7</td>
<td>108.5±3.7</td>
<td>0.285</td>
</tr>
<tr>
<td>Fasting Insulin (µU·mL⁻¹)</td>
<td>25.3±3.1</td>
<td>22.8±2.7</td>
<td>0.249</td>
</tr>
<tr>
<td>Glucose AUC (mg·dL⁻¹·3hr)</td>
<td>13663.6±1423.4</td>
<td>11528.1±1517.1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Log Insulin AUC (log pg·mL⁻¹·3hr)</td>
<td>4.3±0.1</td>
<td>4.16±0.1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Resting FOX (mg·min⁻¹)</td>
<td>49.3±6.1</td>
<td>69.4±7.1</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Data are presented as Mean±SEM. BMI: Body Mass Index, FM: Fat Mass, FFM: Fat Free Mass, IHL: Intrahepatic lipid, VO2max: Maximal Oxygen Consumption, AUC: Incremental Area Under the Curve, FOX: Fat Oxidation
2.3.2 Blood Glucose & Insulin Sensitivity

The plasma glucose and insulin responses to glucose ingestion were significantly reduced (Table 1), and insulin sensitivity, measured by the Matsuda Index, increased following exercise (Figure 1a). Fasting glucose and insulin did not change after the 7-day program (Table 1).

Figure 2-1 Short-term aerobic exercise training increases insulin sensitivity (A) and reduces circulating cytokeratin 18 (CK18) fragments (B), alanine aminotransferase (ALT) (C), and circulating soluble Fas ligand (sFasL) (D). Data are presented as means ± SE. *P < 0.05. OGTT, oral glucose tolerance test; ISI$_{OGTT}$, insulin sensitivity (Matsuda) index; AU, arbitrary units.
2.3.3 Apoptotic Biomarkers

Plasma CK18 (Figure 1b; 558.4±106.8 vs. 323.4±72.5 U\(\cdot\)L\(^{-1}\), \(P<0.01\)) and ALT (Figure 1c; 30.2±5.1 vs. 24.3±4.8 U\(\cdot\)L\(^{-1}\), \(P<0.05\)) were significantly reduced by the intervention, while the reduction in sFasL approached significance (Figure 1d; 66.5±6.0 vs. 63.0±5.7 pg\(\cdot\)mL\(^{-1}\), \(P=0.06\)). However, there was no change in plasma AST (35.1±6.2 vs. 34.5±5.8 U\(\cdot\)L\(^{-1}\), NS), or sFas (6483.2±358.0 vs. 6284.9±315.7 pg\(\cdot\)mL\(^{-1}\), NS). There was a trend towards an association between the intervention-induced change in CK18 and ALT (Figure 2; \(r=0.55, P=0.05\)).

![Figure 2-2 Association between exercise training-induced percent changes in circulating CK18 and ALT in obese individuals with nonalcoholic fatty liver disease (NAFLD) \((r=0.55, P=0.05)\). \(\Delta\), change.](image)
2.3.4 Substrate Oxidation

Resting FOX was increased following the exercise intervention (Table 1). In addition, changes in FOX were significantly correlated with changes in sFasL (Figure 3; rho=−0.65, P<0.05).

Figure 2-3 Association between exercise training-induced changes in basal fat oxidation (FOX) and changes in circulating sFasL (rho = −0.65, P < 0.05).

2.4 Discussion

Exercise currently forms a major component of the treatment recommended by the American Gastroenterological Association (244) for NAFLD, despite a lack of
published evidence on the effectiveness of exercise in treating this disease (245). Here we show, for the first time, that exercise in the absence of weight loss significantly reduces plasma levels of the apoptotic marker, caspase cleaved CK18 fragments, in previously sedentary obese individuals with NAFLD. These data collectively indicate a reduction in the profibrogenic apoptotic state present in NAFLD. This finding is important as hepatocyte apoptosis contributes significantly to the pathogenesis of NAFLD and progression to NASH and liver fibrosis (27). Recently Kistler et al. (245) examined the relationship between self-reported physical activity and the severity of liver fibrosis. They reported that individuals who met the vigorous activity levels recommended by the US Department of Health and Human Services had significantly lower odds of having advanced fibrosis. Our data supports the view that exercise serves a protective function against the pathogenesis of NAFLD.

The changes in plasma CK18 fragments in our data were mirrored by changes in ALT levels suggesting that the reductions we observed in plasma CK18 indeed reflect decreases in hepatocyte apoptosis. CK18 is expressed in epithelial cells and therefore the reductions could theoretically be derived from decreased apoptosis in almost any tissue. However, ALT is an enzyme of the alanine cycle and is predominantly expressed in the liver. For this reason, it has been used for many years as a surrogate biomarker for liver injury. Several previous studies have demonstrated a positive effect of longer term exercise on ALT levels in serum (236, 246, 247). It has also recently been reported that administration of a caspase inhibitor may reduce ALT and CK18 in NAFLD patients in a dose dependent manner (248). Here, we confirm the beneficial effect of exercise on ALT.
levels and show that these effects become apparent within one week of moderate-high intensity exercise. Furthermore, the positive correlation between percent changes in plasma CK18 levels and ALT suggest that improvements in CK18 may be a result of reduced hepatocyte apoptosis.

The exact mechanism by which exercise may reduce apoptosis in NAFLD remains to be elucidated. Insulin resistance is a key component in the pathogenesis of NAFLD (25). Our exercise program resulted in an increase in insulin sensitivity. Exercise mediated improvements in insulin sensitivity are well documented (249-253). Exercise elicits favorable alterations in lipid uptake and metabolism in skeletal muscle (254) and it has been proposed that exercise induced improvements in insulin sensitivity may decrease *de novo* lipogenesis and hepatic triglyceride synthesis (255, 256). We did observe an increase in whole body fat oxidation. Moreover, the alterations in FOX were negatively correlated with changes in sFasL. Collectively, these data support the hypothesis that improved whole body insulin sensitivity and greater fat oxidation may act to reduce lipotoxicity in the liver and thereby reduce the pro-apoptotic stimulus to hepatocytes.

Apoptosis is mediated via two pathways, the extrinsic and intrinsic pathway. In this study we also investigated the effect of exercise on biomarkers of the Fas death inducing pathway, a component of the extrinsic pathway. We found that exercise resulted in a modest reduction in sFasL but not sFas. Previous reports have linked exercise training to reductions in oxidative stress (257). Induction of high levels of ROS subjects cells to oxidative stress, which is believed to play a pivotal role in the
pathogenesis of NAFLD (258-260). While hepatocytes express Fas in large numbers, they do not normally express large numbers of Fas ligand. However, increased ROS production in hepatocytes can cause increased Fas ligand expression, resulting in fratricidal apoptosis (261). Mitochondrial dysfunction is believed to be a major contributor to ROS production in NAFLD (262). Exercise increases mitochondrial function and content principally through HMW adiponectin (122). We have previously demonstrated increases in HMW adiponectin in insulin resistant obese individuals following seven days of moderate-high intensity exercise. Furthermore, the changes in HMW adiponectin were positively correlated with changes in FOX (194). Therefore, improved oxidative capacity and reduced oxidative stress following exercise training may cause a reduction in the generation of Fas ligand within hepatocytes and consequently attenuate the apoptotic signal. However, the relationship between improvements in FOX and ROS production in a NAFLD population following exercise remain to be confirmed.

There is also evidence that ROS increases TNF-α production, which interacts with and activates caspase-8 resulting in permeabilization of the mitochondrial membrane via truncated Bid and induction of cytochrome c release (261). Therefore, exercise may also reduce apoptosis via reductions in intrinsic pathway signaling. Upregulation of the antioxidant defense system can also reduce oxidative stress mediated apoptosis via the intrinsic pathway. Indeed, Sinha-Hikim et al. (263) recently demonstrated that a high fat diet upregulated pro-apoptotic and downregulated anti-apoptotic enzymes BAX and BCL-2, respectively, in a mouse model of NAFLD. However, supplementation with a glutathione precursor significantly reduced the high fat feeding upregulation of BAX and
suppressed activation of caspase 3 in hepatocytes. Cakir et al. (264) recently demonstrated that exercise protects against glutathione depletion in hepatocytes of rats with alcoholic liver disease, however, data from human or NAFLD studies are currently lacking. The possibility that reductions in apoptosis are mediated through downregulation of the intrinsic pathway, on the basis of these data, cannot be excluded.

2.5 Conclusions

Empirical data on the effect of exercise on hepatocyte apoptosis in NAFLD is currently lacking, however, here for the first time we demonstrate that exercise, in the absence of weight loss, reduces a circulating marker of hepatocyte apoptosis in previously sedentary obese individuals with NAFLD. The exact mechanism by which this occurs is currently under investigation and for now our interpretation of the data is limited by the use of whole body indices. Nonetheless, we provide evidence that reductions in apoptosis may be related to increases in insulin sensitivity and reductions in FasL resulting from improvements in oxidative capacity. These findings have significant clinical implications for the prevention of disease progression in NAFLD patients and support the use of exercise as an effective treatment for NAFLD.
CHAPTER 3.
CROSSFIT HIGH INTENSITY POWER TRAINING AMELIORATES INSULIN RESISTANCE AND CARDIO-METABOLIC RISK IN TYPE 2 DIABETES

3.1 Introduction

Epidemiological evidence indicates that type 2 diabetes is an independent risk factor for cardiovascular disease and microvascular complications, and suggests that the rate of cardiovascular disease is approximately 2-fold higher in people with diabetes than without (220, 221). Physical activity remains a cornerstone of treatment and prevention strategies for type 2 diabetes and cardiovascular disease. Current ADA/ACSM physical activity recommendations for type 2 diabetes include at least moderate intensity (40-60% VO$_{2\text{max}}$), aerobic exercise 3-5 times per week, recognizing that additional benefits may be gained from vigorous exercise (>60% VO$_{2\text{max}}$). In addition to aerobic exercise, resistance training 2-3 times per week is also recommended, recognizing greater benefits from this combined training than either aerobic or resistance training alone (202). Such programs typically take more than 5 hours per week to complete. Despite these recommendations, compliance and adherence to exercise advice continues to remain disappointingly low. Although prediabetic and T2D patients report awareness that diet and physical activity can improve their condition, these patients have not applied this advice to their own health (265). In fact, only 42% of patients with T2DM are reported
to have met the 2005 ADA guidelines for physical activity (266). One of the most cited barriers to regular physical activity is lack of time (267).

In order to mitigate this perceived barrier to physical activity, high intensity exercise has been proposed as a time-efficient method for achieving cardio-metabolic health outcomes equivalent to traditional aerobic training programs (268, 269). Such has been the increased popularity of high intensity training amongst exercise specialists and the general public that programs like “boot camp” – a military-styled fitness approach (270) - and high intensity interval training (HIIT) have become mainstays in the top 20 worldwide fitness trends since 2010, with HIIT featured in the top 5 in each year since its initial appearance in 2014 (270). Moreover, Crossfit Inc., a commercial entity which was founded in 2000 and promotes a form of high intensity power training (HIPT) (271), now has more than 11,000 affiliate gyms worldwide (272).

HIIT typically involves repeated, short (usually ~30 second) bouts of “all out” sprint performances with interspersed periods of rest or low intensity exercise (268). While HIIT may be an adequate or even superior alternative to moderate intensity aerobic exercise for metabolic health (273-276), it does not include resistance exercise. CrossFit is defined by constantly varied, high intensity, functional movements including weightlifting, gymnastics, or body weight exercises, and monostructural or endurance exercises. The workouts combine a combination of 2-3 functional exercises such as deadlifts, clean and snatch, overhead press, gymnastic style ring exercises, box jumps, and body weight exercises. In CrossFit, exercise sessions are arranged in a workout of the day (WOD), and are performed either in the shortest amount of time, as many rounds
as possible (AMRAP) in a given time, or for maximal loads (271). Despite its growing popularity, few studies have examined the safety and efficacy of such interventions in the general population (271, 272, 277), and none to our knowledge in individuals with T2D. We therefore examined the effectiveness of a 6 week CrossFit intervention in individuals with T2D. We hypothesized that, given the combined aerobic and resistance components, CrossFit exercise would reduce body fat while maintaining lean tissue mass, and ameliorate insulin resistance and cardio-metabolic risk in individuals with type 2 diabetes.

3.2 Methods

3.2.1 Subject Population

13 overweight/obese, previously sedentary adults (5 males, 8 females; age 53±2 years; BMI 34.5±1.0 kg/m2; mean±SEM) with diagnosed non-insulin dependent type 2 diabetes were recruited from the local community. All participants were screened with a medical history and physical examination, blood and urine chemistry analyses, and a resting and exercise stress test 12-lead electrocardiogram. Individuals were excluded from participation if they 1) were smokers in the past 5 years, 2) had greater than 5 kg weight change in the previous 6 months, 3) undertook regular exercise (>30 min/day, >3 days/week), 4) had contraindications to elevated levels of physical activity as indicated by an electrocardiogram, 5) demonstrated any evidence of current or previous hematological, renal, hepatic, cardiovascular, or pulmonary disease, or 6) were taking medications known to affect our primary outcome variables. Female subjects were
either, postmenopausal and not using any hormone replacement therapy, or premenopausal and in the follicular phase of the menstrual cycle during the testing period. The study was approved by the Institutional Review Board and all subjects provided signed informed consent in accordance with our guidelines for the protection of human subjects.

3.2.2 Exercise Intervention

Subjects participated in a 6-week CrossFit training program at an established Crossfit Inc. affiliate gym. A CrossFit coach led groups of 2-6 subjects in three exercise training sessions per week each lasting one hour in duration. Training sessions included a warm-up, skill practice, and one high-intensity WOD (> 85% HR maximum) ranging in duration from 8-20 minutes. Over the course of 6-weeks, subjects were exposed to an array of functional weightlifting, gymnastics, and endurance movements in various combinations (Table 1). Although the research team gave no specific guidance regarding diet, participants were treated as regular members of the CrossFit affiliate, and coaches were available to provide nutrition guidance as desired. Thus, dietary alterations were at the participants’ discretion. 3-day diet records were issued prior to and in the last week of the exercise intervention to monitor any changes in dietary intake. Furthermore, subjects were instructed to avoid caffeine consumption for 12- and alcohol for 48- hours prior to testing and to consume the same diet containing 250 g carbohydrate on the day prior to the pre- and post-study testing days. Post-testing commenced within 36 hours of the final exercise bout.
Table 3-1 Selected examples of daily workouts performed by patients during the 6-week HIPT intervention.

<table>
<thead>
<tr>
<th></th>
<th>Day 2</th>
<th>Day 11</th>
<th>Day 16</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Warm Up:</strong></td>
<td>• 5 Rollouts</td>
<td>3 sets; 15 reps</td>
<td><em>CrossFit Warmup</em> 3 sets; 10 reps</td>
</tr>
<tr>
<td></td>
<td>• 5 Dumbbell Press</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• 5 Dumbbell Push Press</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Skills:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Box Jump</td>
<td>• Medicine ball clean</td>
<td>• Barbell power clean and jerk</td>
</tr>
<tr>
<td></td>
<td>• Stepping Lunge F&amp;B</td>
<td>• Dumbbell Clean</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Cobra Stretch</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>WOD:</strong></td>
<td>5 sets; 1 min per exercise:</td>
<td><em>Fight Gone Bad</em> 1 min/exercise: 3 sets</td>
<td><em>Grace</em></td>
</tr>
<tr>
<td></td>
<td>• Row for calories</td>
<td>• Row for calories</td>
<td>• Clean and jerk</td>
</tr>
<tr>
<td></td>
<td>• Sit ups</td>
<td>• Wall ball</td>
<td>• 30 reps; ground to overhead; for time</td>
</tr>
<tr>
<td></td>
<td>• Squats</td>
<td>• Sumo deadlift high pull</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Rest</td>
<td>• Push Press</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Box Jump</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Rest</td>
<td></td>
</tr>
<tr>
<td><strong>Cool down:</strong></td>
<td>3 sets:</td>
<td>• Wall ball sit ups (25)</td>
<td>30 reps each:</td>
</tr>
<tr>
<td></td>
<td>• Maximum Plank hold</td>
<td></td>
<td>• Sit up</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Squat</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Flutter kick</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Mountain Climbers</td>
</tr>
</tbody>
</table>

Abbreviations: Reps; repetitions, KB; Kettlebell, GHD; Glute-Hamstring developer, F&B; Front and back.
3.2.3 **Body Composition**

Height, body weight and waist circumference were measured by standard techniques. Whole body adiposity was measured by dual-energy X-ray absorptiometry (model iDXA; Lunar, Madison, WI).

3.2.4 **Aerobic Fitness**

Each participant performed an incremental-graded treadmill exercise test to determine maximal oxygen consumption ($\text{VO}_{2\text{max}}$) as previously described (239). Speed was set between 2 and 5 miles/hr, and the incline of the treadmill increased 2-3% every 2 min until fatigue. Inspired air volumes were measured from pressure changes detected by a bidirectional digital volume sensor (Triple V) pneumotach, and concentrations of $\text{O}_2$ (electrochemical detection) and $\text{CO}_2$ (thermal conductivity detection) were measured using a Jaeger OxyCon Pro/Delta System (Version 4.6, Hoechberg, Germany). At least two of the following criteria were attained to assure a maximum test: plateau in $\text{VO}_2$, heart rate (HR) within 10 beats/min of age-predicted maximum, and/or a respiratory exchange ratio >1.0. $\text{VO}_{2\text{max}}$ testing was conducted on the pre- and post-testing days following an oral glucose tolerance test.

3.2.5 **Insulin Sensitivity and Substrate Metabolism**

Subjects arrived at the Clinical Research Unit following an overnight fast, and lay supine in bed for 30 minutes followed by assessment of non-protein corrected, whole body fat oxidation (FOX) by indirect calorimetry using the following equation; ($\text{FOX} = 1.695(\text{VO}_2) – 1.701(\text{VCO}_2)$) (278). Subsequently, a 75 gram OGGTT was administered.
Baseline blood draws were obtained from an antecubital vein prior to ingestion of the glucose drink. Blood samples were drawn in EDTA tubes at 30, 60, 90, 120, and 180 minutes after ingestion. Total and incremental metabolite responses (area under the curve) during the OGTT were calculated using the trapezoidal rule. Insulin sensitivity during the OGTT (ISI\textsubscript{OGTT}) was calculated using a modified Stumvoll equation (279).

3.2.6 Biochemical Analysis

Plasma analyses were performed on samples which were stored at -80°C immediately following post-draw processing. Glucose was determined using the YSI 2300 STAT Plus analyzer (Yellow Springs, OH), and insulin was determined via radioimmunoassay (Millipore, Billerica, MA). Triglycerides and cholesterol were analyzed using enzymatic methods with an automated platform (Roche Modular Diagnostics, Indianapolis, IN). Fasting plasma HMW adiponectin and resistin were measured at baseline and following the exercise intervention by ELISA (Millipore, Billerica, MA). Creatine kinase activity was measured as a marker of muscle damage (Sigma-Aldrich, St. Louis, MO).

3.2.7 Statistical Analyses

Statistical analysis was performed using on GraphPad Prism 6.0 (Graphpad Software Inc., San Diego CA). Values were tested for normality using the D’Agostino & Pearson omnibus normality test. Pre- to post-intervention changes were assessed using a repeated measures analysis of variance for normally distributed samples. Pre- to post-changes that were not normally distributed were assessed using the non-parametric
Wilcoxon signed rank test. Pearson’s correlation was used to assess associations between normally distributed data. In addition, Spearman’s rank correlation analyses were used to identify relationships between variables that failed the test for normality. Statistical significance was accepted when \( P<0.05 \) and all data are expressed as mean ± SEM.

3.3 Results

3.3.1 Participants

Anthropometric data for the group are summarized in Table 2. 6-weeks of CrossFit HIPT training did not produce significant changes to body weight (\( P=0.09 \)), BMI (\( P=0.11 \)), or fat free mass (\( P=0.63 \)); however fat mass (\( P<0.001 \)) was significantly decreased following the intervention. In addition, aerobic fitness (\( \text{VO}_{2\text{max}} \); \( P<0.001 \)) was increased after the HIPT training program.

Table 3-2 Antropometric and aerobic fitness characteristics before and after six weeks of CrossFit high intensity power training. Data are Mean±SEM

<table>
<thead>
<tr>
<th>Variable</th>
<th>PRE</th>
<th>POST</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M/F)</td>
<td>5/8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Age (years)</td>
<td>53±2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>168.7±2.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>98.2±3.3</td>
<td>96.5±2.5</td>
<td>0.09</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>34.5±1.0</td>
<td>34.0±0.9</td>
<td>0.11</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>110.7±3.7</td>
<td>108.7±3.5</td>
<td>0.11</td>
</tr>
<tr>
<td>DXA Fat Mass (kg)</td>
<td>43.0±2.4</td>
<td>40.7±2.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DXA Fat-Free Mass (kg)</td>
<td>55.2±2.1</td>
<td>55.5±1.9</td>
<td>0.63</td>
</tr>
<tr>
<td>( \text{VO}_{2\text{max}} ) (ml/kg/min)</td>
<td>24.3±1.2</td>
<td>28.6±1.3</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
3.3.2 Insulin sensitivity and substrate metabolism

While there were no significant effects of the intervention on OGTT glucose measures (Figure 1A) including fasting (P=0.11) and 2-hour glucose (P=0.50), and total (tAUC; P=0.22) and incremental (iAUC; P=0.85) glucose area under the curve (Table 3), ISI\textsubscript{OGTT} was increased in all but one individual following CrossFit training (Figure 2). Fasting insulin (P=0.63) and insulin areas under the curve (tAUC P=0.16 and iAUC; P=0.88) were unchanged after the intervention (Table 2.), however, plasma insulin was significantly increased at t=30 min (P<0.05) during the OGTT (Figure 1B), while basal fat oxidation (P<0.05) was significantly increased following the intervention (Table 2).

Table 3-3 Pre- and Post- intervention data for glucose tolerance, OGTT derived indices of insulin sensitivity, substrate metabolism, plasma adipokines and creatine kinase activity, a biomarker for muscle damage.

<table>
<thead>
<tr>
<th>Variable</th>
<th>PRE</th>
<th>POST</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting Glucose* (mg/dL)</td>
<td>173.4±16.3</td>
<td>154.0±16.6</td>
<td>0.11</td>
</tr>
<tr>
<td>2hr Glucose* (mg/dL)</td>
<td>304.7±20.8</td>
<td>290.0±17.5</td>
<td>0.50</td>
</tr>
<tr>
<td>tAUC Glucose* (mg/dL·3h)</td>
<td>50105.8±3527.6</td>
<td>46411.6±3094.3</td>
<td>0.22</td>
</tr>
<tr>
<td>iAUC Glucose (mg/dL·3h)</td>
<td>18896.5±1083.0</td>
<td>18699.9±1050.4</td>
<td>0.85</td>
</tr>
<tr>
<td>Fasting Insulin (µU/mL)</td>
<td>24.1±5.7</td>
<td>26.0±4.7</td>
<td>0.63</td>
</tr>
<tr>
<td>tAUC Insulin* (µU/mL·3h)</td>
<td>11545.3±2304.1</td>
<td>13135.3±3159.8</td>
<td>0.16</td>
</tr>
<tr>
<td>iAUC Insulin* (µU/mL·3h)</td>
<td>9056.1±2137.5</td>
<td>9142.7±2563.9</td>
<td>0.88</td>
</tr>
<tr>
<td>ISI\textsubscript{OGTT} (µmol/kg/min/pM)</td>
<td>0.037±0.003</td>
<td>0.042±0.003</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Resting Fat Oxidation* (g/min)</td>
<td>0.08±0.01</td>
<td>0.10±0.01</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>HMW Adiponectin (ng/mL)</td>
<td>214.4±26.8</td>
<td>288.8±38.4</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Resistin (ng/mL)</td>
<td>6.4±1.5</td>
<td>5.6±1.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Creatine Kinase Activity (U/L)</td>
<td>83.4±5.1</td>
<td>116.2±20.1</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Data are Mean±SEM. *denotes that data was analyzed using the non parametric Wilcoxon signed rank test
Figure 3-1 A. Glucose and B. insulin data during the OGTT before (closed circles) and after (open circles) the six week CrossFit high intensity power training intervention. Data are Mean±SEM. *P<0.05

Figure 3-2 ISI_{OGTT} measures for individual participants pre- and post- intervention. All but one individual increased their insulin sensitivity.
3.3.3 Blood Lipids and Cardiometabolic Risk Factors

Crossfit HIPT resulted in significant reductions in plasma triglycerides (P<0.05) and VLDL cholesterol (P<0.05) (Table 2). There was also a tendency for reductions in total cholesterol (P=0.11) and LDL cholesterol (P=0.15); however, these reductions were not sufficient to achieve statistical significance. The 6-week exercise intervention also resulted in a decrease in diastolic blood pressure (DBP; P<0.01), mean arterial pressure (MAP; P<0.05), and the metabolic syndrome z-score (P<0.001), a metric of metabolic syndrome severity. Systolic blood pressure (P=0.73) was unchanged following the intervention (Table 2).

Table 3-4 Plasma lipid and cardiovascular health measures before and after the 6-week intervention.

<table>
<thead>
<tr>
<th>Variable</th>
<th>PRE</th>
<th>POST</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trig (mg/dl)*</td>
<td>146.7±24.5</td>
<td>110.8±18.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Chol (mg/dl)</td>
<td>176.9±8.4</td>
<td>160.4±9.1</td>
<td>0.11</td>
</tr>
<tr>
<td>VLDL chol (mg/dl)*</td>
<td>29.3±4.9</td>
<td>22.2±3.6</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>LDL chol (mg/dl)*</td>
<td>96.7±7.2</td>
<td>87.1±8.3</td>
<td>0.15</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>133.8±2.1</td>
<td>132.8±3.4</td>
<td>0.73</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>81.0±1.5</td>
<td>75.4±2.3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>98.6±1.5</td>
<td>94.6±2.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Pulse Pressure (mmHg)</td>
<td>52.8±1.8</td>
<td>57.4±2.9</td>
<td>0.06</td>
</tr>
<tr>
<td>Metabolic Syndrome z-score (AU)</td>
<td>6.3±1.0</td>
<td>-0.7±1.3</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are Mean±SEM. *denotes that data was analyzed using the non-parametric Wilcoxon signed rank test

3.3.4 HMW Adiponectin, Resistin, and Creatine Kinase

Plasma adipokines were alternately modified by the exercise intervention. The insulin sensitizing HMW-adiponectin was increased (P<0.01) by the intervention while
the pro-inflammatory, pro-atherogenic adipokine resistin was reduced (P<0.05) after exercise training. Plasma CK activity (P<0.05), a biomarker for muscle damage, was also increased following 6-weeks of CrossFit training.

3.3.5 Correlation analysis

The increase in HMW adiponectin and Fox both correlated with the change in ISI (P<0.01, Figure 3A and B). Moreover, ISIOGTT changes were correlated to differences in both fasting glucose (rho -0.26; P<0.05) and tAUC glucose (rho -0.27; P<0.05). Changes to HMW adiponectin were also associated with alterations in fat mass (rho -0.67; P<0.05), while differences in glucose iAUC were correlated with changes in CK activity (r=0.61; P<0.05).

A

B

Figure 3-3 Correlation between pre- to post- intervention changes in ISI_{OGTT} and A. plasma HMW adiponectin and B. whole body fat oxidation.
3.4 Discussion

Exercise training has long been recognized as a key component in the treatment regimen of patients with T2D (280). Despite this, adherence to traditional exercise programs is low (281, 282), with one of the main barriers to adherence cited as a lack of time (267). Here, we demonstrate for the first time in patients with T2D, the effectiveness of a novel high intensity training modality for increasing insulin sensitivity, fat oxidation, and HMW-adiponectin, while reducing fat mass, plasma triglycerides and cholesterol, metabolic syndrome severity, diastolic blood pressure, and plasma concentration of the pro-inflammatory adipokine resistin over the course of a 6-week intervention. It is important to note that this was achieved with no injuries reported, and greater than 95% compliance with the exercise program. This is significant due to the widespread, and legitimate, concerns expressed within the fitness and scientific community regarding the safety and efficacy of CrossFit-style HIPT training programs for individuals with pre-existing chronic illness (270, 283, 284). The data presented herein, however, indicate that HIPT, performed in a controlled setting and under adequate supervision, can be safe and effective for individuals with T2D and adds to the growing body of literature which suggests that high intensity exercise interventions may offer a time efficient method to achieve outcomes comparable, and perhaps even superior to traditional aerobic exercise programs.

Glucose control remains a major focus in the management of patients with T2D (285). Traditional, long duration, moderate intensity aerobic exercise programs have proven extremely effective at improving insulin sensitivity (286), lowering HbA1c
(REF), and regulating plasma glucose levels (287). Indeed, we have observed improvements of ~25% in OGTT derived indices of insulin sensitivity with as little as 7 days of moderate intensity aerobic exercise (288). However, these interventions lack a resistance training component and weight loss is often accompanied by a loss of lean tissue (289-292). Increasing recognition of the role of lean mass to the regulation of blood glucose in T2D (293) has prompted the ADA to add 2-3 days of resistance training per week to their physical activity recommendations (293) and has led some to speculate that resistance exercise may improve glucose control via an alternative mechanism than aerobic exercise (294). Nonetheless, while the addition of resistance exercise training to physical activity recommendations is a welcome step, the added exercise burden is unlikely to increase adherence to exercise recommendations. We were therefore interested to understand whether the combination of aerobic and resistance training performed at high intensity would result in similar improvements in insulin sensitivity to those we have previously observed in individuals with T2D (209, 295) while preserving the lean mass sparing benefits of resistance training. Thus, the 15% improvement in insulin sensitivity observed in this study suggests a positive outcome. Nonetheless, we were surprised that we did not observe significant reductions in glucose area under the curve following the exercise intervention. While it is possible that our sample size was not sufficient to detect changes in glucose area under the curve, several previous studies have suggested that high intensity exercise programs may result in improvements in HbA1c (296, 297), and improvements in glucose homeostasis measured by continuous glucose monitoring (298) without apparent differences in glucose AUC.
Given that the current population consisted of relatively young, newly diagnosed diabetics with few medications, it is unlikely that drug interactions were responsible for this anomaly. We therefore considered whether muscle damage might have contributed to transient impairments in glucose uptake. Previous research indicates that muscle damage from eccentric exercise transiently reduces insulin sensitivity (299) and there have been isolated reports of rhabdomyolysis associated with CrossFit-style exercise (300). However, while eccentric exercise induced muscle damage is an expected, acute response, training adaptations rapidly result in a resistance to exercise induced muscle damage (301). Nonetheless we did observe elevated CK activity in plasma after the 6-week training intervention. Although the increases in CK activity were modest relative to the increases present in rhabdomyolysis, we did observe a positive correlation between alterations in CK activity and changes glucose AUC which suggest that a longer intervention may be required to develop a resistance to HIPT induced muscle damage and that ISI OGTT may actually underestimate the magnitude of change in glucose homeostasis achievable with this type of intervention.

Individuals with T2D are at increased risk for all cause premature mortality and death from cardiovascular disease (302). A key predictor for all-cause mortality in T2D is cardiorespiratory fitness (302). VO2max, a measure of maximal oxygen uptake, is the gold standard for assessing cardiorespiratory fitness. In the current study we observed an 18% mean increase in VO2max following the six-week intervention. This result represents an outcome superior to both standard sprint interval training performed 3 days per week and endurance training performed 5 days per week for six weeks in healthy individuals as
described by Burgomaster et al. (303), as well as a recent report of an 8-week HIIT intervention in T2D patients (304). Indeed, the increase in cardiorespiratory fitness in the current study was greater in magnitude to the 11.3% increased observed during a 12 week, 3 day per week, combined aerobic and resistance training program in individuals with T2D (191). While this is the first study to our knowledge to assess changes in VO\(_{2}\text{max}\) associated with HIPT in patients with T2D, our data suggest that early fitness gains through CrossFit HIPT may exceed those achievable through traditional programs.

Previous research indicates that each 1 mL/kg/min increment improvement in VO\(_{2}\text{max}\) was associated with ~7% lower risk of metabolic syndrome (305). Here we demonstrate that CrossFit HIPT induced improvements in fitness were associated with a ~110% decrease in the metabolic syndrome z-score, a marker of metabolic syndrome severity (306). Previous research from our lab has demonstrated a ~33% decrease in metabolic syndrome severity following a 12-week eucaloric diet and aerobic exercise intervention (5 days per week, 60 min per day) in obese pre-diabetic patients (307), suggesting that CrossFit HIPT may be more effective than traditional aerobic exercise. However, that view is tempered somewhat by the 3-fold reduction in metabolic syndrome z-score observed in individuals with impaired glucose tolerance by Malin et al. (308) using a 12-week, 3 day per week aerobic exercise intervention. It is worth noting, however, that the participants in that study had markedly lower z-scores compared to the participants in the current study and 6-weeks of CrossFit HIPT was sufficient to reduce the z-scores of the current cohort equivalent to the post intervention measures in the UMASS study. Components of the metabolic syndrome z-score include fasting plasma
glucose, triglycerides, waist circumference, mean arterial pressure, and high density lipoprotein content. We observed significant improvements in triglycerides and mean arterial pressure, while fasting glucose and waist circumference tended towards a significant reduction. The improvement in MAP was provoked by a significant reduction in diastolic blood pressure of ~5.5mmHg following the intervention. Notably, this was achieved in a cohort where significant hypertension was not present. We did not observe changes to systolic blood pressure or heart rate, however, the duration of the exercise may have been too short to obtain these outcomes. Nonetheless, the reduction in diastolic blood pressure represents a significant reduction in mortality risk, especially stroke risk (309).

Reductions in fat mass and plasma triglycerides also contributed to the lowering of cardiometabolic risk and this was associated with reductions in VLDL cholesterol. The mechanism for decreased plasma lipids is unclear, though improved adipose tissue health is likely to be a factor. Our observation of increased HMW adiponectin and reduced plasma resistin support this idea. Enhanced whole body fat oxidation may also have contributed to reductions in cardiometabolic risk. Increased fat oxidation is a common adaptation to endurance exercise and our data suggests that this adaptation is preserved in CrossFit HIPT. This increase in fat oxidation may be attributable, at least in part, to the increases in HMW adiponectin. HMW adiponectin is a known insulin sensitizer and increases oxidative capacity by signaling through Sirt1 and AMPK to increase mitochondrial biogenesis (122). Indeed the consistent relationship between HMW-adiponectin, fat oxidation, and insulin resistance following exercise training
programs (190, 194, 310, 311), a relationship that is replicated in the current study, support the hypothesis that mitochondrial adaptations are central to the reductions in insulin resistance and cardiometabolic risk in individuals with and without T2D, though the exact mechanism remains elusive (312, 313).

Limited compliance with food diaries represents a significant limitation to this study, though based on the diaries where a complete data set is available, total caloric intake was not altered. However, it does appear that there may have been a shift in macronutrient intake such that fat consumption may have increased at the expense of carbohydrate ingestion. This may have been influenced by the availability of nutritional guidance from within the CrossFit community. Although CrossFit Inc. does not provide definitive dietary guidance, there is a tendency for promotion and adoption of “Paleolithic” style dietary habits amongst the community (271), and although coaches were not instructed to give any dietary guidance to participants, the participants were not restricted from accessing any information provided by their coaches or at CrossFit, Inc. Whether changes in dietary habits contributed to the outcomes in this study are unknown and will require future assessment.

In summary, this pilot study suggests that CrossFit HIPT, performed under controlled, supervised, conditions may be safe and effective for individuals with T2D. Moreover, CrossFit HIPT may provide a time efficient method for reducing the burden of T2D that may be superior to traditional exercise programs.
CHAPTER 4.

EXERCISE TRAINING DECREASES ACTIVATION OF THE MITOCHONDRIAL FISSION PROTEIN DYNAMIN-RELATED PROTEIN-1 IN INSULIN RESISTANT HUMAN SKELETAL MUSCLE

(Published in Journal of Applied Physiology, August 2014; 117(3):239-45)

4.1 Introduction

Increased intramyocellular lipids (106, 107) and lipid intermediates, including diacylglycerols (DAGs) and ceramides (108), combined with impaired fat oxidation (109) suggests the presence of mitochondrial dysfunction in the pathogenesis of insulin resistance. However, despite significant attention, no consensus has thus far emerged regarding a mitochondrial defect antecedent to the development of insulin resistance (for review see (312) and (314)).

One aspect of mitochondrial physiology that has heretofore received comparatively less attention is the potential role of altered mitochondrial dynamics in the pathogenesis of insulin resistance. Mitochondria are highly dynamic organelles that continually fuse and divide. In skeletal muscle, mitochondria form as a highly reticular branched network (132) with morphological characteristics that differ between muscle fiber type (315) and muscle mitochondrial subpopulations (316). It is the balance between the processes of fusion, fission and mitophagy that define the reticular nature of the mitochondrial network (134, 135). Recently, greater knowledge has emerged
regarding the protein machinery that regulates these processes. Outer mitochondrial membrane fusion is regulated by the activity of the mitofusin proteins (Mfn1 and Mfn2) (141, 317) while fusion of the inner mitochondrial membrane is coordinated by the activity of Optic atrophy 1 (OPA1) (141). For fission to occur, evidence suggests that Fission-1 (Fis1) (138, 139) binds to the outer mitochondrial membrane and recruits the dynamin-like GTPase protein dynamin related protein-1 (Drp1) (139), which, when activated, pinches off a portion of the mitochondrial network (318-320) The excised mitochondrial fragment may then be tagged for autophagy by the recruitment of PTEN induced putative kinase 1 (PINK1) and Parkin or may, after a period, rejoin the mitochondrial network by the action of the fusion proteins (143). Crucially, drp1-mediated fission events are associated with a transient loss of mitochondrial membrane potential in the excised mitochondrial portion (143).

Dysregulation of mitochondrial dynamics has been implicated in an increasing number of disease states (134, 150-152), including type 2 diabetes (153, 154). However, data linking alterations in mitochondrial dynamics and skeletal muscle insulin resistance are limited. Muscle mitochondria from patients with type 2 diabetes, observed by electron microscopy, are smaller in size when compared to non-diabetic controls (110), a morphological characteristic that is partially rescued by exercise training (155), while protein expression studies indicate a pro-fragmentary environment in muscle from obese individuals (156) and in type 2 diabetes (157). Furthermore, Bach et al. (156) reported a 25% reduction in the mitochondrial network in skeletal muscle of obese Zucker rats, compared to wild type, in the absence of differences in mitochondrial mass. In the same
study, fibroblasts treated with an Mfn2 antisense sequence exhibited decreased glucose oxidation and oxygen consumption. Mfn2 repression is also associated with decreased rates of pyruvate and palmitate oxidation in L6E9 muscle cells (158). A recent report by Jheng et al. (147) provides evidence that Drp1-mediated mitochondrial fission results in mitochondrial fragmentation, loss of mitochondrial membrane potential, increased oxidative stress, decreased ATP content, and insulin mediated glucose uptake in C2C12 cells pre-incubated with palmitic acid. Moreover, Drp1 knockdown experiments reversed these alterations. These findings were duplicated in \textit{ob/ob} mice. In this case, mice treated with Mdivi-1, a Drp1 inhibitor, recovered tubular mitochondrial percentage and membrane potential, decreased oxidative stress to baseline levels, and increased insulin mediated glucose uptake. Collectively, these data provide strong evidence implicating drp1-mediated mitochondrial fission as a likely contributing factor to insulin resistance. However, there are currently no data that link this process to insulin resistance in humans.

It has been postulated that alterations in energy balance may contribute to changes in mitochondrial network structure such that increased energy demand/starvation leads to a more highly reticular mitochondrial network (321, 322), while increased energy supply results in a more highly fragmented network (147, 148, 323, 324). Since aerobic exercise interventions are an effective strategy for increasing energy demand and simultaneously enhancing insulin sensitivity and fat oxidation in humans, it is possible that a mitochondrial mechanism for improvement in insulin sensitivity may reside in alterations in the mitochondrial fission pathway. However, no studies have, hitherto, reported on the effect of exercise on activation of Drp1-mediated mitochondrial fission in insulin
resistant humans. Indeed, if Drp1-mediated mitochondrial fission is implicated in insulin resistance, we would expect to see reductions in Drp1 activation following an aerobic exercise intervention. Further, alterations in Drp1 activation would likely be related to changes in fat oxidation and insulin sensitivity. In order to test this hypothesis, we examined the effect of a 12-week aerobic exercise intervention on skeletal muscle Drp1 activation, at Ser\textsuperscript{616} phosphorylation (the form required for mitochondrial fission activity) (325), insulin sensitivity, and fat oxidation in obese insulin resistant individuals.

4.2 Methods

4.2.1 Participants

Seventeen (m/f: 10/7) older (Age; 66±1 years), previously sedentary (individuals exercising for 20 min or more at least 2 times per week were excluded), non-smoking, obese (BMI: 34.6±0.8 kg/m\textsuperscript{2}) adults were recruited from the Greater Cleveland community to undergo a 12-week aerobic exercise intervention. Medical screenings excluded individuals with heart, kidney, liver, thyroid, intestinal, and pulmonary diseases as well as individuals taking medications known to affect the outcome variables of the study. Resting 12-lead electrocardiograms and submaximal exercise stress tests excluded individuals with any contraindication to physical activity. All women were postmenopausal and not using hormone replacement therapy. Participants had also been weight stable for at least the previous 6 months. The Cleveland Clinic Institutional Review Board approved the study and all subjects provided informed consent in accordance with guidelines on the protection of human subjects.
4.2.2 Intervention

Participants performed 60 minutes (20 minutes of cycle ergometry and 40 minutes of treadmill walking) of supervised aerobic exercise at ~80-85% HR_max on 5 days per week for 12 weeks. Compliance with exercise intensity was monitored using a heart rate monitor (Polar Electro, Woodbury, NY). Three-day food records were used to monitor dietary intake changes during the intervention.

4.2.3 Inpatient control period

Pre- and post-intervention measures were controlled during a 3-day in-patient stay in the Clinical Research Unit at the Cleveland Clinic. During the inpatient control periods participants were provided with a weight maintenance isocaloric diet (total kcal/d = RMR x 1.25; 55% carbohydrate, 35% fat, 10% protein) derived from indirect calorimetry measures conducted at the beginning of the inpatient control period. All metabolic measurements were conducted during the inpatient control period and within 24 hours of the last exercise bout.

4.2.4 Body Composition

Height and body weight were measured using a stadiometer and digital scale. Whole body adiposity was measured by dual energy X-ray absorptiometry (model iDXA; Lunar, Madison, WI).

4.2.5 Aerobic Fitness

Each participant performed an incremental graded treadmill exercise test to determine maximal oxygen consumption (VO_2max), as previously described (239).
Exhaled air was continuously sampled online with the use of an automated system (Jaeger Oxycon Pro; Viasys, Yorba Linda, CA).

4.2.6 Insulin Sensitivity and Substrate Metabolism

Insulin sensitivity measurements were obtained after an overnight fast using a 2-hour euglycemic-hyperinsulinemic clamp (90 mg·dL⁻¹, 40 mU·m²·min⁻¹), as previously described (326). Briefly, a primed (3.28 mg·kg⁻¹) continuous (0.036 mg·kg⁻¹·min⁻¹) infusion of [6,6-²H₂]-glucose began at t = -120 min and continued throughout the procedure for estimation of endogenous glucose production. At t = 0, the insulin infusion commenced and glucose was infused at a variable rate. Arterialized venous blood was sampled at 5 minute intervals (YSI 2300; STAT Plus, Yellow Springs, OH) and glucose infusion was adjusted according to the calculations of De Fronzo et al. (327). Insulin sensitivity was calculated as insulin-stimulated glucose disposal rate (GDR, mg·kg⁻¹·min⁻¹) divided by plasma insulin (µU·mL⁻¹) during the last 40 minutes of the clamp (326). HOMA-IR {\(\frac{\text{Fasting plasma glucose (FPG)} \times \text{fasting plasma insulin (FPI)}}{405}\) (328) was also measured as an estimate of insulin resistance. Indirect calorimetry (Vmax Encore; Viasys, Yorba Linda, CA) measures were performed prior to the clamp procedure for determination of respiratory exchange rate (RER) and substrate metabolism. Furthermore, protein metabolism was estimated from overnight, timed measurements of urinary nitrogen excretion as previously described (329). Protein corrected fat oxidation was calculated using the Frayn equation (1.67 \(\text{VO}_2\) – 1.67 \(\text{VCO}_2\) – 1.92 n) (330).
4.2.7 **Skeletal Muscle Biopsy**

Basal muscle specimens were obtained from the vastus lateralis before and after the intervention. The muscle was dissected free from visible fat and connective tissue. The tissue was immediately frozen and stored in liquid nitrogen until subsequent analysis. Biopsies and indirect calorimetry measurements were taken immediately prior to initiation of insulin infusion.

4.2.8 **RNA Extraction**

RNA was extracted from human muscle with TRI Reagent (Sigma, St Louis, MO). Briefly, 10-20 mg of muscle tissue was homogenized in 1 ml of TRI Reagent at 4°C with repetitive short bursts. Homogenized tissue was incubated at room temperature for 5-10 minutes, followed by centrifugation at 12,000xg for 10 minutes at 4°C. RNA was separated into an aqueous phase using 0.1 ml of 1-bromo-3-chloropropane, and precipitated with 0.5 ml of isopropanol. Isolated RNA was washed with 1 ml of 75% ethanol, air dried, and dissolved in 40 µl nuclease free water. RNA concentration and purity was determined using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). RNA integrity was also randomly assessed using an Agilent bioanalyzer (Agilent, Santa Clara, CA). Isolated RNA was aliquoted and stored at -80°C until further analysis.

4.2.9 **cDNA Synthesis**

Prior to cDNA synthesis, RNA samples were treated with DNase I for 15 minutes at room temperature to remove any contaminating DNA (Invitrogen, Carlsbad, CA). One
microgram of total RNA was reverse transcribed into cDNA (iScript cDNA synthesis kit, Biorad, Hercules, CA) using a PX2 Thermal Cycler (Thermo Scientific, Wilmington, DE). The reaction volume was 20 µl and synthesis was performed at 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min, respectively. cDNA samples were stored at -20°C until later analysis.

4.2.10 qRT-PCR Primer Pairs

Primer pairs for target genes were obtained from PrimerBank database (pga.mgh.harvard.edu/primerbank/; see Table 1). All primers were checked for specificity to the genes of interest by Blast analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>GenBank Accession no</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNM1L</td>
<td>AGGTTGCCCGTGACAAATGA</td>
<td>ATCAGCAAAAGTCGGGTTGTT</td>
<td>NM_012063</td>
</tr>
<tr>
<td>FIS1</td>
<td>GTCCAAGAGACGCAGCTTTG</td>
<td>ATGCCCTTACGGATGTCATCATT</td>
<td>NM_016068</td>
</tr>
<tr>
<td>PARK2</td>
<td>GTGTTTGTCAAGGTGTCACTCCA</td>
<td>GAAAATCACACGCAAAGTGTT</td>
<td>NM_152410</td>
</tr>
<tr>
<td>MFN1</td>
<td>ATGACCTGGTTGTGAAGAGACAGT</td>
<td>AGCACATCAGCAGCTTAGGCAAAC</td>
<td>NM_033540</td>
</tr>
<tr>
<td>MFN2</td>
<td>CACATGGAGGCGTTGTAGCAGG</td>
<td>TTGAGCAGCTCTTATTAGCAAGC</td>
<td>NM_001127600</td>
</tr>
<tr>
<td>PINK1</td>
<td>AGTGTTTGACCTACAGCGAAACCTGAT</td>
<td>ATCTTTGTCTAACCCTACAGATGAGAT</td>
<td>NM_032409</td>
</tr>
<tr>
<td>OPA1</td>
<td>AGCCCTGCAATTTTTGG</td>
<td>AGCCGATCCTAGGATAGATAAGC</td>
<td>NM_130837</td>
</tr>
</tbody>
</table>

MFN1; Mitofusin 1, MFN2; Mitofusin 2, OPA1; Optic atrophy 1; FIS1; Fission 1, DNM1L; Dynamin1-like protein, PINK1; PTEN induced putative kinase 1, PARK2; Parkin 2.

4.2.11 Semi-Quantitative Real Time PCR Analysis

Determination of relative mRNA expression was performed in duplicate on an MX3000P QPCR system (Agilent Technologies/Stratagene, La Jolla, CA) using 10 ng of cDNA as the template and the Brilliant II SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA). The human GAPDH gene was used as an internal standard (331).
Relative change in mRNA abundance was calculated using the comparative $\Delta \Delta Ct$ method (332). Briefly, the threshold cycle (Ct) for GAPDH was subtracted from the Ct for the gene of interest to adjust for variations in mRNA/cDNA generation efficacy to generate the $\Delta Ct$ value. Pre-intervention was used as the baseline and the fold induction of the target gene at post-intervention was calculated as an exponential of the negative value of the subtraction of $\Delta Ct$ at pre-intervention from $\Delta Ct$ at post intervention ($2^{-\Delta\Delta Ct}$) (332).

4.2.12 Tissue Homogenization and Western Blot Analysis

Muscle homogenates were prepared by grinding muscle tissue with ice-cold lysis buffer (Invitrogen, Camarillo, CA) in the presence of protease inhibitor cocktail, 5 mM phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO) and Phos-STOP (Roche Applied Sciences, Indianapolis, IN)]. Samples for Western blot were prepared from supernatants after centrifugation of homogenates for 10 minutes at 14,000 x g. Protein concentrations were measured using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL). Fifty micrograms of muscle homogenate was solubilized in Laemmli sample buffer containing 5% $\beta$-mercaptoethanol and boiled for 5 minutes. Proteins were separated by 4-20% Novex Tris Glycine SDS-PAGE Electrophoresis (Invitrogen, Camarillo, CA), and transferred to polyvinylidene fluoride membrane (Biorad, Hercules, CA), and blocked with 5% bovine serum albumin in phosphate-buffered saline with 0.1% Tween-20 (PBST) for 1 hour. Membranes were then incubated overnight with anti-phospho Drp1 (Cell Signaling Technology, Danvers, MA; catalog no. 3455), anti-Drp1 (Cell Signaling Technology, Danvers, MA; catalog no. 8570) and anti-actin (Santa Cruz Biotechnologies Inc., Dallas, TX) antibodies. Membranes were washed in PBST and incubated with anti-
rabbit horseradish peroxidase-conjugated antibodies (GE Healthcare, Piscataway, NJ; catalog no. NA931). Immunoreactive proteins were visualized by enhanced chemiluminescence reagent (ECL Prime; GE Healthcare, Piscataway, NJ) and quantified by densitometric analysis using Image Quant TL software (GE Healthcare, Piscataway, NJ).

4.2.13 Statistical Analyses

Values were tested for normality using the D’Agostino and Pearson omnibus normality test on GraphPad Prism 4.0 (Graphpad Software, San Diego CA). Pre- to post-intervention changes were assessed using repeated measures ANOVA for normally distributed samples. A Wilcoxon signed-rank test was used to determine changes in gene expression. Linear regression analysis was used to determine associations between normally distributed data. In addition, Spearman’s rank correlation analyses were used to identify relationships between variables that failed the normality test [Δ Insulin Sensitivity]. Statistical significance was accepted when $P<0.05$. These analyses were carried out using StatView for Windows 5.0.1 (SAS Institute, NC), and all data are expressed as mean±SE.

4.3 Results

4.3.1 Participant Characteristics

Subject characteristics are summarized in Table 2. The intervention resulted in a significant 10.4±1.1% decrease in body weight. This was primarily due to a decrease in
fat mass (20.9±3.4% reduction) while fat free mass was largely preserved (1.9±0.8% reduction). \( \text{VO}_{2\text{max}} \) was also increased following the intervention \( (P<0.001) \).

<table>
<thead>
<tr>
<th>Table 4-2 Pre- and Post-intervention subject characteristics.</th>
</tr>
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<tbody>
<tr>
<td><strong>Subject Characteristics</strong></td>
</tr>
<tr>
<td>( n ) (m/f)</td>
</tr>
<tr>
<td>Age (years)</td>
</tr>
<tr>
<td>Weight (kg)</td>
</tr>
<tr>
<td>BMI (kg( \text{m}^{-2} ))</td>
</tr>
<tr>
<td>FM (kg)</td>
</tr>
<tr>
<td>FFM (kg)</td>
</tr>
<tr>
<td>FPG (mg( \text{dL}^{-1} ))</td>
</tr>
<tr>
<td>FPI (µU( \text{ml}^{-1} ))</td>
</tr>
<tr>
<td>HOMA-IR</td>
</tr>
<tr>
<td>Insulin Sensitivity (mg( \text{kg}^{-1} \text{min}^{-1} )µU( \text{ml}^{-1} ))</td>
</tr>
<tr>
<td>Basal Fat Oxidation (mg( \text{kg}^{-1} \text{min}^{-1} ))</td>
</tr>
<tr>
<td>( \text{VO}_{2\text{max}} ) (mL( \text{kg}^{-1} \text{min}^{-1} ))</td>
</tr>
</tbody>
</table>

Data are mean±SEM BMI: Body Mass Index, FM: Fat Mass, FFM: Fat Free Mass, FPG; Fasting Plasma Glucose, FPI; Fasting Plasma Insulin, HOMA-IR; Homeostasis Model Assessment of Insulin resistance, \( \text{VO}_{2\text{max}} \): Maximal Oxygen Consumption.

### 4.3.2 Insulin Sensitivity and Substrate Metabolism

Fasting glucose was decreased following the intervention \( (P<0.01) \) while fasting insulin approached a significant decrease \( (P=0.06) \). This translated into a significant reduction in HOMA-IR \( (P<0.05) \). Insulin sensitivity derived from the clamp was also increased following the intervention \( (P<0.001) \), as was basal fat oxidation \( (P<0.01) \) (Table 2).
4.3.3 Protein Expression

Exercise training resulted in a significant reduction in Drp1 phosphorylation at Ser$^{616}$ (ratio of phosphorylated Drp1 to total Drp1) ($P=0.01$) (Figure 1). Total Drp1 (ratio of Drp1 to actin) protein expression was not increased by the exercise intervention (Pre vs Post: 0.91±0.22 vs 1.02±0.13, $P=0.63$).

![Figure 4-1 Representative blots of phosphorylated-Drp1 at Ser$^{616}$, total Drp1 and actin and densitometric analysis of Phosphorylated Drp1 (pDrp1), total Drp1 (Drp1) and actin. Exercise resulted in a reduction in the Drp1 Ser$^{616}$ phosphorylation ($P=0.01$). Data are mean±SEM. *P<0.05](image)
4.3.4 Gene Expression

DNM1L is the gene that codes for Drp1, and its expression was also significantly upregulated ($P<0.01$) following the intervention. Further, exercise training increased OPA1 gene expression ($P=0.05$), while there was a trend towards an increase in expression of both MFN1 ($P=0.08$) and MFN2 ($P=0.07$) genes. The expression of FIS1 was unchanged and no change was observed in the expression of PINK1 or PARK2 genes (Figure 2).

![Figure 4-2 Expression of genes related to mitochondrial dynamics. Exercise resulted in increased expression of both OPA1 ($P<0.05$) and DNM1L ($P<0.01$), but no change in the expression of the other genes. MFN1; Mitofusin 1, MFN2; Mitofusin 2, OPA1; Optic atrophy 1; FIS1; Fission 1, DNM1L; Dynamin1-like protein, PINK1; PTEN induced putative kinase 1, PARK2; Parkin 2.](image-url)
4.3.5 Correlations

There was a significant correlation between changes in Drp1 Ser\textsuperscript{616} phosphorylation and fat oxidation ($r=-0.58$, $P<0.05$) (Figure 3), and also changes in Drp1 Ser\textsuperscript{616} phosphorylation and insulin sensitivity ($\rho=-0.52$, $P<0.05$) (Figure 4).

![Figure 4-3 A. Correlation between change in Drp1 Ser\textsuperscript{616} phosphorylation and whole body fat oxidation following the exercise intervention ($r=-0.58$, $P<0.05$).](image)

![Figure 4-3 B. Correlation between changes in the ratio of phosphorylated Drp1 Ser\textsuperscript{616} to total Drp1 and insulin sensitivity following the exercise intervention ($\rho=-0.52$, $P<0.05$).](image)

4.4 Discussion

Our data show that a 12-week aerobic exercise intervention that induced significant weight loss in obese adults improved peripheral insulin sensitivity and fat oxidation in parallel with alterations in basal Drp1 activation. Since phosphorylation of Drp1 at Ser\textsuperscript{616} increases mitochondrial fission activity (325) our data suggest that Drp1-mediated mitochondrial fission may be decreased following exercise training. Furthermore, this downregulation of Drp1 activity was significantly correlated with improvements in fat oxidation and insulin sensitivity. These data are novel because they
provide the first *in vivo* evidence in humans that support the findings of recent research using cell and animal models, which suggest that Drp1-mediated mitochondrial fission may be an important determinant of skeletal muscle insulin resistance.

Much has been made in recent years of the potential role of mitochondria in the development of diabetes. Despite this, no mechanism has thus far emerged linking impairments in mitochondrial function to insulin resistance. The hypothesis that mitochondrial fission may result in mitochondrial dysfunction and insulin resistance is intriguing, as this mechanism would require no intrinsic mitochondrial respiratory chain defect and can also account for how mitochondrial dysfunction could be induced without reductions in mitochondrial mass or content.

The precise mechanism by which Drp1 activation may mediate improvements in insulin resistance and fat oxidation is unclear. However, multiple studies have reported that Drp1-mediated mitochondrial fission is associated with a transient loss of mitochondrial membrane potential in the excised mitochondrial fragment (325, 333-335). Dispersal of mitochondrial membrane potential may result in accelerated respiration but this necessarily requires suppression of ATP production (336). Consequently, higher basal rates of mitochondrial fission may result in increased uncoupling and a corresponding reduction in overall mitochondrial network efficiency. Interestingly, data from Conley et al. (337) suggest that sedentary individuals indeed exhibit higher mitochondrial respiration and uncoupling compared to active subjects, while Chavez et al. (338) demonstrated decreased mitochondrial membrane potential in the presence of decreased insulin sensitivity in healthy subjects following lipid infusion. Furthermore,
increased uncoupling is evident in several obesity-related pathologies (339, 340). Therefore, it is conceivable that Drp1 activation may result in loss of membrane potential through sensitization of the mitochondrial permeability transition pore (mPTP) (131, 341). Importantly, activation of the mPTP in this case would potentially result in increased ROS production despite a loss of membrane potential (131, 342-344) rather than membrane hyperpolarization as has previously been suggested (345). Furthermore, such an event would likely result in sufficient permeabilization of the mitochondrial membrane to factors such as fatty-acyl CoAs and acylcarnitines, which under normal circumstances would be sequestered in the mitochondria, and have the ability to interfere with the insulin signaling pathway (123). This postulation is supported by recent work of Taddeo et al. (130), which suggests that opening of the mPTP may indeed be an important novel mediator of insulin resistance. In that study inhibition of the mPTP restored glucose uptake in muscle cells, although the increase in GLUT-4 expression on the cell membrane appeared to be independent of the classical insulin signaling pathway. Although we did not directly measure membrane potential in vivo in the present study, it is reasonable to infer that the exercise training mediated reductions in basal Drp1 activation are likely accompanied by increased mitochondrial membrane potential, decreased mitochondrial permeability within the mitochondrial population, and improved mitochondrial efficiency and reduced insulin resistance.

Further to this hypothesis, Jheng et al. (147) recently reported that in C2C12 cells, pre-incubation with palmitic acid induced Drp1-mediated mitochondrial fragmentation, higher levels of oxidative stress, reduced ATP production and reduced
insulin-mediated glucose uptake. Knockdown of Drp1 prevented these palmitate-induced effects. Furthermore, ob/ob mice treated with Mdivi-1, a Drp1 inhibitor, recovered tubular mitochondrial percentage and membrane potential, decreased oxidative stress to baseline levels and increased insulin-stimulated glucose uptake. The data presented herein indicate that exercise training may also reduce Drp1-mediated mitochondrial fission. Additionally, these alterations correlate with changes in fat oxidation and insulin sensitivity. Taken together, these data corroborate the hypothesis that mitochondrial fission may be a determinant of insulin resistance in obesity. However, further research is required to determine whether Drp1 activation and its downstream effects, either directly or indirectly, regulate insulin sensitivity in human skeletal muscle.

Our data are also consistent with previous research suggesting that energy balance may be a key determinant of mitochondrial dynamics activity (147, 148, 321-324). The weight loss observed in the current study confirms that individuals were placed in energy deficit. The increased expression of fusion related genes combined with total Drp1 protein expression suggests that either the exercise training itself, or the energy deficit, initiated a pro-fusion state within the skeletal muscle. However, this finding cannot be confirmed without imaging the mitochondrial network status, something that was outside the scope of the current investigation.

Interestingly, DNM1L, the gene that codes for Drp1, was significantly upregulated following the intervention. It is possible that the increase in DNM1L gene expression is related to the last exercise bout. It was previously reported that total Drp1 protein content was increased in skeletal muscle of lean healthy males 24 hours after a
single bout of high intensity interval training, and peaked 24 hours after the 3rd exercise session, however, total Drp1 protein expression trended downward thereafter for up to 2 weeks of training (346). Nevertheless, we cannot discount the possibility that the effects observed in the current study are in part due to an acute rather than chronic effect of exercise on gene expression. Interestingly, the increase in DNM1L gene expression in the current study was not reflected in total Drp1 protein expression, suggesting that Drp1 in skeletal muscle may undergo post-transcriptional regulation.

In summary, this study provides new in vivo evidence that altering energy balance through an aerobic exercise intervention may result in decreased Drp1 activity and a profusion environment in skeletal muscle of older obese individuals with insulin resistance and that these alterations are associated with improvements in fat oxidation and insulin sensitivity. These findings support the hypothesis that elevated mitochondrial fission may result in mitochondrial dysfunction and insulin resistance, and that exercise training may cause improvements in insulin sensitivity through inhibition of this pathway. The data presented here also suggest that a more complete investigation into the role of Drp1 activation and its downstream effects on mitochondrial morphology, mitochondrial function, and insulin sensitivity is warranted and may result in novel approaches for therapeutic treatment of insulin resistance and T2D.
CHAPTER 5.

TARGETING MITOCHONDRIAL FISSION RESTORES MITOCHONDRIAL COUPLING EFFICIENCY AND FUNCTION, AND INSULIN SIGNALING IN A C2C12 CELL MODEL OF LIPID-INDUCED INSULIN RESISTANCE

5.1 Introduction

We have previously shown that phosphorylation of dynamin-related protein-1 (Drp1) at serine residue 616 is reduced by 12 weeks of endurance exercise training in obese individuals with prediabetes (163). Moreover, the changes in Drp1 phosphorylation were associated with changes in whole body fat oxidation and peripheral insulin sensitivity, suggesting that mitochondrial fission may be altered in insulin resistance. Indeed, Jheng et al. (147) demonstrated that inhibition of mitochondrial fission under conditions of lipid oversupply using a pharmacological approach resulted in normalization of insulin-mediated glucose uptake and a reduction in ROS production. These data indicate that mitochondrial fission in conditions of nutrient excess may mediate the effects of lipid on insulin mediated glucose uptake.

Although the mechanism by which mitochondrial fission might exert this effect is not known, Drp1-mediated mitochondrial fission is associated with a reduction in mitochondrial membrane potential (143). We speculated that mitochondrial fission, in the presence of lipid oversupply, may be an adaptive mechanism to reduce coupling efficiency in the presence of lipid oversupply, thereby acting as a pressure valve to dissipate energy and switch off nutrient uptake in the face of an energy imbalance (163).
In order to test this hypothesis, we assessed whether modulation of mitochondrial fission in the presence of lipid-induced insulin resistance would result in alterations in mitochondrial function and coupling efficiency.

5.2 Materials and Methods

5.2.1 Cell culture

C2C12 myoblasts (American Type Culture Collection, Manassas, VA) were maintained at subconfluent conditions in DMEM (4.5 g/l glucose), supplemented with 10% FBS and antibiotics. Myotube differentiation was induced by switching growth media to DMEM, supplemented with 2% horse serum, and maintained for 5–6 days with medium changes every other day. DMEM (4.5 g/l glucose), supplemented with 10% FBS and antibiotics. Myotube differentiation was induced by switching growth media to DMEM, supplemented with 2% horse serum, and maintained for 2-3 days with medium changes every other day. Cells were serum starved in low glucose DMEM (1 g/L) supplemented with 1% Bovine Serum Albumin (BSA) prior to lipid treatment. After 16 hours, cells were washed with warm PBS and the media was replaced with the glucose free DMEM containing either 500 µM BSA-conjugated palmitic acid or 500 µM oleic acid. 1% BSA in DMEM served as the treatment control. For assessment of insulin signaling, 100nM insulin was added to the relevant wells 15 minutes before the termination of the experiment. At the end of the experiment, cells were washed three times in ice cold PBS, then collected and lysed in cell extraction buffer (Invitrogen, Carlsbad, CA) in the presence of protease inhibitors (Sigma) and PhosSTOP (Roche
5.2.2 Western blotting

Twenty-five to fifty micrograms of cell lysate was solubilized in Laemmli sample buffer containing 5% β-mercaptoethanol and boiled for 5 minutes. Proteins were separated by 4-12% or 4-20% Novex Tris Glycine SDS-PAGE Electrophoresis (Invitrogen, Camarillo, CA), and transferred to polyvinylidene fluoride membranes (Biorad, Hercules, CA), and blocked with 5% bovine serum albumin in phosphate-buffered saline with 0.1% Tween-20 (PBST) for 1 hour. Membranes were then incubated overnight with anti-phospho Drp1 (Ser616) (Cell Signaling Technology, Danvers, MA; catalog no. 3455), anti-Drp1 (Cell Signaling Technology, Danvers, MA; catalog no. 8570), anti-phospho Akt (thr308) (Cell Signaling Technology, Danvers, MA; catalog no. 9275S, anti-Akt (Cell Signaling Technology, Danvers, MA; catalog no. 9272S) and anti-GAPDH (Santa Cruz Biotechnologies Inc., Dallas, TX catalog no. sc-32233) antibodies. Membranes were washed in PBST and incubated with anti-rabbit horseradish peroxidase-conjugated antibodies (GE Healthcare, Piscataway, NJ; catalog no. NA931). Immunoreactive proteins were visualized by enhanced chemiluminescence reagent (ECL Prime; GE Healthcare, Piscataway, NJ) and quantified by densitometric analysis using ImageJ (347). Samples were run in duplicate from at least three independent experiments.
5.2.3 2-Deoxyglucose uptake

We assessed the timecourse of 2-deoxyglucose uptake upon palmitic acid stimulation. Following 4 hours of treatment cells were incubated in Krebs-Ringer HEPES buffer (KRH; in mM: 20 HEPES, pH 7.4, 136 NaCl, 4.7 KCl, 1.25 MgSO4, and 1.25 CaCl2) with 100 nM insulin for 1 h. Post-1-h insulin stimulation, the glucose uptake experiment commenced by adding 0.5·Ci/ml 2-deoxy-D-[3H]-glucose (PerkinElmer, Waltham, MA) and 100·M 2-deoxy-D-glucose (Sigma, St. Louis, MO) in KRH buffer. After 5 min, cells were washed four times with ice-cold PBS and lysed with 0.5% SDS. Cell-associated radioactivity was measured, and non-carrier-mediated glucose uptake was assessed by parallel incubations in the presence of 10·M cytochalasin B (Sigma). The protein content was determined with a bicinchoninic acid (BCA) assay. C2C12 glucose uptake was expressed as specific uptake after subtracting the total uptake with nonspecific background (nmoles mg protein⁻¹·min⁻¹).

5.2.4 Confocal Microscopy

The effects of palmitate on mitochondrial morphology and membrane potential cells were cultured on cell culture treated µslides and treated with palmitate containing media for 4 hrs as described. To assess mitochondrial morphology, treated treated cells were incubated for 20 minutes with 150 nM of MitoTracker Green FM (Life Technologies, Grand Islands, NY) and maintained in serum-free DMEM media. For assessment of ΔΨm, cells were treated with the ΔΨm-sensitive fluorescent probe TMRM (0.05 µM) and imaged by confocal microscopy (excitation, 543 nm; emission, above 560 nm). Confocal image stacks were captured with Spectral Laser Scanning Confocal
Microscopy for assessment of form factor and aspect ratio (morphology) and TMRM-MTG ratio, using ImageJ software, and coefficient of colocalization ($\Delta\Psi_m$ – Volocity Imaging Software. Perkin Elmer, MA).

5.2.5 mDivi1 treatment

In order to assess the effects of mitochondrial fission inhibition the treatment wells were spiked with either dimethyl sufoxide (DMSO) or 150µM Mitochondrial division inhibitor-1(Mdivi-1;Enzo Life Sciences, Plymouth Meeting, PA) dissolved in DMSO one hour prior to termination of the palmitate treatment.

5.2.6 Drp1 knockdown experiments

Early passage C2C12 cells were transfected with lentiviral particles generated in HEK293T cells using the Sigma-Aldrich MISSION pLKO.1-puro lentiviral shRNA system. pLKO.1-puro containing shRNA for mouse Drp1 (Sigma-Aldrich: Clone ID: NM_152816.1-1101s1c1) or MISSION Non-Target shRNA Empty Vector, together with packaging plasmids were transfected to HEK293T cells. The HEK293T conditioned media containing viral particles was collected for 3-5 days. The media was then filtered and added to C2C12 cells intermittently with normal growth media for 2-3 days. In addition, a non-viral treated plate was maintained as a control. Following treatment with viral media C2C12 cells were cultured in growth media containing 1µg/mL puromycin and grown until the non-viral treated cells are killed. shRNA efficiency was measured by Western blot.
5.2.7 XF24 Analysis

Basal respiration, spare respiratory capacity and mitochondrial coupling efficiency were assessed using the XF14 extracellular flux analyzer (Seahorse Bioscience, North Billerica, MA). Following 16 hours in low glucose, serum free media C2C12 cells were washed with XF Assay media and placed in a 0% CO2 incubator for 1 hour while the system calibrated. XF assay media was then replaced with treatment media containing XF assay media with sodium pyruvate and with 500 µM BSA-conjugated palmitic acid or FFA free BSA control media. Where appropriate, after 3 hours of incubation, DMSO or mDivi1 was injected from port A to a concentration of 150 µM. After 4 hours of treatment a mitochondrial stress was performed according to a standard protocol (348). Once complete, cells were washed 3x in ice cold PBS and lysed with cell extraction buffer (Invitrogen, Carlsbad, CA) in the presence of protease inhibitors (Sigma) and PhosSTOP (Roche Applied Science, Indianapolis, IN). Lysate was collected by centrifugation at 12,000 g for 10 min at 4°C. Oxygen consumption rates were corrected for protein content, measured by BCA (Pierce Biotechnology, Rockford, IL).

5.3 Statistical Analysis

Statistical analysis was performed using on GraphPad Prism 6.0 (Graphpad Software Inc., San Diego CA). Values were tested for normality using the D’Agostino & Pearson omnibus normality test. Treatment outcomes were assessed for differences using students unpaired t-test. Data that were not normally distributed were assessed using the
non-parametric Mann-Whitney U test. Statistical significance was accepted when \( P<0.05 \) and all data are expressed as mean ± SEM.

5.4 Results

5.4.1 Time course experiments reveal mitochondrial fragmentation and loss of \( \Delta \Psi \text{m} \) in a manner temporally consistent with the onset of insulin resistance.

The effect of palmitate treatment on mitochondrial morphology and \( \Delta \Psi \text{m} \) in C2C12 cells stained with the non-membrane potential dependent dye MitoTracker® Green FM, and the \( \Delta \Psi \text{m} \) dependent dye, tetramethylrhodamine methyl Ether (TMRM) was assessed by confocal microscopy. Palmitate produced a progressive shift from tubular to fragmented mitochondria (Figure 5-1 and 5-2) that was associated with a decrease in TMRM staining, suggesting a loss of \( \Delta \Psi \text{m} \) (Figure 5-2). To confirm that the loss of TMRM was an effect of treatment rather than time we compared the colocalization correlation between lipid treated cells and a BSA control at 4 hours post-treatment. Coefficient of colocalization was significantly reduced in the palmitate treated cells (\( P<0.05 \))( Figure 5-2). These data indicate that treatment rather than time is the main driver of mitochondrial membrane depolarization. These effects were mirrored by reductions in 2-Deoxyglucose uptake (Figure 5-3).

5.4.2 Palmitic acid, but not oleic acid, results in increased Drp1 (Ser616) and reduced Akt (thr 308) phosphorylation.

Drp1 activation by phosphorylation at Serine residue 616 was significantly elevated by palmitic acid, however, we did not observe a similar effect induced by treatment with
oleic acid (Figure 5-5). Similarly, insulin signaling to Akt (thr308) was inhibited only by palmitic acid (Figure 5-6).

Figure 5-1. Representative images of mitochondrial structures in C2C12 cells stained with 150nM MitoTracker® Green (MTG) and 50nM TMRM (red), and treated with 0.5mM palmitic acid. Images were collected at baseline (t=0), before lipid treatment, and after 0.5, 1, 2, 3, and 4 hours.
Figure 5-2. Mitochondria become progressively less branched (form factor) and more rounded in shape (aspect ratio) indicating increased mitochondrial fragmentation over time with palmitate treatment. Loss of red dye indicates a loss of TMRM, a membrane potential dependent dye. Depolarization occurred within 30 min to the end of the experiment. To confirm that this was a treatment effect we compared the colocalization correlation of MTG and TMRM with 4 hours of palmitic acid treatment versus BSA control. *P<0.05

Figure 5-3. 2-Deoxyglucose uptake following palmitic acid treatment.
Figure 5-4. Palmitic acid but not oleic acid treatment increases Drp1 (Ser616) phosphorylation in C2C12 cells.

Figure 5-5. Palmitic acid but not oleic acid reduces Akt (thr308) phosphorylation in C2C12 cells (white bars – -Insulin; black bars – +Insulin).
5.4.3 Basal respiration, spare respiratory capacity, and mitochondrial coupling efficiency are altered by palmitic acid treatment.

4 hours of palmitic acid treatment increased basal respiration in C2C12 cells (Figure 5-6). This increase in basal respiration was associated with a reduction in spare respiratory capacity. This effect may be explained, in part at least by the reduction in mitochondrial coupling efficiency.

![Figure 5-6. A. Basal Respiration, B. Spare Respiratory Capacity, and C. Coupling Efficiency are altered in C2C12 cells treated with palmitic acid.](image)

5.4.4 Inhibition of mitochondrial fission by mDivi1 and Drp-1 KO restore insulin mediated Akt (thr308) phosphorylation in palmitic acid treated C2C12 cells.

Insulin mediated Akt phosphorylation was reduced (P=0.06) in vehicle treated C2C12 cells following 4 hours of palmitic acid treatment. However, 1 hour of incubation in the mitochondrial division inhibitor, mDivi1, was sufficient to restore insulin mediated Akt signaling to that of the vehicle control (Figure 5-7). We saw a similar effect in Drp1
KO cells - shRNA knockdown efficiency was 85.4±2.0 % - compared to the empty vector control (Figure 5-8).

Figure 5-7. The mitochondrial fission inhibitor (mDivi1) restores insulin mediated Akt (thr308) signaling in C2C12 cells treated with palmitic acid (white bars – - Insulin; black bars – +Insulin).
Figure 5-8. Drp1 KO restores insulin mediated Akt (thr308) signaling in C2C12 cells treated with palmitic acid (white bars – BSA control; black bars – PA treatment).

5.4.5 Inhibition of mitochondrial fission restores normal mitochondrial respiratory capacity and coupling efficiency in palmitic acid treated C2C12 myotubes

Elevated basal oxygen consumption and reduced spare respiratory capacity were apparent in the vehicle treated cells. However, 1-hour treatment with mDivi1 normalized
the alterations in mitochondrial function. In addition, mitochondrial coupling efficiency, which was reduced in the vehicle treated cells, was also normalized by pharmacological inhibition of mitochondrial fission (Figure 5-9)

![Figure 5-9. Inhibition of mitochondrial fission with the mitochondrial fission inhibitor (mDivi1) normalizes mitochondrial respiration and coupling efficiency in C2C12 cells treated with palmitic acid. (white bars – BSA control; black bars – PA treatment).](image-url)
5.5 Discussion

Increased intramyocellular lipids (106, 107) and lipid intermediates, including diacylglycerols (DAGs) and ceramides (108), combined with impaired fat oxidation (109) suggests the presence of mitochondrial dysfunction in the pathogenesis of insulin resistance. Yet despite significant research focus over the past 20 years no consensus has emerged regarding a mitochondrial mechanism in insulin resistance. Recent evidence has suggested that dysregulated mitochondrial dynamics may result in alterations in insulin signaling in the presence of elevated saturated fats (147), with a possible role for Drp1-mediated mitochondrial fission (147, 163, 349). Here we confirm that Drp1 (Ser616) phosphorylation is increased in a C2C12 cell model of skeletal muscle palmitate induced insulin resistance. Conversely, we show that oleic acid, which exerts a protective effect against induction of insulin resistance, had no effect on Drp1-phosphorylation. In addition, these effects are associated with reduced ΔΨm, and increased mitochondrial fragmentation consistent with previous literature examining the role of mitochondrial fission in insulin resistance (147, 350). Importantly, we also demonstrate, for the first time, that palmitate increases basal respiration, and reduces both spare respiratory capacity and mitochondrial coupling efficiency. Furthermore, inhibition of mitochondrial fission restores normal mitochondrial efficiency and respiration. These data suggest that one of the key contributors to reduced oxidative capacity in insulin resistance may be reduced coupling efficiency that contributes to and increased basal respiration.

While there is general consensus that individuals with insulin resistance have reduced oxidative capacity, either due to impaired electron transport chain activity, or
reduced mitochondrial mass, the debate regarding whether reduced oxidative capacity can contribute to insulin resistance remains unresolved. Some argue that decreased mitochondrial content alone is a mediator of insulin resistance (351, 352) others make the case that despite reduced content there remains sufficient capacity to meet the energy demands of sedentary insulin resistant individuals (313). There is little doubt, indeed, that the capacity for ATP generation in sedentary and obese individuals, even in the presence of reduced mitochondrial mass, is sufficient to meet the energy demands. Moreover, if ATP generating capacity was impaired in insulin resistance to such a degree that energy demand could not be met we would expect an upregulation nutrient uptake pathways including the insulin signaling cascade. In support of this, oligomycin, an inhibitor of ATP synthase, activates AMPK (353). However, ATP production is a tightly regulated process such that the rate of ATP production is a function of cellular ATP demand rather than nutrient supply. Therefore, an alternative view posits that nutrient overload causes a build-up of intermediary metabolites, products of incomplete oxidation such as acyl-CoA and acylcarnitines, in the mitochondria (123) and these moieties inhibit insulin signaling directly (98), or indirectly by forming lipid species (124) that activate inflammation (125, 126), oxidative stress (127) and stress kinases such as the PKCs (128, 129).

Continuous delivery of substrate to the electron transport system leads to hyperpolarization of the mitochondrial membrane (159) and increased localized ROS production and oxidative stress (160-162). Sustained, elevated ROS production can have negative consequences for cellular integrity (354). Several studies implicate increased
mitochondrial ROS production with activation of Drp1 at serine 616 and subsequent loss of membrane potential (149, 355). We propose that in the presence of a nutrient overload, activation of Drp1 is an adaptive mechanism to uncouple respiration from ATP production in an attempt to resolve the energy imbalance in the short term, and divert nutrients away from uptake and oxidation and towards storage, hence downregulation of insulin signaling. However, in the face of chronic nutrient excess in obesity, where storage capacity is also exceeded, hyperglycemia and insulin resistance ensue. The presence of elevated basal respiration, in association with reduced mitochondrial coupling efficiency, an effect that is resolved by inhibition of mitochondrial fission in palmitate induced insulin resistance, supports this hypothesis.

del Campo et al. (356) published data showing that a double negative Drp1 knockdown did not affect insulin signaling, yet mitochondrial fragmentation induced by genetic knockdown of Mfn2 and OPA1 did result in reduced insulin stimulated Akt phosphorylation at thr473, though not the upstream phosphorylation residue thr308. The authors propose that fragmented mitochondria have reduced insulin mediated calcium uptake, which is a necessary step in Akt (thr473) phosphorylation - and subsequently reduced insulin stimulated glucose uptake. Indeed, reduced mfn2 content in skeletal muscle of insulin resistant individuals has been reported (156, 357). In addition, there is evidence that oxidative, slow twitch muscle fibers contain a more elongated mitochondrial network when compared to glycolytic, fast twitch muscle fibers (144). Oxidative muscle fibers are also more insulin sensitive, and have lower oxygen consumption rates than glycolytic fibers (358). This supports an association between
mitochondrial fragmentation, reduced respiration and insulin sensitivity at least under ambient conditions. In lipid-induced insulin resistance, however, the mechanism appears to be somewhat different. We demonstrate that reductions in insulin signaling were upstream of Akt (ser473) at Akt (thr308) consistent with previous data in palmitate induced insulin resistance. Furthermore, inhibition of mitochondrial fission by the mitochondrial fission inhibitor mDivi1, and knockdown of Drp1 by shRNA, restored insulin stimulated Akt (thr308) phosphorylation.

These data therefore, support a role for Drp1-mediated mitochondrial fission in the induction of skeletal muscle lipid induced insulin resistance via an adaptive response in the presence of lipid oversupply and suggest that targeting mitochondrial fission in obesity may represent a novel therapeutic target for preventing obesity associated metabolic disease.
CHAPTER 6. **Summary, Conclusions, and Future Directions**

The prevalence of obesity has been increasing rapidly over the past 30 years in the United States and it has rapidly become a worldwide epidemic. Recent evidence has shown that based on a BMI ≥ 30, approximately one-third (78.6 million or 34.9%) of U.S. adults are obese (359). Overall, this pandemic can be attributed to current societal trends in the United States reflecting a hazardous health environment that is promoted by excess caloric intake coupled with a sustained decline in physical activity. These conditions have led to a dramatic increase in the rates of obesity-associated disease, including NAFLD and type 2 diabetes (T2D).

Exercise has long been utilized for the treatment of metabolic disease and to permit better understanding of the mechanisms that contribute to obesity associated disease. The studies contained in this document continue that tradition. Thus, we have demonstrated that short term exercise in individuals with NAFLD, in the absence of weight loss, improved whole body insulin sensitivity, and reduced plasma biomarkers of hepatocyte apoptosis. Given that apoptosis is a major contributor to progression of NAFLD, this study suggests that increasing physical activity levels may result in better outcomes for individuals with NAFLD even without altering body composition. Furthermore, it provides evidence that pharmacological approaches that target insulin resistance in NAFLD may be sufficient to halt the progression of the disease.
Epidemiological evidence indicates that T2D is an independent risk factor for cardiovascular disease and microvascular complications, and suggests that the rate of cardiovascular disease is approximately 2-fold higher in people with diabetes than without (220, 221). A 2010 joint statement from the American Diabetes Association (ADA) and the American College of Sports Medicine (ACSM) recommended moderate intensity (40-60% heart rate max, HRmax) aerobic exercise 3 times per week, in addition to resistance training 2-3 times per week, for the management of T2D (202). The statement also recognized that higher exercise intensity (>60% HRmax) predicts better blood glucose control than exercise volume (202). Despite these recommendations, compliance and adherence to diet and exercise advice is disappointingly low. One of the most cited barriers to regular physical activity is lack of time (360-362). Therefore, we also examined a novel exercise approach that combines both aerobic and resistance exercises into short, high intensity workouts. This approach resulted in improvements in cardiometabolic health that were at least consistent, if not greater, than the effects shown by others using long duration, moderate intensity exercise.

Public opinions related to an individual’s capacity for physical activity has traditionally been conservative. Indeed, one only needs to consider the furor surrounding women’s participation in marathon events as late as the 1960s to see evidence of this. Similarly, there has been significant concern expressed regarding the safety and effectiveness of these types of high intensity exercise in obesity, or in metabolically unhealthy individuals. However, we have demonstrated that, at least where adequate supervision and coaching is present, this type of exercise is well tolerated by individuals
with T2D and conferred significant health benefits despite the comparatively shorter time burden. Future studies will be required to determine if the effects of this combined high intensity aerobic and resistance training approach improves cardiometabolic health via the established, discrete mechanisms associated with aerobic and resistance exercise in isolation, or whether we may uncover novel adaptations associated with the novel exercise paradigm.

Finally, our observations of altered substrate metabolism, increased HMW-adiponectin and reduced apoptosis following exercise interventions led us to suspect mitochondrial involvement in the pathogenesis of insulin resistance and metabolic disease. Once again, we took an exercise based approach in order to assess whether mitochondrial fission may be a factor in obesity associated insulin resistance. The data generated from this study supported an association between altered mitochondrial fission in insulin resistance and prompted us to explore this mechanism further in a cell culture model of lipid induced insulin resistance and allowed us to develop a more complete hypothesis for mitochondrial involvement in insulin resistance.

Yet, many questions still remain unanswered. For example, we demonstrated an increase in mitochondrial respiration in association with insulin resistance and reduced coupling efficiency. However, most studies, including those contained in this document suggest that fat oxidation is impaired in insulin resistance and increased by exercise. Our mitochondrial data, however, suggests the opposite. This paradox might be explained by an increase in CO₂ production from a corresponding increase in glycolysis. This would also serve to reduce intracellular energy supply by producing less ATP than
mitochondrial oxidation. Yet data linking altered mitochondrial dynamics with increased glycolysis in insulin resistance are lacking.

Furthermore, fatty acid intermediates are thought to be responsible for the inhibition of insulin signaling via activation of PKCs. There is evidence that this requires mitochondrial lipid overload, however, the mechanism by which these molecules then escape the mitochondria to exert their downstream effects on insulin resistance remains to be elucidated. Future studies should therefore test whether mitochondrial fission results in decreased production of DAG and ceramides, and facilitates release of acyl-CoAs and acylcarnitines from the mitochondrial matrix to the cytosol. In addition, since we propose that increased mitochondrial lipid metabolism may drive the production of ROS and subsequent activation of Drp1 (Ser616), targeting of mitochondrial lipid uptake by etomoxir or acipimox and measuring the subsequent effects on Drp1 phosphorylation will provide an additional means of testing the mitochondrial overload-mitochondrial fission hypothesis.

While cell culture data are important for exploration of molecular pathways and provide useful tools such as genetic manipulation of protein expression, translation of these findings to clinical therapies is the ultimate goal of biomedical research. Thus, these findings need to be replicated in clinical studies. Evaluations of mitochondrial dynamics in healthy humans undergoing a lipid infusion have not yet been done and would present opportunity to confirm in humans that Drp1-mediated mitochondrial fission is present during the induction of insulin resistance in healthy muscle, while reversing insulin resistance by alternative methods to exercise such as diet and bariatric
surgery also present an opportunity to test this hypothesis. Indeed, since one of the major arguments against mitochondrial involvement in insulin resistance remains that diet induced weight loss results in reversal of insulin resistance in the absence of apparent changes in mitochondrial mass or function, although it should be mentioned that improvements in whole body fat oxidation do occur, it will be necessary to conduct a study in individuals with insulin resistance whereby comparable caloric deficit is induced either by increasing physical activity or by reducing caloric intake. Based on the data presented and the ideas presented in this document, we expect to observe that both interventions will result in similar changes in insulin resistance yet it would also provide an opportunity to demonstrate that alterations in mitochondrial dynamics represent a common underlying mechanism that ties the change in energy balance with the resolution of insulin resistance. The converse, ie. increased caloric intake vs. reduced physical activity would likely achieve the same outcome.
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Appendix 1.

Meeting Abstracts

Title
Anti-apoptotic effect of short term exercise in obese individuals with NAFLD

Conference: 29th Annual Scientific Meeting of the Obesity-Society Location: Orlando, FL Date: OCT 01-05, 2011

Authors

Introduction
Obesity is a leading cause of chronic liver disease in the US today. Progression of non-alcoholic fatty liver disease (NAFLD) is marked by increased levels of hepatocyte apoptosis, indicated by elevations in plasma caspase-3 generated CK-18 fragments. Strategies aimed at halting the progression of apoptosis may therefore result in better outcomes for obese individuals with NAFLD.

Methods
We examined the effect of a short-term exercise program on plasma CK-18 in 11 obese (34.9±1.3 kg·m⁻²) adults (59±3 years) with NAFLD (>5% intrahepatic lipid (IHL) assessed by ¹H MR spectroscopy). Exercise consisted of treadmill walking for 60 min per day on 7 consecutive days at ~85% of maximal heart rate. Additionally, subjects underwent an oral glucose tolerance test before and after the exercise intervention. The Matsuda Index was used to assess insulin sensitivity. Fasting CK-18 was measured before and after the intervention, and liver enzymes (ALT and AST) were assessed at baseline.

Results
At baseline both ALT and AST correlated with plasma CK-18 levels (\(rho=0.70, P<0.05;\) \(rho=0.80, P<0.05\)). Exercise training improved VO₂max (21.7±5.3 vs. 23.5±4.7 ml·kg·min⁻¹, \(P<0.05\)) and insulin sensitivity (1.31±0.12 vs. 1.66±0.15, \(P<0.05\)), and decreased CK-18 concentrations in all subjects (630.8±370.3 vs. 345.4±277.1 U·L⁻¹, \(P<0.001\)), but did not significantly alter body weight (97.6±4.0 vs. 97.7±3.8 kg) or IHL (17.4±2.6 vs. 16.7±7.2 %).

Conclusion
Our results support the use of CK-18 as an indicator of the severity of liver disease and suggest an anti-apoptotic effect of exercise training in the liver of obese individuals with NAFLD. Exercise may therefore provide a clinically important therapeutic approach to halt the progression of obesity-related liver disease.
Lifestyle Intervention Reduces Skeletal Muscle Dynamin-Related Protein-1 (Drp1) Activation in Obese Insulin Resistant Humans

Ciaran E. Fealy, Anny Mulya, and John P. Kirwan

Conference: Joint Annual Meeting of the ASPET/BPS at Experimental Biology (EB) Location: Boston, MA Date: APR 20-24, 2013

Mitochondria play a central role in cellular bioenergetics and the regulation of metabolic homeostasis. Defects in mitochondrial dynamics, the processes of fission, fusion and mitochondrial autophagy, may contribute to metabolic disease including insulin resistance and type 2 diabetes. Drp1 is a key regulator of mitochondrial fission. We hypothesized that an exercise/diet lifestyle intervention would decrease Drp1 mediated mitochondrial fission, increase fat oxidation, and contribute to increased insulin sensitivity in older (66±1 years) obese (BMI: 34.6±0.8 kg/m²) adults. Seventeen subjects performed supervised exercise for 60 min/d, 5 days/week at 80-85% of max heart rate, and were provided a eucaloric diet for 12 weeks. Insulin sensitivity was measured by hyperinsulinemic-euglycemic clamp and fat oxidation was determined by indirect calorimetry. Skeletal muscle biopsies were obtained from the vastus lateralis muscle prior to each clamp. The lifestyle intervention increased insulin sensitivity 2.1±0.2 fold (P<0.01) and fat oxidation 1.3±0.3 fold (P<0.01). Phosphorylation of Drp1 at Ser^{616} was reduced (pDrp1:tDrp1: 0.58±0.14 vs 0.81±0.15, P<0.05) following the intervention. Furthermore, reductions in the pDrp1:tDrp1 ratio were negatively correlated with
increases in fat oxidation \( r=-0.58, P<0.05 \) and insulin sensitivity \( \rho=-0.52, P<0.05 \).

Our data suggest that lifestyle-mediated improvements in substrate metabolism and insulin sensitivity in obese insulin resistant adults may be regulated through decreased activation of mitochondrial fission protein Drp1.
High Intensity CrossFit Exercise Increases β-Cell Function and Insulin Sensitivity in Adults with Type 2 Diabetes

Fealy CE, Nieuwoudt S, Scelsi AR, Malin SK, Foucher JA, Pagadala M, Li M, Rocco M, Burguera B, Kirwan JP

Conference: 75th Scientific Sessions of the American-Diabetes-Association Location: Boston, MA Date: JUN 05-09, 2015

CrossFit (CF) is a novel fitness paradigm that integrates simultaneous aerobic and resistance training in sets of constantly varied functional movements performed at high intensity in workouts that range from ~8-20 min/session. We hypothesized that CF would be a safe and effective exercise mode for managing type 2 diabetes (T2D). For this pilot study we recruited 12 overweight/obese participants (4 males, 8 females; 53±2 years; BMI 34.9±1.1 kg/m²) with T2D (treated with medications, but not on insulin) to participate in a 6 week (3d/wk) supervised CF program. Body composition (iDXA), fat oxidation (Fox; indirect calorimetry), aerobic fitness (VO₂max), and adipokines (HMW adiponectin) were assessed at baseline and 24 hours after the last exercise session at the end of the program. An oral glucose tolerance test was used to derive measures of β-cell function (insulinogenic index (IGI) and the disposition index (DI)) and insulin sensitivity (ISI – Solomon index). Compliance with the training program was >95% and no injuries or adverse events were reported. CF training resulted in a significant reduction in fat mass (43.8±2.5 vs 41.6±2.2 kg; P<0.01), and an increase in Fox (0.08±0.01 vs 0.10±0.01
g/min; P=0.05), VO2max (2379±129 vs 2796 ml/min; P<0.001), and HMW adiponectin (214.4±26.8 vs 288.8±38.4 ng/mL; P<0.01). Importantly, CF training increased β-cell function (8.1±2.0 vs 12.3±3.0 AU; P<0.05, and DI: 0.33±0.08 vs 0.54±0.13 AU; P<0.05), and ISI (0.037±0.003 vs 0.042±0.003 AU; P<0.05) with a trend towards lower fasting glucose (166.8±16.2 vs 146.0±15.8 mg/dL; P=0.13). The increase in HMW adiponectin and Fox both correlated with the change in ISI (rho 0.75; P<0.05, rho 0.81; P<0.01, respectively). These data suggest that CF may be a safe and effective exercise mode for managing T2D. The increase in β-cell function and insulin sensitivity addresses a key defect in T2D and is consistent with improvements observed after more traditional aerobic exercise programs in overweight/obese adults with T2D.
Appendix 2.

Informed Consent Documents
The Cleveland Clinic  
Informed Consent to Participate in a Research Study

Study title: Effects of Short Term Aerobic Exercise Training on Insulin Resistance and Inflammatory Cytokine Secretion in Nonalcoholic Fatty Liver Disease (NAFLD)

The purpose of the informed consent is to provide you with information to help you decide whether you wish to participate in this research study. Your decision is completely voluntary and will not affect your medical care if you choose not to participate. It is important for you to understand the research risks and benefits, be able to ask questions and understand alternatives. Please carefully review this document.

- This is a research study, not standard treatment
- Your participation is completely voluntary
- You must carefully weigh the risks and benefits of participating in this research

1. INFORMATION ON THE RESEARCH

Why Are You Being Asked To Take Part In This Research?

You are being invited to participate in a research study because you have Nonalcoholic Fatty Liver Disease (NAFLD) which is a condition of excess fat accumulation in your liver caused by being overweight and/or obese and lack of regular exercise. Before you can decide whether or not to volunteer for this study, you must understand the purpose of this study, how this study may help you, any risks to you, and what is expected of you. This information gathering process is called informed consent. The study is being conducted through the Departments of Gastroenterology and Hepatology, at the Cleveland Clinic.

Why Is This Study Being Done?

The purpose of the study is to evaluate the effects of a short term exercise (7 consecutive days) intervention on insulin resistance and inflammation in Nonalcoholic Fatty Liver Disease (NAFLD), a disease that is the result of excess fat accumulation in your liver.

How Many People Will Take Part In The Study?

Approximately 12 sedentary overweight/obese adults will participate in this study.
What Is Involved In The Study?

Phase 1 (Screening)

These tests may be performed over 1 to 3 days depending on your schedule. The order of these tests may vary. As part of the screening you will complete a physical examination, medical history, resting electrocardiogram (ECG, a non-invasive measure of the electrical activity of your heart), blood sample (13 cc, or 2½ teaspoons), and an exercise stress test (all procedures are described below).

Medical History and Physical (approximately 1 hour):
You will have a medical history and physical exam, and a resting electrocardiogram.

Questionnaires (30 minutes): You will be asked to complete 4 questionnaires, described below:

Medical History (a checklist about current and past medical information)

NMR procedure Screening Form (a checklist about any metal that may be implanted into your body)

Minnesota Leisure Time Physical Activity (a checklist of 60 physical or recreational activities you may have participated in over the last 12 months)

Seven Day Physical Activity Recall (9 questions about your sleep and physical activity in the past week)

24 Hour Diet Record (a checklist of foods/drinks within the last 24 hours)

Exercise Stress Test (approximately 1 hour):

This test evaluates whether your heart is healthy and safe for exercise. You will be encouraged to exercise as hard as you can, but safely. During this test, adhesive pads will be attached to your chest and leads (wires) will be connected to a machine that measures the electrical activity in your heart. The procedure is generally painless. Males that are particularly “hairy” in the chest area may need to have their chests shaved in order to ensure proper conductivity of the leads.

Next, you will walk on a treadmill, with the incline (slope/angle) progressively increased, until fatigue, breathlessness, and/or symptoms indicate to the medical staff, or yourself, that you should stop the exercise.
Phase 2 (Diet / Weight Stabilization and Pre-Program Testing)

If the cardiologist from this research study considers your heart healthy to exercise, you will be eligible to participate in the study. These tests will be performed once before and once after the diet and exercise program. During this period we will control your diet and activity levels. To control for diet and physical activity, you will be instructed on how to follow a weight maintenance diet for a 2-week period and to maintain your normal daily activities. During the last day of this period, you will be admitted as an inpatient and reside in the CRU for an overnight stay (arrive before 5 pm). Also during this time you will be asked to complete a series of tests that include: (1) Body Composition Assessment, (2) Exercise Capacity Test, and (3) Metabolic Tests.

1. Body Composition:

   **Waist to Hip Ratio (WHR):** To determine if you have a healthy waist to hip ratio, we will perform three measurements using a tape measure to assess the circumference of your hips at the widest part of your buttocks. Then two other measurements will be performed just under the ribs (natural waist) and at the level of the belly button (umbilicus).

   The accumulation of body fat in specific body regions has been linked to health problems. For example, people with "apple-shaped" bodies (more weight around the waist) face more health risks than people with "pear-shaped" bodies who carry more weight around the hips.

   **Computer Tomography (CT) scan:** We will measure the amount of fat distributed around your waist using a CT scan: This test is similar to an x-ray and involves a small amount of radiation exposure. During this test you will lie on your back and a picture will be taken of your abdomen. This picture will allow us to measure the fat inside your abdomen. This test will take about 1 hour.

   **Proton-Nuclear Magnetic Resonance Spectroscopy (NMR):** We will also measure the amount of fat within your liver using Proton-Nuclear NMR: This is similar to the CT scan but uses a different piece of equipment that does not involve radiation. You will lie on your back and separate pictures will be taken of your liver. This will allow us to measure the amount of fat within the liver. This test will take about 1 hour. This particular test is unlike a typical NMR in that it uses different methods to look at the body's internal organs. This test is being done for research purposes. It is not a diagnostic imaging examination.

   **Dual Energy Absorptiometry (DEXA):** This test is used to measure the amount of fat distributed throughout your whole body and will allow us to measure your percent body fat. During this test, you will lay on your back on a table for 10-15 minutes while a scanner passes over your body. This test also involves a very small amount of radiation exposure.
2. **Weight Stabilization Diet:**

Before beginning the exercise program you will be provided with instruction on how to follow a weight maintenance diet for two weeks. In the last three days of the diet and activity standardization period you will be asked to eat approximately the same amount of calories as your normal everyday diet with only two exceptions. You cannot drink alcohol 48 hours prior to any of the metabolic tests and cannot eat or drink foods that contain caffeine for at least 12 hours prior to testing. Common foods containing caffeine include: coffee, tea, soda and chocolate. If you have questions on foods containing caffeine, please ask one of the team members prior to your diet standardization period. In addition, we ask that you refrain from any structured exercise for 24 hours prior to testing and limit exercise to your normal everyday activities.

3. **Exercise Capacity Test:**

This test is used to evaluate your current level of physical fitness. You will be encouraged to walk on a treadmill for as long as you can, safely. During this test you will breathe into a mask with a tube attached (pneumotach) so that all of the air you breathe in and out, will pass through the pneumotach and into a machine alongside the treadmill. The machine allows us to measure your oxygen consumption and provides a measure of your maximum oxygen capacity ($\text{VO}_{2\text{max}}$), or maximum exercise capacity. During this test your heart’s activity will be monitored with an ECG (as described above) and blood pressure measurements will be taken frequently. You will walk on a treadmill, with the incline progressively increased, until fatigue, breathlessness, and/or symptoms indicate to the medical staff, or yourself, that you should stop the exercise. The test will be repeated during the post-testing period ($\approx 9$ days after the first test.) to evaluate the effects of the exercise program. The pictures below show what a typical test looks like.
4. Metabolic Tests

Oral Glucose Tolerance Test (approximately 4 hours):

You will be asked to come to the Clinical Research Unit (Unit M51) on the morning of the test. You will need to be fasted at the time of your arrival, which means nothing to eat or drink (except for sips of water) for at least 10 hours before the test. This is a standard 2-hour test that is used to detect diabetes mellitus. An indwelling plastic catheter (needle) will be placed in a vein in your arm. This needle will remain in place for about 3 hours. Once the needle is in place, an initial blood sample will be taken. This sample will tell us your fasting blood glucose (sugar) level, calcium, phosphorus, cholesterol and triglyceride (fats) levels, your complete blood count, and your thyroid (a gland in your neck) function.

Once this sample has been obtained, you will drink an orange flavored beverage that contains a known amount of glucose (sugar). Over the next 3 hours, we will measure your body's tolerance to the glucose (oral glucose tolerance test, OGTT). Blood glucose and insulin levels will be measured again at ½, 1, 1½, 2 and 3 hours. The amount of blood that will be obtained for this test will be approximately 30 cc (or 6¼ teaspoons). During this test you will complete questionnaires to assess your diet and activity levels over the previous 12 months.

Indirect Calorimetry (approximately ½ hour):

This test will calculate your resting metabolic rate (the amount of energy that your body requires over 24 hours while at rest). We will place you in a hospital bed or a reclining chair in the CRU. Once you are comfortable, a clear plastic hood with two tubes will be placed over your head. One tube allows fresh air to flow in while another tube sends the air that you breathe out into an instrument that measures oxygen and carbon dioxide. We will ask you to breathe normally under the hood for approximately 15-20 minutes. A computer then analyzes your oxygen and carbon dioxide readings and calculates your resting metabolic rate. This will allow us to determine your body’s fuel preference. The following photo shows the test device:
**Urine collection:** Urine will be collected from 06:00 until completion of the calorimetry measures. Total volume and time of urine collection are recorded and analyzed for urea nitrogen and subsequent protein oxidation.

### Phase 3 (Diet Monitoring and Exercise Intervention)

During the week of your exercise program, you will need to complete a Participant Daily Intake Form every day for seven days. This form has 8 questions that ask you about what you ate and drank during the day.

You will meet with a member of the research staff at the CRU for approximately 1 hour once before you begin your exercise program to familiarize yourself with the facility, the exercise equipment and the goals of the study (exercise prescription). You will also have time during the study or after the study is completed to ask any questions about continuing your exercise program or to share any “likes” or “dislikes” of the study.

**Exercise Intervention:**

During the 7-day program you will exercise for 7 consecutive days for 50-60 minutes each day. The exercise will consist of walking/jogging on a treadmill or pedaling a stationary bike. Most of the exercise sessions will be conducted at the Fitness Center in the Walker Building, while the exercise stress test, and maximum aerobic capacity tests will be conducted at the CRU at the Cleveland Clinic.

You will be instructed to keep your heart rate within a prescribed limit (85% of maximum) and you will be provided with a heart rate monitor and watch to make it easy to check your heart rate. You should notify the investigators if there is any change in your medications (prescription, over-the-counter, herbal products) during the study period.

### Phase 4 (Post-Program Testing)

At the end of the 7-day Exercise Program you will be admitted as an inpatient to the CRU overnight. During this time we will again control your diet and activity level. You will then repeat the tests performed at the beginning of the program.

**How Long Will You Be In The Study?** You will be in the study for ≈ 2 weeks.
Study Schematic:

<table>
<thead>
<tr>
<th>Health and Physical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting 12 lead ECG</td>
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<tr>
<td>Graded Exercise Test</td>
</tr>
<tr>
<td>Blood Chemistries</td>
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<tr>
<td>Nutritional Counseling</td>
</tr>
<tr>
<td>Diet/Activity Questionnaires</td>
</tr>
</tbody>
</table>

Screening

Baseline Testing

| OGTT, Urine |
| Body Composition |
| NMR, DEXA, WHR |
| Indirect Calorimetry |
| VO\textsubscript{2}\text{max} |

Intervention

| OGTT, Urine |
| Body Composition |
| NMR, DEXA, WHR |
| Indirect Calorimetry |
| VO\textsubscript{2}\text{max} |

Post Intervention testing

2. RISKS AND DISCOMFORTS

What Are The Risks Of The Study?

Your participation in this study may involve the following risks:

Venipuncture/catheter placement (blood sampling): When a needle is inserted into a vein there will be some temporary pain and possible bruising. Infection is very rare as your skin is cleansed prior to needle insertion and only sterile needles are used. Some people also feel dizzy when they have their blood drawn.

Oral Glucose Tolerance Test: The sugar drink tastes very sweet and it can make some people feel nauseated or even vomit.

Exercise Testing on the Treadmill: You might experience fatigue or shortness of breath. You might also feel like your heart is pounding very fast or very hard and experience dizziness or chest pain. You could stumble and fall off the treadmill. If you have any of these experiences, tell the research team. Also, when the mask is on your face, this might feel uncomfortable.
**Indirect Calorimetry:** For some people, the plastic canopy over their heads makes them feel claustrophobic or anxious. This feeling is temporary and will go away when the canopy is removed. The canopy is “see through” and does not restrict movement.

**Cumulative Radiation Exposure:** This research study involves exposure to radiation from CT and DEXA scans. The amount of radiation exposure is equivalent to 3% of the exposure doses for clinical determinations, and is within the guidelines recommended by the Food and Drug Administration for radioactive research.

**CT Scan:** This technology uses radiation to take a picture of whatever is being scanned. The amount of radiation you will be exposed to in the CT scan is about what you receive naturally over the course of a year in the general environment. Such doses of radiation may be potentially harmful, but the risks are so small that they are difficult to measure. If you have already had many x-rays, you should discuss the potential added risk from more radiation with the researchers before agreeing to be in the study.

**DEXA Scan:** You will be exposed to a small amount of radiation. The amount is similar to that which a person living in Ohio, would be exposed to from natural sources over a 2-month period. Such doses of radiation may be potentially harmful, but the risks are so small that they are difficult to measure. If you have already had many x-rays, you should discuss the potential added risk from more radiation with the researchers before agreeing to be in the study.

**NMR Scan:** Because the NMR machine is a large magnet, it can move iron-containing objects in the room during your examination. Unexpected movement of such objects could possibly harm you. Precautions have been taken to minimize such an event from happening. Loose metal objects, like pocketknives or key chains, are not allowed in the NMR room. If you have a piece of metal in your body, such as a fragment in your eye, aneurysm clips, ear implants, spinal nerve stimulators, or a pacemaker, dental braces, or a cochlear implant, you cannot have an NMR. You must inform the research team if you have any of these implants in your body. In addition there is a small risk of a claustrophobic reaction during the procedures.

**ECG:** A small amount of adhesive might remain on your skin when the pads are removed or the adhesive might pull on hair in the area. The adhesive is easily removed with alcohol or soap and water.

**Questionnaires:** You might find it boring or time-consuming to complete the questionnaires. Also, some of the questions might be stressful. You don’t have to answer any question that makes you feel uncomfortable.

**Exercise Program:** You might find it taxing or burdensome to have to come to the Cleveland Clinic for 7 consecutive days to do the exercise program. You might have to make special arrangements with work or family to have the time off to participate. The exercise might make you tired and cause temporary muscle soreness. You could also feel dizziness, shortness of breath or have chest pain, abnormal blood pressure or abnormal
heart rhythms while exercising. You could stumble and fall off the treadmill or bike. If you have any of these experiences, tell the research team immediately.

Additionally, there may be risks that we do not know about.

3. BENEFITS

Are There Benefits To Taking Part In The Study?

Benefits include obtaining knowledge of current health status as determined by medical history and physical examination, assessment of current physical fitness status, improved knowledge of exercise and access to personal data collected during the study. Your glucose tolerance may improve as a result of participating in this study and your liver fat may be reduced, but these changes cannot be guaranteed.

Societal benefits may include new knowledge about the immediate effects of exercise on control of blood sugar. Information from this study may help us learn the most effective exercise protocol to prevent the development of, and/or help, in the treatment of insulin resistance or type 2 diabetes mellitus (T2DM) and nonalcoholic fatty liver disease (NAFLD).

4. ALTERNATIVES

What Other Options Are There?

This is a research project. You may decline to participate. The procedures described in this consent form are not needed for your medical care and they do not treat or cure diabetes, obesity, and/or fatty liver disease. We do not guarantee that you will lose weight if you participate in this research study.

5. PRIVACY AND CONFIDENTIALITY

Will Your Information Be Kept Private?

The medical and research information recorded about you will be used within the Cleveland Clinic and/or disclosed outside the Cleveland Clinic as part of this research. Tests and procedures done solely for this research study may be placed in your medical record to indicate your participation in this study. Upon completion of the study, you may have access to the research information if contained in the medical record. Your access to research information about you will be limited while the study is in progress. Preventing this access during the study keeps the knowledge of study results from affecting the reliability of the study. This information will be available should an emergency arise that would require your treating physician to know this information to treat you best.
Your research information may be disclosed to the National Institutes of Health, the research study Sponsor and its agents, the Cleveland Clinic research review staff, the U.S. Food and Drug Administration, and other outside collaborators or laboratories that are participating in this study, if any, that are listed as follows: Department of Health and Human Services Agencies, National Committee for Quality Assurance. The Cleveland Clinic also may use and disclose this information for treatment and payment reasons. The Cleveland Clinic must comply with legal requirements that mandate disclosure in unusual situations. Otherwise, the information recorded about you as part of this research will be maintained in a confidential manner. It is possible that information disclosed about you outside the Cleveland Clinic could be re-disclosed and no longer protected by federal privacy laws.

Your research information may be used and disclosed indefinitely, but you may stop the use and disclosure of personal information at any time by writing to John Kirwan, PhD at Cleveland Clinic, 9500 Euclid Avenue, Cleveland, Ohio 44195. If you do so, any information previously disclosed cannot be withdrawn. The Cleveland Clinic will not use or disclose the information collected in this study for another research purpose without your written permission, unless the Cleveland Clinic Institutional Review Board gives permission after ensuring that appropriate privacy safeguards are in place. The Institutional Review Board is a committee whose job is to protect the safety and privacy of research subjects.

If you choose not to sign this consent form, you will not be permitted to participate in this research study.

6. RESEARCH RELATED INJURIES

What Happens If An Injury Occurs?

If physical injury occurs due to your involvement in this research, medical treatment is available, but your medical insurance must pay the cost of treatment. Such medical treatments that are not covered by your medical insurance shall not be paid by the sponsor, the National Institutes of Health. Compensation for lost wages and/or direct or indirect losses are not available. The Cleveland Clinic will not voluntarily provide compensation for medical expenses or any other compensation for research-related injuries. Further information about research-related injuries is available by contacting the CCF Institutional Review Board at (216) 444-2924.

7. COSTS

What Are The Costs?

There is no cost to you or your insurance company for participation in this study.
The sponsor will pay for the procedures and extra study specific tests that are not routine and only being performed because you are participating in this study. The Cleveland Clinic will not pay for the costs of procedures, tests, visits and hospitalizations in connection with this study. For your participation in this study you will be compensated $150.00.

8. VOLUNTARY PARTICIPATION

What Are Your Rights As A Participant?

Taking part in this study is completely voluntary. You may choose to leave the study or decline participation at any time. Leaving the study will not result in any penalty or loss of benefits to which you are entitled.

We will tell you about new information that may affect your health, welfare, or willingness to stay in this study.

9. QUESTIONS

Whom Do You Call With Questions Or Problems?

If you have any questions about the research or develop a research-related problem, you should contact:

John Kirwan, PhD at 216-444-3412
Jacob Haus, PhD 216-445-4864
Amy Patrick-Melin, MEd 216-444-8460

After hours: Call GI Fellow at 216-444-2000 pager #25555

If you have questions about your rights as a research subject, you should contact the Cleveland Clinic Institutional Review Board at (216) 444-2924.

Cleveland Clinic Clinical Research Unit (CRU) study participants may also contact the CRU Research Subject Advocate (RSA), Carmen Paradis, MD at 216-445-2767 or 216-444-2200 and ask the operator to page beeper #28848 with regard to questions about study participation and research subject protections.
10. SIGNATURE

I have read the informed consent (the above information) and I have had the informed consent verbally explained to me. I have had all my questions answered to my satisfaction. I understand that my participation is completely voluntary and that I may decline my participation at any time during the course of the study. Signing this form does not waive any of my legal rights. I understand that a copy of this consent will be provided to me. By signing below, I agree to take part in this research study.

____________________________________  ________________
Subject Signature                                         Date

____________________________________
Printed name of Subject

Statement of Person Conducting Informed Consent Discussion

I have discussed the above points with the subject. It is my opinion that the subject understands the risks, benefits, and procedures involved with participation in this research study.

____________________________________  ________________
Signature of person obtaining consent                                Date

____________________________________
Printed name of person obtaining consent
Consent to Participate in a Research Study

Study title: **EFFECTS OF CHRONIC (12-WEEKS) EXERCISE TRAINING AND DIET ON BODY COMPOSITION AND INSULIN RESISTANCE**

Please carefully review this document. The purpose of this is to provide you with information to help you decide whether you wish to participate in this research study. Your decision is completely voluntary and will not effect your medical care if you choose not to participate. It is important for you to ask questions and understand the research risks, benefits and alternatives.

- This is research, not standard treatment
- Your participation is voluntary
- You must weigh the risks and benefits to yourself of participating in this research

Your health care provider may be an investigator in this research study, and as investigator, is interested in both your welfare and in the conduct of the study. Before entering this study or at any time during this research, you may ask for a second opinion about your care from another doctor who is in no way associated with the research study. You are not under any obligation to participate in any research project offered by your doctor.

1. INFORMATION ON THE RESEARCH

   **Why Are You Being Asked To Take Part In This Research?**

You are being invited to participate in a research study. Before you can decide whether or not to volunteer for this study, you must understand the purpose of this study, how this study may help you, any risks to you, and what is expected of you. This process is called informed consent. The study is being conducted through the Departments of Gastroenterology and Pathobiology, Cleveland Clinic Foundation, and the Departments of Nutrition, and Physiology & Biophysics at Case Western Reserve University.

   **Why Is This Study Being Done?**

The purpose of the study is to evaluate the effect of a high-or low-glycemic diet with exercise on insulin resistance, or type 2 diabetes, in older men and women. A food’s glycemic index is a ranked number based on its immediate effect on blood glucose (blood sugar) levels. A carbohydrate food that breaks down quickly during digestion has a high glycemic index and blood sugar quickly rises. On the other hand, carbohydrates that break down slowly release glucose little by little into the blood stream. These have a low glycemic index. It is not clear at this time which diet/exercise regimen is better. In this study, you will be assigned to one of two
groups (high- or low-glycemic diet) based on chance using a method of selection called randomization (similar to a coin toss).

This study will provide information for older men and women regarding:

- The possible benefits of a 12-week diet/exercise training program on the regulation of glucose (sugar) by insulin.
- The changes that may occur in skeletal muscle that can control how glucose (sugar) is regulated by insulin after a diet/exercise program.
- The changes that may occur in body fat and its distribution in response to a diet/exercise program.

How Many People Will Take Part In The Study?

Approximately 80 people will participate in this study.

What Is Involved In The Study?

Phase 1 (Screening)
These tests may be performed over 1 to 3 days depending on your schedule. The order of these tests may vary. As part of the screening you will complete a physical examination, medical history, resting electrocardiogram (ECG, a non-invasive look at the electrical activity of your heart), blood samples (6 cc, or 1¼ teaspoons) and urine sample, an oral glucose tolerance test (12 cc, or 2½ teaspoons of blood), resting metabolic rate, and an exercise stress test (see below).

Questionnaires (30 minutes): We will ask that you complete 6 questionnaires, described below:

- Medical History (a checklist about current and past medical information)
- Minnesota Leisure Time Physical Activity (a checklist of 60 activities you may have participated in during the last 12 months)
- Seven Day Physical Activity Recall (9 questions about your sleep and physical activity in the past week)
- Mini-Mental State Questionnaire (32 questions about your memory, attention, and language skills)
- Block 98 Dietary Questionnaire (approximately 150 questions about the food you typically eat and how often you eat it)
- Patient Food Consumption (20 questions about the foods you like and dislike, as well as a list of foods and drinks that we would consider for your research diet)
Medical History and Physical (approximately 1 hour):
You will have a medical history and physical exam, and a resting electrocardiogram.

Exercise Stress Test (approximately 1 hour):
This test is to evaluate whether your heart is healthy and safe for exercise. You will be encouraged to exercise as hard as you can safely. During this test, adhesive pads will be attached to your chest and leads (wires) will be connected to a machine that can measure the electrical activity in your heart. The procedure is generally painless and you will not be exposed to radiation.

Next, you will walk on a treadmill, with the incline (slope/angle) progressively increased, until fatigue, breathlessness, and/or symptoms indicate to the medical staff, or yourself, that you should stop the exercise.

Oral Glucose Tolerance Test (approximately 4 hours):
You will be asked to come to the General Clinical Research Center (GCRC, Unit M51) on the morning of the test. You will need to arrive fasting, which means nothing to eat or drink (except for sips of water) for at least 10 hours before the test. This is a standard 3-hour test that is used to detect diabetes. An indwelling plastic catheter (needle) will be placed into your arm. This needle will remain in place for about 3 hours. Once the needle is in place, an initial blood sample will be taken. This sample will tell us your fasting blood glucose (sugar), calcium, phosphorus, cholesterol and triglyceride (fats) levels, your complete blood count, and thyroid (a gland in your neck) function.

Once this sample has been obtained, you will drink an orange flavored beverage that contains a known amount of glucose (sugar). Over the next 3 hours, we will measure your body's tolerance to the glucose (oral glucose tolerance test). Blood glucose and insulin levels will be measured again at ½, 1, 1½, 2 and 3 hours. The amount of blood that will be obtained for this test will be approximately 12 cc (or 2½ teaspoons). During this test you will complete questionnaires to assess your diet and how active you have been over the previous 12 months.

Indirect Calorimetry (approximately ½ hour):
We will place you in a hospital bed or a reclining chair in the GCRC. Once you are comfortable, a clear plastic hood will be placed over your head. This hood has two tubes. One tube allows fresh air to flow in while another tube sends the air that you breathe out into an instrument that measures oxygen and carbon dioxide. We will ask you to continue breathing under the hood for approximately 30 minutes. A computer then analyzes your oxygen and carbon dioxide readings and calculates your resting metabolic rate. This will allow us to determine your caloric needs for the duration of the study. See photo of device:
Phase 2 (Diet / Weight Stabilization and Pre-Program Testing)
If your heart is considered healthy for exercise, you will be eligible to participate in the program tests. These tests will be performed once before and once after the diet and exercise program. During this period we will control your diet and activity levels and complete a series of tests that include: (1) Body Composition Assessment, (2) Exercise Capacity Test, (3) Metabolic Tests. A morning resting blood pressure will be obtained during this period. To control for diet and physical activity, you will be instructed on how to follow a weight maintenance diet for a 2-week period. During the last 3-days of this period, you will be admitted as an inpatient and reside in the GCRC (arrive before 10:00pm) on the first two nights and at 5:00 pm on the third night.

1. **Body Composition:** We will measure the amount of fat distributed around your waist using a **Computer Tomography (CT) scan:** The image obtained will allow us to measure the fat inside your abdomen. You will not be given contrast agent (dye) for this test. This test will take about 1 hour.

   We will also measure the amount of fat within your muscle cells and liver using **Proton-Nuclear Magnetic Resonance Spectroscopy (NMR):** This is similar to the CT Scan but uses a different piece of equipment. You will again lie on your back and a picture will be taken of your leg muscles and liver. This will allow us to measure the amount of fat within the muscle and liver cells. This test will take about 1 hour.

   **Anthropometrics (body measurements):** We will also determine the distribution of fat on your body through skinfold and tape measures. We will use a caliper that will measure the amount of fat beneath the skin by calculating the thickness of skin at seven (7) different sites on the body (for example, thigh, chest, arm).

   **Dual Energy Absorptiometry (DEXA):** During this test, you will lay on your back on a table for 10-15 minutes while a scanner passes over your body. This will allow us to measure your percent body fat.
2. **Weight Stabilization Diet**: Before beginning the special study diet and exercise program you will be provided with instruction on how to follow a weight maintenance diet for two weeks.

3. **Exercise Capacity Test**: This test will be used to evaluate your current level of physical fitness. You will be encouraged to walk on a treadmill as much as you can, safely. During this test you will breathe into a mouthpiece and a clip will be placed over your nose so that all of the air you breathe in and out, will pass through the mouthpiece and into a machine alongside the treadmill. The machine allows us to measure your oxygen consumption and provides a measure of your maximum oxygen capacity (\(\text{VO}_{2\text{max}}\)), or maximum exercise capacity. During this test your heart’s activity will also be monitored with an ECG (as described above). You will walk on a treadmill, with the incline progressively increased, until fatigue, breathlessness, and/or symptoms indicate to the medical staff, or yourself, that you should stop the exercise. The test will be repeated at 4-week intervals during the exercise training program.

4. **Metabolic Tests**

   * **Indirect Calorimetry**: as described above.

   * **Glucose Production by the Liver, Insulin Sensitivity, and Muscle Biopsy**: On the morning of the test, we will put a needle into a vein located on the back of your hand. This needle will stay in place for the duration of the study (about 4 hours) and will be used to obtain blood samples (this eliminates the need for repeated needle sticks). Your hand will be kept warm (131-140°F) in a warming box, for the duration of this study, in order to warm the blood in your vein to be more like the blood in your artery. We will insert another needle (catheter) into one of the large veins in your arm. This needle will be for infusions of insulin and glucose. Therefore, you will have a total of two needles (intravenous catheters) in place for approximately 4 hours.

   If the skin on your hand feels irritated or too warm, tell the research team, so as to avoid a possible skin burn. See RISKS section of this consent form.

   * **Measurement of Glucose Production by the Liver**: After placement of an intravenous line in your arm, we will inject glucose labeled with a tracer (~2 drops) called dideuterated glucose. The tracer contains a very small amount of heavy hydrogen (\(^2\text{H}_2\)), which makes the glucose more identifiable. A small percentage of heavy hydrogen is naturally present in your body (0.015%). We add to this natural pool and measure its concentration in the blood. This allows us to determine how insulin is controlling liver glucose production.

   Dideuterated glucose is not radioactive. The infusion of dideuterated glucose through one indwelling catheter is continued during the entire 4-hour test. For a 30 minute period during the first two hours we will place a clear plastic hood over your head (as described above) so that we can measure the oxygen and carbon dioxide levels in your breath. By analyzing these levels it is possible to estimate whether you are using the sugar for energy or storing it as glycogen.
**Insulin Sensitivity Test:** This test measures your body's sensitivity to insulin. For 2 hours, insulin and glucose are infused through a catheter in your arm. After the initial blood samples for measurement of insulin and glucose are drawn, insulin is infused at a constant rate. Your blood sugar is measured every 5 minutes, and a 20% glucose infusion is adjusted to keep your blood sugar at a normal level (90 mg/dl) throughout the test. The test is called a "glucose clamp" because the blood sugar is "clamped" at a normal level.

With the aid of a computer the amount of glucose you need from minute to minute is calculated. The total amount of glucose infused during the test plus the amount of glucose your liver produces is an estimate of how sensitive you are to insulin. The total length of time for the entire procedure is approximately 4 hours. During the last 30 minutes of the test we will again place a clear plastic hood over your head so that we can measure the oxygen and carbon dioxide levels in your breath. Blood removed during this test will amount to ~4 oz. or about 8 tablespoons.

We may need to draw a sample of blood within a few days prior to your insulin sensitivity test to check your blood sugar.

**Muscle Biopsy:** Two muscle biopsy samples will be taken, one before and one at the end of each clamp procedure. The biopsy involves removal of a very small piece of muscle (1/3 the size of an eraser on a pencil) by way of a needle inserted into your thigh through a quarter inch skin incision. Local anesthesia will be used to numb the area where the incision will be made. Muscle biopsies will be performed on both legs, alternating right and left. Three to five days after the biopsy, you will return to the GCRC for the purpose of determining whether the skin where the biopsy was taken is healing properly. The biopsy involves the following:

First, the skin on the outside portion of your lower thigh will be cleansed with an iodine solution. Once thoroughly cleansed and dry, a small amount of numbing agent (about 3-4cc, or less than ½ teaspoon of Lidocaine), will be injected into the area to be biopsied. If you are allergic to Lidocaine or drugs in the ‘caine’ family (e.g., Novocaine), tell the research team.

Once the area is sufficiently numbed, a small incision will be made (approximately 5mm, or less than ¼ of an inch) and a biopsy needle will be inserted in order to obtain approximately 100-150mg or about 0.3oz of muscle. Once the biopsy has been completed, slight pressure will be applied to the biopsy area to minimize any bleeding. The area will then be cleansed and Steri-Strips (a type of bandage) will be applied to the biopsy site. An ACE bandage will be wrapped over the site and you will be asked to wear this bandage for the next 24 hours to reduce the risk of bleeding.

**Phase 3 (Diet and Exercise Intervention)**

**Dietary Intervention:** You will be randomly assigned to one of two dietary groups, either a low- or a high-glycemic diet. During the 12-week program, all of your meals will be prepared in the GCRC kitchen and provided for you. You will come to the GCRC each morning during
the week and pick up your pre-packaged meals for the day. Weekend meals will be prepared and packed for carry out on Fridays. You will be instructed to consume only the food and drink provided by the dietitian throughout the study. Do not share your meals with others or supplement your meals with food or drinks that are not provided by the GCRC.

During the weeks of your special diet, you will need to complete a Participant Daily Intake Form every day. This form has 8 questions that ask you about what you ate and drank during the day.

You will also be asked to complete a questionnaire that measures how hungry, thirsty, nauseous and full you feel. This will need to be completed before and 2-hours after breakfast, lunch and dinner, for one day and will be repeated every 3 weeks.

Once a week, you will meet with a member of the research staff at the GCRC for 1-2 hours, along with other participants in the study, to learn how to better control your diet and exercise, share experiences, and ask any questions you may have.

**Exercise Intervention:** During the 12-week program you will exercise 5 days each week for 50-60 minutes each day. The exercise will consist of walking/jogging on a treadmill and pedaling a stationary bike. The exercise sessions will be conducted at the Cleveland Clinic GCRC or the Fitness Center in the Walker Building. You will be instructed to keep your heart rate within a prescribed limit (85% of maximum) and you will be provided with a heart rate monitor and watch to make it easy to check your heart rate. You should notify the investigators if there is any change in your medications (prescription, over-the-counter, herbals) during the period of this study. All of your exercise sessions will be recorded in an exercise training log.

**Phase 4 (Post-Program Testing)**
At the end of the 12-week Diet/Exercise Program you will be admitted to the GCRC as an inpatient for a 3-day period. During this time we will again control your diet and activity level. You will then repeat the tests performed at the beginning of the program.

**Study Flowsheet:**

- **Screening**
  - Medical History
  - Resting 12-Lead EKG
  - Blood and Urine Chemistries
  - Exercise Stress Test
  - Oral Glucose Tolerance Test
  - Medical History, Dietary and Physical Activity Questionnaires

- **Randomization**
  - Low – Glycemic Group
  - High – Glycemic Group

- **Baseline Testing**
  - Diet / Weight Stabilization
  - Before Week 1
    - Clamp Procedure
    - Muscle Biopsy (Pre / Post Clamp)
    - Body Composition
    - CT Scan
    - DEXA
    - NMR Spectroscopy
    - VO2max

- **Intervention**
  - Diet Aerobic Exercise
  - Nutrition Counseling
  - Dietary Questionnaires

- **Post Testing**
  - Diet / Weight Stabilization
  - End of Week 12
    - Clamp Procedure
    - Muscle Biopsy (Pre / Post Clamp)
    - Body Composition
    - CT Scan
    - DEXA
    - NMR Spectroscopy
    - VO2max
How Long Will You Be In The Study?

You will be in the study for approximately 14-16 weeks.

2. RISKS AND DISCOMFORTS

What Are The Risks Of The Study?

Your participation in this study may involve the following risks:

Venipuncture/catheter placement (blood sampling): When a needle is inserted into a vein there will be some temporary pain and possible bruising. Infection is very rare as your skin is cleansed prior to needle insertion and only sterile needles are used. Some people also feel dizzy when they have their blood drawn.

Oral Glucose Tolerance Test: The sugar drink tastes very sweet and it can make some people feel nauseated or even vomit.

Exercise Testing on the Treadmill: You might experience tiredness or shortness of breath. You might feel like your heart is pounding very fast or very hard. You might feel dizzy or experience chest pain. You could stumble and fall off the treadmill. If you have any of these experiences, tell the research team. Also, when the breathing tube is in your mouth and you are wearing the nose clip, this might feel uncomfortable.

Indirect Calorimetry: For some people, the plastic canopy over their heads makes them feel anxious. This is temporary and the feeling will go away when the canopy is removed.

CT Scan: This technology uses radiation to take a picture of whatever is being scanned. The amount of radiation you will be exposed to in the CT scan is about what you receive naturally over the course of a year, when exposed to the general environment. Such doses of radiation may be potentially harmful, but the risks are so small that they are difficult to measure. If you have already had many x-rays, you should discuss the potential added risk from more radiation with the researchers before agreeing to be in the study.

DEXA Scan: You will be exposed to a small amount of radiation. The amount is similar to that which a person living in Ohio, would be exposed to from natural sources over a 2-month period. Such doses of radiation may be potentially harmful, but the risks are so small that they are difficult to measure. If you have already had many x-rays, you should discuss the potential added risk from more radiation with the researchers before agreeing to be in the study.

ECG: The procedure is generally painless and you will not be exposed to radiation. A small bit of adhesive might remain on your skin when the pads are removed. This adhesive is easily
removed with alcohol or soap and water.

**NMR Scan:** Because the NMR machine acts like a large magnet, it could move iron-containing objects in the room during your examination. Unexpected movement of such objects could possibly harm you. Precautions have been taken to minimize such an event from happening. Loose metal objects, like pocketknives or key chains, are not allowed in the NMR room. If you have a piece of metal in your body, such as a fragment in your eye, aneurysm clips, ear implants, spinal nerve stimulators, or a pacemaker, dental braces, or a cochlear implant, you cannot have an NMR. You must inform the research team if you have any of these implants in your body.

**Insulin Clamp Study:** You could react to the infusion of glucose or insulin. These reactions could include low blood sugar, an increase in blood pressure, flushing and/or sweating. When your hand is in the “hot box”, there is a slight risk of skin discomfort or burn. Tell the research team if your hand is uncomfortable when it is in the box.

**Questionnaires:** You might find it boring or time-consuming to complete the questionnaires. Also, some of the questions might be stressful. You don’t have to answer any question that makes you feel that way.

**Nutritional Counseling Sessions:** You might find the weekly counseling sessions time-consuming. You might feel uncomfortable talking about your diet, weight, or exercising in front of people you don’t know in the group setting.

**Tracer Study:** The isotopes are natural products and are not approved by the US Food and Drug Administration (FDA) for human use. They have been used in the research setting for many years by researchers studying metabolism, without adverse effects. They are sterilized (to kill bacteria and viruses) prior to use and tested for sterility prior to administration. There is a slight risk of an allergic reaction.

**Special Diet:** You might find it burdensome to adhere to a special diet. You might have to fight cravings to eat or drink items that are not part of the approved and provided research diet.

**Exercise Program:** You might find it burdensome to have to come to the Cleveland Clinic once a day, 5 days a week for 12 weeks, to do the exercise program. You might have to make special arrangements with work or family to have the time off to participate. The exercise might make you tired and your muscles might feel temporarily sore. You might also feel dizzy, short of breath or have chest pain, abnormal blood pressure or heart rhythm while exercising. You could stumble and fall off the treadmill or bike. If you have any of these experiences, tell the research team.

**Muscle Biopsy:** You will feel pain, cramping, or bleeding where the sample is taken. Infection is very rare as your skin is cleansed with alcohol and the needle used is sterile. It is very rare, but you could have an allergic reaction to the lidocaine that is used to numb your skin. Tell the research team if you are allergic to any drugs in the “-caine” family (for example, lidocaine, novocaine).
Activity is good for your muscle after the biopsy. Walking is required after the biopsy procedure to help prevent additional stiffness and blood clot formation. (The development of a blood clot is related to inactivity, and may occur in less than 1% of biopsy procedures). A nurse or one of the investigators will call you within 24 hours to follow-up on how your biopsy is healing and to ask if you are having any pain or discomfort that might limit your activity.

**Body Fat Measurements with Calipers:** It is common to feel slight, temporary discomfort when the calipers pinch your skin.

**Urine collection:** there are no risks to collecting a urine sample in a sterile cup.

Additionally, there may be risks that we do not know about.

### 3. BENEFITS

**Are There Benefits To Taking Part In The Study?**

Benefits include knowledge of current health status as determined by medical history and physical examination, assessment of current physical fitness status, improved level of fitness, improved knowledge of nutrition and weight control, and access to personal data collected during the study. Your glucose tolerance may improve as a result of participating in this study.

The benefit to society will include new knowledge pertaining to the effects of a high-or low-glycemic diet combined with exercise on control of blood sugar. Data from this study may help to determine the most effective dietary components to prevent the development and/or help in the treatment of type 2 diabetes.

### 4. ALTERNATIVES

**What Other Options Are There?**

This is a research project. You may decline to participate. The procedures described in this consent form are not needed for your medical care and they do not treat or cure diabetes or obesity. We do not guarantee that you will lose weight if you participate in this research study.

### 5. PRIVACY AND CONFIDENTIALITY

**Will Your Information Be Kept Private?**

The medical and research information recorded about you will be used within the Cleveland Clinic and/or disclosed outside the Cleveland Clinic as part of this research. Tests and procedures done solely for this research study may be placed in your medical record to indicate
your participation in this study. Upon completion of the study, you may have access to the research information if contained in the medical record.

Your access to research information about you will be limited while the study is in progress. Preventing this access during the study keeps the knowledge of study results from affecting the reliability of the study. This information will be available should an emergency arise that would require your treating physician to know this information to treat you best.

Your research information may be disclosed to the National Institutes of Health, the research study Sponsor and its agents, the Cleveland Clinic research review staff, the U.S. Food and Drug Administration, and other outside collaborators or laboratories that are participating in this study, if any, that are listed as follows: Department of Health and Human Services Agencies, National Committee for Quality Assurance. The Cleveland Clinic also may use and disclose this information for treatment and payment reasons. The Cleveland Clinic must comply with legal requirements that mandate disclosure in unusual situations. Otherwise, the information recorded about you as part of this research will be maintained in a confidential manner. It is possible that information disclosed about you outside the Cleveland Clinic could be re-disclosed and no longer protected by federal privacy laws.

Your research information may be used and disclosed indefinitely, but you may stop these uses and disclosures at any time by writing to John Kirwan, PhD or Latina Brooks, PhD, at The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, Ohio 44195. If you do so, any information previously disclosed cannot be withdrawn. The Cleveland Clinic will not use or disclose the information collected in this study for another research purpose without your written permission, unless the Cleveland Clinic Institutional Review Board gives permission after ensuring that appropriate privacy safeguards are in place. The Institutional Review Board is a committee whose job is to protect the safety and privacy of research subjects.

If you choose not to sign this consent form, you will not be permitted to participate in this research study.

6. RESEARCH RELATED INJURIES

What Happens If An Injury Occurs?

If physical injury occurs due to your involvement in this research, medical treatment is available, but your medical insurance must pay the cost of treatment. Such medical treatments that are not covered by your medical insurance shall not be paid by the sponsor, the National Institutes of Health. Compensation for lost wages and/or direct or indirect losses are not available. The Cleveland Clinic will not voluntarily provide compensation for medical expenses or any other compensation for research-related injuries. Further information about research-related injuries is available by contacting the Institutional Review Board at (216) 444-2924.
7. COSTS

What Are The Costs?

There is no cost to you or your insurance company for participation in this study.

The sponsor, NIH, will pay for the procedures and extra study specific tests that are not routine and only being performed because you are participating in this study. The Cleveland Clinic will not pay for the costs of procedures, tests, visits and hospitalizations in connection with this study.

For your participation in this study you will be compensated $500, made in four payments of $125. The first payment will be made after completion of the initial set of tests and prior to beginning the exercise period. The second and third payment will be provided on weeks 6 and 12 of the exercise-training program. The fourth payment will be provided on completion of all tests associated with the post-exercise period. You may choose to receive all of the money in a single payment at the end of the study.

8. VOLUNTARY PARTICIPATION

What Are Your Rights As A Participant?

Taking part in this study is voluntary. You may choose not to take part or may leave the study at any time. Leaving the study will not result in any penalty or loss of benefits to which you are entitled.

We will tell you about new information that may affect your health, welfare, or willingness to stay in this study.

9. QUESTIONS

Whom Do You Call With Questions Or Problems?

If you have any questions about the research or develop a research-related problem, you should contact:

John Kirwan, PhD at 216-444-3412

Latina Brooks, PhD at 216-444-3445

After hours: Call GI Fellow at 216-444-2000 pager #25555

If you have questions about your rights as a research subject, you should contact the Institutional Review Board at (216) 444-2924.

Cleveland Clinic Foundation General Clinical Research Center (GCRC) study participants may also contact the GCRC Research Subject Advocate (RSA), Katrina A. Bramstedt, PhD at 216-
444-0204 or 216-444-2200 and ask the operator to page beeper #25339 with regard to questions about study participation and research subject protections.

10. SIGNATURE

I have read and have had verbally explained to me the above information and have had all my questions answered to my satisfaction. I understand that my participation is voluntary and that I may stop my participation in the study at any time. Signing this form does not waive any of my legal rights. I understand that a copy of this consent will be provided to me. By signing below, I agree to take part in this research study.

_____________________________  ___________
Subject Signature Date

_____________________________
Printed name of Subject

Statement of Person Conducting Informed Consent Discussion

I have discussed the above points with the subject. It is my opinion that the subject understands the risks, benefits, and procedures involved with participation in this research study.

_____________________________  ___________
Signature of person obtaining consent Date

_____________________________
Printed name of person obtaining consent
Study Title: Effects of a CrossFit lifestyle intervention on insulin sensitivity, cardiometabolic risk factors, and quality of life in obese type 2 diabetic individuals

Principal Investigator: John P. Kirwan, Ph.D.

Carefully review this consent document. The purpose of a consent document is to provide you with information to help you decide whether you wish to participate in research. Your decision is completely voluntary and will not affect your medical care if you choose not to participate. It is important for you to ask questions and understand the research risks, benefits and alternatives.

Please note:
- You are being asked to participate in a research study
- Carefully consider the risks, benefits and alternatives of the research
- Your decision to participate is completely voluntary

Your doctor may be an investigator in this research study, and as a research investigator, is interested in both your welfare and in the conduct of the research study. Before entering this study or at any time during this research, you may ask for a second opinion about your care from another doctor who is not involved with the research study. You are not under any obligation to participate in any research project offered by your doctor.

Conflict of Interest Disclosure:
One or more of the investigators conducting this study serve as paid speakers, consultants or advisory committee members for the company that is paying for this research or a company that makes products used in this study. These financial interests are within permissible limits established by the Cleveland Clinic Conflict of Interest Policy. If you have any questions regarding conflicts of interest, please ask your study doctor or call the Institutional Review Board at 216-444-2924.

1. INFORMATION ON THE RESEARCH

Why Are You Being Asked To Take Part In This Research?
You are being asked to participate in this research study because you have been diagnosed with type 2 diabetes. Exercise and dietary intervention are well-established treatment methods for type 2 diabetes. Before you can decide whether or not to volunteer for this study, you must understand the purpose of this study, how this study may help you, any risks to you, and what is expected of you. This information gathering process is called informed consent. The study is being conducted through the Department of Pathobiology at the Cleveland Clinic.

Why Is This Study Being Done?
The purpose of this study is to evaluate the effect of a CrossFit lifestyle intervention on insulin sensitivity in overweight and obese men and women with type 2 diabetes. The CrossFit lifestyle
intervention involves exercise training and dietary instructions. Exercise and diet are known treatments for type 2 diabetes; however, many patients find it difficult to adhere to recommended regimens of aerobic and resistance exercise training. CrossFit combines aerobic and resistance training in high-intensity workouts utilizing a variety of movements such as Olympic weightlifting and body-weight and endurance exercises. One example of a CrossFit workout is seen below:

*Spend 1 minute at each station and complete as many repetitions as possible. After completing the 3 stations, rest for 1 minute. Repeat entire cycle 5 times and record total repetitions:*

- Row (1 calorie = 1 repetition)
- Sit-up
- Squat

CrossFit affiliate gyms around the world provide exercise and dietary guidance to members, yet this program has not been investigated for those with type 2 diabetes.

This study will investigate how CrossFit lifestyle interventions impact individuals with type 2 diabetes regarding:

- The regulation of glucose (sugar) by insulin.
- The changes that may occur in risk factors for cardiac and metabolic disease
- The changes that may occur in aerobic fitness.
- The changes that may occur in quality of life

How Many People Will Take Part In The Study?

About 12 people will take part in this study.

What Is Involved In The Study?

Phase 1: (Screening)

These tests may be performed over 1 to 3 days depending on your schedule. The order of these tests may vary. As part of the screening you will complete a physical examination, medical history, resting electrocardiogram (ECG, a non-invasive measure of the electrical activity of your heart), blood sample (13 cc, or 2½ teaspoons), and an exercise stress test. These procedures will be done in the Clinical Research Unit (Unit M51) of the Cleveland Clinic. All procedures are described below:

Medical History and Physical (approximately 1 hour): You will have a medical history and physical exam, and a resting electrocardiogram.

Questionnaires (60 - 90 minutes): You will be asked to complete 3 questionnaires, described below. These questionnaires may be administered at various times during the study depending on your schedule.

Medical History: You will complete a checklist about current and past medical information.

Minnesota Leisure Time Physical Activity: You will complete a checklist of 60 physical or recreational
activities you may have participated in over the last 12 months.

Seven Day Physical Activity Recall: You will be asked 9 questions about your sleep and physical activity in the past week.

Urine Pregnancy Test: You will be asked to provide a urine sample for a pregnancy test if you are a female between the ages of 18 and 60 years old.

Exercise Stress Test (approximately 1 hour): This test evaluates whether your heart is healthy and safe for exercise. You will be encouraged to exercise as hard as you can, but safely. During this test, adhesive pads will be attached to your chest and leads (wires) will be connected to a machine that measures the electrical activity in your heart (cardiac monitor). The procedure is generally painless. Males that are particularly “hairy” in the chest area may need to have their chests shaved in order to ensure proper conductivity of the leads.

Next, you will walk on a treadmill, with the incline (slope/angle) progressively increased, until fatigue, breathlessness, and/or symptoms indicate to the medical staff or yourself, that you should stop the exercise.

If the cardiologist from this research study reviews your exercise stress tests and determines you are heart healthy to exercise, you will be eligible to participate in the study.

Phase 2: (Baseline Testing)

These baseline tests will be performed once before and once after the 6-week CrossFit program. These tests will be performed in the Clinical Research Unit (Unit M51) of the Cleveland Clinic.

You should refrain from taking any medications (including diabetic medications) 24 hours before this pre-program testing. You should bring your medications with you to take after testing is complete. You will be provided with a standardized mixed meal (55% carbohydrate, 30% fat, 15% protein) that you should consume the evening before testing. You will need to be fasted at the time of your arrival, which means nothing to eat or drink (except for sips of water) for at least 12 hours before the test. You cannot drink alcohol or eat or drink foods that contain caffeine for at least 24 hrs prior to testing. Common foods containing caffeine include: coffee, tea, soda and chocolate. If you have questions on foods containing caffeine, please ask one of the team members prior to your diet standardization period. In addition, we ask that you refrain from any structured exercise for 24 hrs prior to testing and limit exercise to your normal everyday activities.

In addition to obtaining your resting heart rate and blood pressure, during this time you will be asked to complete a series of tests that include: (1) Body Composition Assessment; (2) Exercise Capacity Test, (3) Metabolic Tests, and (4) Questionnaires.

1. Body Composition:
**Body Mass Index (BMI):** To determine your body mass index, a measure commonly used to assess obesity, we will measure your body weight as well as your height.

**Waist to Hip Ratio (W:H):** To determine if you have a healthy waist to hip ratio, we will perform three measurements. We will use a tape measure to assess the circumference of your hips at the widest part of your buttocks. Then two other measurements will be performed just under the ribs (natural waist) and at the level of the belly button (umbilicus).

The accumulation of body fat in specific body regions has been linked to health problems. For example, people with "apple-shaped" bodies (more weight around the waist) face more health risks than people with "pear-shaped" bodies who carry more weight around the hips.

**Dual Energy Absorptiometry (DXA) (approximately 30 minutes):** This test is used to measure the amount of fat distributed throughout your whole body and will allow us to measure your percent body fat. During this test, you will lay on your back on a table for 10-15 minutes while a scanner passes over your body. This test also involves a very small amount of radiation exposure.

2. **Exercise Capacity Test (approximately 30 minutes):**

   This test is used to evaluate your current level of physical fitness. You will be encouraged to walk on a treadmill for as long as you can, safely. During this test you will breathe into a mask with a tube attached (pneumotach) so that all of the air you breathe in and out will pass through the pneumotach and into a machine alongside the treadmill. The machine allows us to measure your oxygen consumption and provides a measure of your maximum oxygen capacity (VO$_{2\text{max}}$), or maximum exercise capacity. During this test your heart’s activity will be monitored with an ECG (as described above) and blood pressure measurements will be taken frequently. You will walk on a treadmill, with the incline progressively increased, until fatigue, breathlessness, and/or symptoms indicate to the medical staff, or yourself, that you should stop the exercise. The pictures below show what a typical test looks like.

3. **Metabolic Tests:**
**Indirect Calorimetry (approximately 30 minutes):** This test will calculate your resting metabolic rate (the amount of energy that your body requires over 24 hours while at rest). We will place you in a hospital bed or a reclining chair in the CRU. Once you are comfortable, a clear plastic hood with two tubes will be placed over your head. One tube allows fresh air to flow in while another tube sends the air that you breathe out into an instrument that measures oxygen and carbon dioxide. We will ask you to breathe normally under the hood for approximately 15-20 minutes. A computer then analyzes your oxygen and carbon dioxide readings and calculates your resting metabolic rate. This will allow us to determine your body’s fuel preference. The following photo shows the test device:

![Test Device Image]

**Urine Collection:** You will be asked to supply a urine sample for a pregnancy test if you are a female between the ages of 18 and 60 years old.

**Blood Draw:** A blood sample (13 cc, or 2½ teaspoons) will be drawn from your arm in order to measure levels of metabolic factors and lipids in your blood.

**Oral Glucose Tolerance Test (approximately 3 hours):** You will be asked to come to the Clinical Research Unit (Unit M51) on the morning of the test. You will need to be fasted at the time of your arrival, which means nothing to eat or drink (except for sips of water) for at least 12 hours before the test. This is a standard 3-hour test that is used to detect diabetes. An IV will be placed into a vein in your arm. This IV will remain in place for about 3 hours. Once the IV is in place, an initial blood sample will be taken.

Once this sample has been obtained, you will drink an orange flavored beverage that contains a known amount of glucose (sugar). Over the next 3 hours, we will measure your body's response to the glucose (oral glucose tolerance test, OGTT). Blood samples will be taken again at ½, 1, 1½, 2 and 3 hours. The amount of blood that will be obtained for this test will be approximately 110 cc (or 7.5 tablespoons).

**4. Questionnaires (60 - 90 minutes):** You will be asked to complete 2 questionnaires, described below. These questionnaires may be administered at various times during the study depending on your
schedule.

24 Hour Diet Record: You will be asked to record the amount of foods and beverages and the time they were consumed over a 24-hour period. You will then be asked to repeat this for 3 days, including two weekdays (Monday-Friday) and one weekend day (Saturday or Sunday).

SF-36 Quality of Life Questionnaire: You will be asked to complete a 36-item questionnaire assessing your physical and mental health. Four additional questions will be added to this questionnaire after the CrossFit lifestyle intervention to assess your satisfaction with the program.

Phase 3: 6 Weeks of CrossFit Lifestyle Intervention (Intervention Period)

Following the baseline testing phase you will begin the intervention phase which will consist of 6 weeks of CrossFit training. You will attend a local CrossFit affiliate gym, Great Lakes CrossFit (http://greatlakescrossfit.com/) which is located at 5075 Taylor Drive Bedford Heights, OH 44128. As standard procedure with any new member of Great Lakes CrossFit, you will also be required to sign a waiver of liability before participating in exercise training at this affiliate gym. There, certified CrossFit trainers will lead groups of 2-6 subjects at a time in three CrossFit exercise training sessions per week each lasting one hour in duration. These training sessions will include a warm-up, skill work, and one high-intensity workout (ideally > 85% HR maximum). Over the course of six weeks, you will be exposed to many different weightlifting, gymnastics, and endurance exercises. Workouts will also vary in length. After each session, the CrossFit trainers will record the weights you used, the time it took you to perform the workout, and the number of repetitions you completed in a training log. The workout you perform in the second session will be repeated in the last session to assess your improvements over 6 weeks. You will be asked to wear heart rate (HR) monitors during each training session. In addition to the exercise training, the CrossFit trainers will provide you with information about the Paleolithic diet and guidance for implementing this diet. Briefly, you will be counseled to eat a diet of lean meats, vegetables, nuts, and seeds. You will be asked to complete the 24 Hour Diet Record (described above) again at weeks 2, 4, and 6 during this period. You will also be encouraged to join the existing community of Great Lakes CrossFit and take part in its social events (i.e. potluck dinners) and online community (i.e. sharing of workouts and recipes). This is not necessary for the research.

Phase 4: (Post-Intervention Testing)

At the end of the 6-week intervention period you will again be asked to consume a standard evening meal that we will provide and undergo a 12-hour fast before testing. You will then repeat the tests performed at the beginning of the program as described previously in Phase 2. These tests will be performed in the Clinical Research Unit (Unit M51) of the Cleveland Clinic.

The research team will also contact you by telephone 6 months after completion of the study in order to determine whether you have continued with CrossFit or an exercise and/or dietary regimen, why you have or have not continued, and any other information you would like to share about your experience.
Study Schematic:

**Screening**
- Medical History and Physical Exam
- Resting 12-lead EKG
- Exercise Stress Test
- Blood and Urine Chemistries (CMP, Lipid, CBC, TSH, HbA1c, Pregnancy)
- Activity Questionnaires

**Baseline Testing**
- Oral Glucose Tolerance Test
- VO_{2max}
- Blood and Urine Chemistries (CMP, Lipid, CRP, UUN)
- Indirect Calorimetry
- Resting HR, BP
- Body Composition (BMI, W:H, DXA)
- Nutrition & QOL Questionnaires

**CrossFit Lifestyle Intervention**
- CrossFit Exercise
- Nutrition Counseling and Questionnaires

**Post-Intervention Testing**
- Oral Glucose Tolerance Test
- VO_{2max}
- Blood and Urine Chemistries (CMP, Lipid, CRP, UA)
- Indirect Calorimetry
- Resting HR, BP
- Body Composition (BMI, W:H, DXA)
- Nutrition & QOL Questionnaires
- 6 Month Follow-Up

**How Long Will You Be In The Study?**
Your participation in this study will last 8-10 weeks.
2. RISKS AND DISCOMFORTS

What Are The Risks Of The Study?

Your participation in this study may involve the following risks:

**Venipuncture/catheter placement (blood sampling):** When a needle is inserted into a vein there will be some temporary pain and possible bruising. Infection is very rare as your skin is cleansed prior to needle insertion and only sterile needles are used. Some people also feel dizzy when they have their blood drawn.

**Pregnancy Test (Urine HCG):** There are no known risks related to testing your urine.

**Insulin Sensitivity Tests:** You could react to the ingestion of glucose. These reactions could include nausea, low blood sugar, an increase in blood pressure, flushing and/or sweating.

**Exercise Testing on the Treadmill:** You might experience fatigue or shortness of breath. You might also feel like your heart is pounding very fast or very hard and experience dizziness or chest pain. You could stumble and fall off the treadmill. If you have any of these experiences, tell the research team. Also, when the mask is on your face, this might feel uncomfortable.

**Indirect Calorimetry:** For some people, the plastic canopy over their heads makes them feel claustrophobic or anxious. This feeling is temporary and will go away when the canopy is removed. The canopy is “see-through” and does not restrict movement.

**Questionnaires:** You might find it boring or time-consuming to complete the questionnaires. There is the potential risk of loss of confidentiality. Every effort will be made to keep your information confidential, however, this cannot be guaranteed. Some of the questions we will ask you as part of this study may make you feel uncomfortable.

**Cumulative Radiation Exposure from DXA scans:** One of the risks associated with radiation exposure is cancer. The natural incidence of fatal cancer in the U.S. is about 1 chance in 5. Everyday radiation exposure from natural occurring background radiation (sun, radon exposure in the home) is approximately 3mSv per year. In this research study, you will be receiving four DXA scans. Four DXA scans amount to 0.004 mSv. This radiation exposure risk is equal to less than two days of natural background radiation or what you might experience from an airplane flight of 2 or more hours. This amount of radiation is so low as to make an accurate risk estimate meaningless. There is also no chance for skin injury. If you have already had many x-rays you should discuss the potential added risk from more radiation with the researchers before agreeing to be in the study.

**ECG:** A small amount of adhesive might remain on your skin when the pads are removed or the adhesive might pull on hair in the area. The adhesive is easily removed with alcohol or soap and water.

**Exercise Program:** You might find it taxing or burdensome to have to travel to the CrossFit affiliate gym 3 times per week for 6 weeks to complete the exercise program. You might have to make special arrangements with work or family to have the time off to participate. The exercise might result in fatigue and muscle soreness. You might also experience discomfort, dizziness, shortness of breath,
chest pain, abnormal blood pressure or abnormal heart rhythms while exercising. You could stumble and fall or drop equipment on yourself while exercising. If you have any of these symptoms/experiences, tell the CrossFit trainers and research team immediately.

**Unforeseen Risks:** There may be risks related to the study that are unknown at this time. You will be notified of any significant new findings that become known that may affect your willingness to continue in the study.

3. **BENEFITS**
   **Are There Benefits To Taking Part In The Study?**

   Benefits include obtaining knowledge of current health status as determined by medical history and physical examination, assessment of current physical fitness status, improved knowledge of exercise, and access to personal data collected during the study. Your insulin sensitivity may improve as a result of participating in this study and you may experience other improvements in health status such as weight loss, but these changes cannot be guaranteed.

   Societal benefits may include new knowledge for developing lifestyle interventions to decrease insulin resistance in type 2 diabetic individuals. Information from this study may help to determine the most effective strategies to prevent the development and/or help in the treatment of obesity-related insulin resistance.

4. **ALTERNATIVES**
   **What Other Options Are There?**

   This is a research project. You may decline to participate. The procedures described in this consent form are not needed for your medical care and they do not treat or cure diabetes or obesity.

5. **PRIVACY AND CONFIDENTIALITY**

   The medical and research information recorded about you will be used within the Cleveland Clinic and/or disclosed outside the Cleveland Clinic as part of this research. Tests and procedures done solely for this research study may be placed in your medical record to indicate your participation in this study. Upon completion of the study, you may have access to the research information if contained in the medical record.

   Your access to research information about you will be limited while the study is in progress. Preventing this access during the study keeps the knowledge of study results from affecting the reliability of the study. This information will be available should an emergency arise that would require your treating physician to know this information to treat you best.

   Your research information may be disclosed to the Cleveland Clinic research review staff, the U.S. Food and Drug Administration, and other outside collaborators or laboratories that are participating in this study, if any, that are listed as follows: Department of Health and Human Services Agencies, National Committee for Quality Assurance. The Cleveland Clinic also may use and disclose this information for treatment and payment reasons. The Cleveland Clinic must comply with legal requirements that mandate disclosure in unusual situations. Otherwise, the information recorded about
you as part of this research will be maintained in a confidential manner. It is possible that information disclosed about you outside the Cleveland Clinic could be re-disclosed and no longer protected by federal privacy laws.

Your research information may be used and disclosed indefinitely, but you may stop the use and disclosure of personal information at any time by writing to John Kirwan, PhD at Cleveland Clinic, 9500 Euclid Avenue, Cleveland, Ohio 44195. If you do so, any information previously disclosed cannot be withdrawn. The Cleveland Clinic will not use or disclose the information collected in this study for another research purpose without your written permission; unless the Cleveland Clinic Institutional Review Board gives permission after ensuring that appropriate privacy safeguards are in place. The Institutional Review Board is a committee whose job is to protect the safety and privacy of research subjects.

If you choose not to sign this consent form, you will not be permitted to participate in this research study.

6. RESEARCH RELATED INJURIES  
   What Happens If An Injury Occurs?

In the event you are injured as a result of participation in this research, medical care is available to you. The costs of such medical care will be billed to you or your insurance company. There are no plans to provide compensation for lost wages, direct or indirect losses. The Cleveland Clinic will not voluntarily provide compensation for research related injury. You are not waiving any legal rights by signing this form. Further information about research related injury is available by contacting the Institutional Review Board at 216-444-2924. As standard procedure with any new member of Great Lakes CrossFit, you will also be required to sign a waiver of liability before participating in exercise training at this affiliate gym.

7. COSTS  
   What Are The Costs?

There is no cost to you or your insurance company for participation in this study.

The sponsor will pay for the procedures and extra study specific tests that are not routine and only being performed because you are participating in this study. The Cleveland Clinic will not pay for the costs of procedures, tests, visits and hospitalizations in connection with this study.

For your participation in this study you will be compensated $150.00. You will receive $50 for the completion of baseline testing and the remaining $100 for the completion of the post-intervention testing. If you withdraw from the study before the pre-program (Phase 2) testing is complete, you will not receive compensation.

8. VOLUNTARY PARTICIPATION  
   What Are Your Rights As A Participant?
Taking part in this study is voluntary. You will be told of any new, relevant information from the research that may affect your health, welfare, or willingness to continue in this study. You may choose not to take part or may leave the study at any time. Withdrawing from the study will not result in any penalty or loss of benefits to which you are entitled. If you decide to withdraw from the study you should discuss with your study doctor your decision to ensure a safe withdrawal.

9. QUESTIONS
Whom Do You Call With Questions Or Problems?

If you have any questions about the research or develop a research-related problem, you should contact:

John Kirwan, Ph.D.  216-444-3412

Julianne Filion, BSN, RN  216-445-5553

Julie Foucher  248-767-0284

After hours:

Julie Foucher  248-767-0284

If you have questions about your rights as a research subject, you should contact the Cleveland Clinic Institutional Review Board at (216) 444-2924.

Cleveland Clinic Foundation Clinical Research Unit (CRU) study participants may also contact the CRU Research Subject Advocate (RSA), Lara Danziger-Isakov, M.D. at 216-636-1077 or 216-444-2200 and ask the operator to page beeper # 28152 with regard to questions about study participation and research subject protections.

10. SIGNATURE

Statement of Participant

I have read and have had verbally explained to me the above information and have had all my questions answered to my satisfaction. I understand that my participation is voluntary and that I may stop my participation in the study at any time. Signing this form does not waive any of my legal rights. I understand that a copy of this consent will be provided to me. By signing below, I agree to take part in this research study.

______________________________
Printed name of Participant

______________________________
Statement of Person Conducting Informed Consent Discussion

I have discussed the information contained in this document with the participant and it is my opinion that the participant understands the risks, benefits, alternatives and procedures involved with this research study.

________________________________________
Printed name of person obtaining consent

________________________________________
Signature of person obtaining consent   Date