From PHYSIOLOGY AND PHARMACOLOGY
Karolinska Institutet, Stockholm, Sweden

REGULATORS OF GLUCOSE AND LIPID METABOLISM IN SKELETAL MUSCLE AND SERUM

IMPLICATIONS FOR OBESITY AND TYPE 2 DIABETES

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Stockholm 2012

Published by Karolinska Institutet. Printed by Karolinska University Service US-AB.

ISBN 978-91-7457-952-9

Happiness lies in the joy of achievement and the thrill of creative effort.
Franklin D. Roosevelt.

To my Wife

ABSTRACT

Type 2 diabetes mellitus (T2DM) has become a growing worldwide problem of public health importance. Insulin resistance is commonly associated with obesity and a key factor mediating the progression to T2DM. The failure of insulin-sensitive peripheral tissues to respond to insulin results in an increase in serum glucose levels that leads to an impaired homeostatic state. Skeletal muscle plays a crucial role in maintaining glucose metabolism. Impairments in both glucose and lipid metabolism arising from a dysregulation of hormones, free fatty acids, or other factors contribute significantly to the pathogenesis of T2DM.

The roles of several circulating metabolites in the development of insulin resistance have been described. However the molecular mechanisms involved in skeletal muscle insulin resistance remain poorly defined. Furthermore, the biological interactions between skeletal muscle, novel circulating factors, and lifestyle factors such as exercise in the regulation of glucose and lipid metabolism need to be investigated. This thesis aims at examining the role of novel regulators of glucose and lipid metabolism, uncovering the molecular targets involved in the development of skeletal muscle insulin resistance, and describing their clinical implications in obesity and T2DM.
Physical exercise has beneficial effects on glucose and lipid metabolism and hence improves cardiovascular risk factors. In Study I, we report differential effects of Nordic walking (low-moderate intensity exercise) on cardiovascular risk factors in normal and impaired glucose tolerant individuals. We provide evidence to support the recommendation of a more intense and supervised exercise modality for significant improvements in cardiovascular risk factors.

Fibroblast growth factor (FGF)-21 is a member of the FGF family that plays a role in a variety of endocrine functions, including the regulation of glucose and lipid metabolism. Observations from animal models have suggested a potential therapeutic role of this growth factor in T2DM. In Study II, we provide evidence for direct effects of FGF-21 in skeletal muscle glucose uptake. Using cell-surface photolabeling of human myotubes, we report enhanced glucose transporter-1 abundance at the cell membrane, coincident with increased basal and insulin-stimulated glucose uptake. We further confirm a paradoxical increase in serum FGF-21 in T2DM in humans, and identify BMI as the strongest independent predictor of FGF-21 serum levels. The mechanisms controlling the metabolic actions of FGF-21 are currently being resolved.

Signal transducer and activator of transcription factor 3 (STAT3) is involved in cytokine- and nutrient-induced insulin resistance. The role of STAT3 in the development of skeletal muscle insulin resistance and T2DM pathogenesis is incompletely defined. In Study III, we report an increased STAT3 phosphorylation in T2DM. Using palmitate and STAT3 specific siRNA treatment of myotubes *in vitro*, we provide evidence for the role of STAT3 in the development of lipid-induced skeletal muscle insulin resistance.

Collectively, the work presented in this thesis contributes to the understanding of various regulators of glucose and lipid metabolism from the whole body physiology context to molecular mechanisms in skeletal muscle. Metabolic alterations result from the interplay between biological processes within the cells, tissues and organs. These alterations may translate into ill health such as T2DM. Information from Translational studies like the ones presented in this thesis will help to identify molecules with both clinical significance and therapeutic potential.

LIST OF PUBLICATIONS

I. Fritz T, Caidahl K, Krook A, Lundström P, **Mashili F**, Osler M, Szekeres F, Östenson CG, Wändell P, Zierath JR. Effects of Nordic walking on cardiovascular risk factors in overweight individuals with type 2 diabetes, impaired or normal glucose tolerance. Diabetes Metab Res Rev. Aug 8 2012. doi: 10.1002/dmrr.2321. [Epub ahead of print].

II. **Mashili FL**, Austin RL, Deshmukh AS, Fritz T, Caidahl K, Bergdahl K, Zierath JR, Chibalin AV, Moller DE, Kharitonenkov A, Krook A. Direct effects of FGF-21 in human skeletal muscle: implications for type 2 diabetes and obesity. Diabetes Metab Res Rev 27:286-97, 2011.

III. **Mashili FL**, Chibalin AV, Krook A, Zierath JR. Constitutive STAT3 phosphoryation in skeletal muscle contributes to skeletal muscle insulin resistance in type 2 diabetes. *In press* Diabetes Oct 5 2012 [Epub ahead of print].

CONTENTS

LIST OF ABBREVIATIONS

2-DOG	2-Deoxyglucose
AMP	Adenosine monophosphate
AMPK	Activated protein kinase
AS160	Akt substrate of 160 kDa
ATP	Adenosine triphosphate
BMI	Body mass index
caMKK	Ca^{2+}/calmodulin-dependent protein kinase kinase
C-NMR	Carbon-nuclear magnetic resonance
EDL	Extensor digitorum longus
FFA	Free fatty acids
FGF	Fibroblast growth factor
GAPDH	Glyceraldehyde phosphate dehydrogenase
GLUT	Glucose transporter
HOMA-IR	Homeostatic model of assessment for insulin resistance
IDF	International diabetes federation
IGT	Impaired glucose tolerance
IRS	Insulin receptor substrate proteins
JAK	Janus activated kinase
mTOR	Mammalian target of rapamycin
NGT	Normal glucose tolerance
OGTT	Oral glucose tolerance test
PBS	Phosphate buffered saline
PI3K	Phosphatidylinositol 3 kinase
PKB	Protein kinase B (or Akt)
PKC	Protein kinase C
siRNA	Small interfering ribonucleic acid
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activator of transcription factor
T2DM	Type 2 diabetes mellitus
WHO	World health organization

1 INTRODUCTION

1.1 EPIDEMIOLOGY

The prevalence of diabetes has increased rapidly all over the world. According to the International Diabetes Federation (IDF), the number of people with diabetes in the world is estimated to be 346 million. This number is projected to grow to 552 million by the year 2030 (Whiting et al., 2011). Type 2 diabetes (T2DM) accounts for 90% of all diabetes cases. Therefore, the continuously growing epidemic reflects an increased incidence and prevalence of T2DM. Epidemiologically, T2DM is well-documented in populations in the United States and Europe, notably among Native Americans, Pacific Islanders, people of Asian Indian origin, Hispanics and African Americans. The prevalence of T2DM grew substantially in these populations and ethnic groups during the 20th century (Acton et al., 2003; Harris et al., 1998). Increased intake of high-caloric foods and physical inactivity has contributed to the development and progression of T2DM (Berlin and Colditz, 1990; Powell et al., 1987).

Increased incidence of obesity is closely linked to the rising prevalence of T2DM. Between 70 to 90 percent of T2DM is attributable to obesity. Approximately 197 million people have impaired glucose tolerance globally, mostly due to obesity and the associated metabolic syndrome. Obesity and T2DM are rare in communities where a traditional lifestyle has been preserved (Fall, 2001; King and Rewers, 1993; Swai et al., 1993a; Swai et al., 1993b). By contrast, urbanization along with westernization has become a major driving force for these co-morbidities, mostly in developing countries where the rate of growth of T2DM is rapid (Fall, 2001; Haslam and James, 2005; Wild et al., 2004). Prevention of obesity is therefore an early strategy to slow the rapidly growing prevalence of T2DM and its associated cardiovascular complications, which exert a great socio-economic toll on affected countries.

The serious cardiovascular complications of obesity and diabetes are overwhelming. In developing countries, this creates a double burden of disease since these countries are already straining under the burden of communicable diseases (Ramaiya, 2005). The risk of cardiovascular complications is higher among obese people (Lee, 2003; Wannamethee et al., 2011c; Wild et al., 2004), and the effect of diabetes on cardiovascular complications is more severe among people of most ethnic minorities in western countries and in the populations of the developing countries, where an increased waist-to-hip ratio strongly predicts ischemic heart disease and stroke (Lee, 2003; Wannamethee et al., 2011b, c). Approximately 3.6% deaths per year are attributable to diabetes complications (Barcelo et al., 2003). Around 2.5% to 25% of the annual health care budgets are directed to direct health care costs for diabetes and according to estimates derived from a number of Latin America countries, the indirect costs might be as much as five times the direct costs (Barcelo et al., 2003).

1.2 OBESITY AND T2DM

Generally overweight and obesity are defined as abnormal or excessive fat accumulation that can cause health problems. According to the World Health Organization (WHO), individuals with a body mass index (BMI) greater or equal to 30 kg/m^2 are considered obese, while those with BMI greater or equal to 25 kg/m^2 are categorized as overweight (Alberti et al., 1998; Klein et al., 2007; Pi-Sunyer et al., 1998). Waist circumference, a good surrogate marker for visceral fat, may reflect obesity. However due to different standards on how to measure it, the use of waist circumference in the definition of obesity is debatable (Klein et al., 2007). Different criteria are used in the diagnosis of T2DM, but in general, the oral glucose tolerance

test (OGTT) has been widely employed in the clinical diagnosis of T2DM. The choice of detection method is of particular relevance in T2DM diagnosis since, in the earlier stages of disease progression, individuals with "pre-diabetes" can present normal fasting glucose values. With an OGTT, impaired glucose tolerance is easily apparent from an abnormal response following a glucose challenge. Furthermore, an OGTT provides both the fasting and postprandial glucose levels, which reflect liver and skeletal muscle phenotypes, respectively. WHO defines diabetes as fasting plasma glucose ≥ 7.0 mmol/l (126 mg/dl) or plasma glucose ≥ 11.1 mmol/l (200 mg/dl) 2 hours after an oral glucose load.

1.3 PATHOPHYSIOLOGY AND NATURAL HISTORY OF T2DM

T2DM is a chronic, progressive disease characterized by hyperglycemia, insulin resistance, and importantly, decreased β-cell number and secretory function (Harris et al., 1998; Haslam and James, 2005; Wild et al., 2004). It progresses from an early 'pre-diabetes' stage, which is often asymptomatic, with insulin resistance, to a relatively mild postprandial hyperglycemia, before ultimately developing into overt diabetes, requiring pharmacological intervention. A clear understanding of the pathogenesis of T2DM is a cornerstone to its management. Effective treatment regimens should be directed at the pathological characteristics within the different stages of the disease.

The metabolic defects underlying T2DM are insulin resistance, β-cell dysfunction and impaired hepatic glucose production (Wild et al., 2004). Insulin resistance is both a primary defect and an early feature in the development of T2DM, and tends to manifest in liver as well as in peripheral tissues such as skeletal muscle and adipocytes (Lee, 2003; Wild et al., 2004). While insulin resistance in liver is characterized by the inability of insulin to suppress glucose production, insulin resistance in peripheral tissues is characterized by the inability of tissues to take up glucose in response to insulin. Skeletal muscle is a key player in glucose metabolism and accounts for 75% of the whole body insulin stimulated glucose uptake (Hjeltnes et al., 1998; Wannamethee et al., 2011b; Wannamethee et al., 2011d; Zierath et al., 1998). Defects resulting in skeletal muscle insulin resistance originate from both genetic and environmental etiology (Jones et al., 2011; Wild et al., 2004).

Insulin resistance is a primary defect in T2DM. It is early trigger for the progression of normal glucose tolerance (NGT) into impaired glucose tolerance (IGT) and finally to frank T2DM. At an early stage, β-cells compensate for nominal insulin resistance by secreting more insulin. Elevated levels of secreted insulin counteract the effect of hyperglycemia that result from decreased glucose disposal at the periphery. Thus, in "pre-diabetes", normal glycemia is maintained by hyperinsulinemia (Reiber et al., 1993; Wannamethee et al., 2011e). This compensation is able to maintain normal glucose levels for several years. Continuous assault from harmful metabolites such as free fatty acids (FFA) worsens the insulin resistance, demanding for even higher levels of insulin to maintain physiological fasting glucose levels (Fall, 2001; Kitange et al., 1993). Although the fasting glucose levels are within physiological limits at this stage, response to a glucose challenge is impaired, resulting in a state of impaired glucose tolerance (Figure 1). In addition to the deleterious effects of insulin resistance, Current evidence point to the genetic defect in β-cells as an important trigger in the pathogenesis of T2DM (Griffen et al., 2001; Moran et al., 2012). Genome wide association studies (GWAS), have provided a new insight on the role of β-cell function in the pathogenesis of T2DM, reviewed in (McCarthy, 2009; McCarthy and Zeggini, 2009), further highlighting the important role of the β-cell in T2DM pathogenetis.

Initially, IGT is characterized by mild postprandial hyperglycemia, but as the degree of insulin resistance worsens, more global derangements in insulin production

occur that result in progressive hyperglycemia (Wannamethee et al., 2011e). Clinically, IGT represents an essentially asymptomatic but potentially pathologic stage in a continuum between normal glucose metabolism and the development of overt T2DM. IGT is a suitable predictor for both T2DM and cardiovascular diseases. In fact, several cardiovascular complications such as macroangiopathies may already be present at this stage indicating that macroangiopathies might not be secondary to diabetes but rather contribute to development of the diabetes phenotype (Wannamethee et al., 2011a).

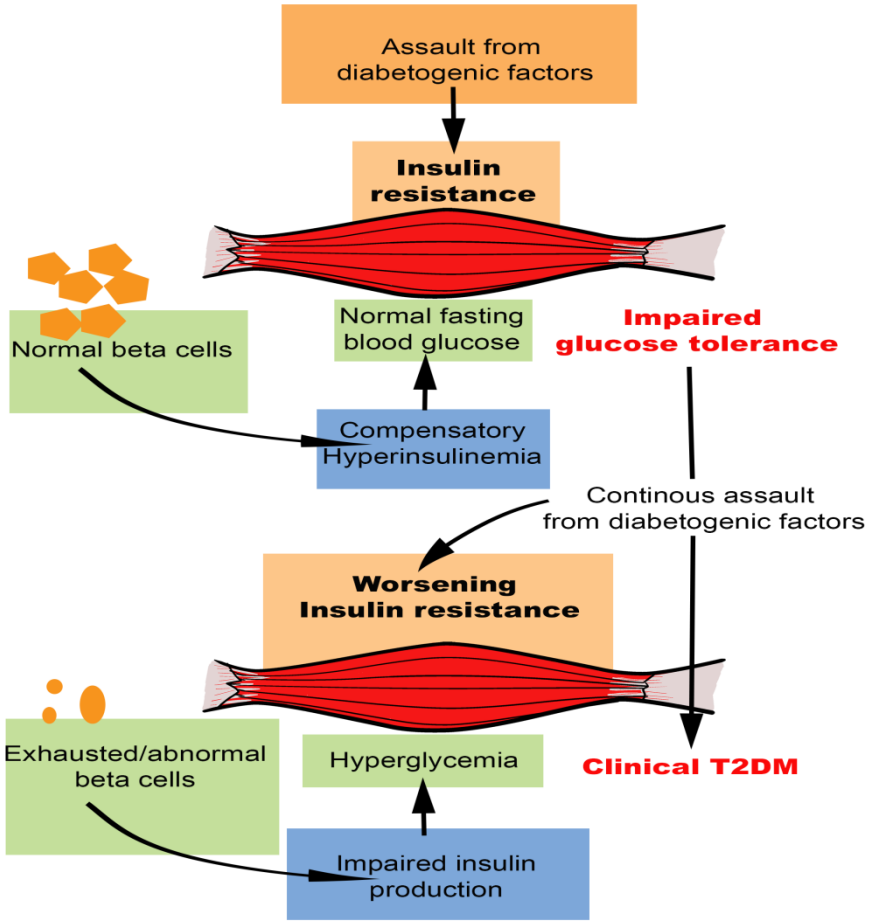

Figure 1: Progression of IGT to clinical T2DM in skeletal muscle. Insulin resistance in peripheral tissues including skeletal muscle is an important trigger for the progression of IGT into T2DM. Initially, β cells compensate by producing more insulin that normalizes the glycemia. At this stage, however, the body displays an impaired response to a glucose challenge (IGT). Worsening insulin resistance exhausts β cell function, resulting in impaired insulin production. Insufficient insulin production fails to counteract the increasing insulin resistance and, hence, a state of hyperglycemia that triggers clinical T2DM ensues.

Progression of IGT to T2DM is marked by diminished β-cell function and concomitant reduction in insulin secretion (Wannamethee et al., 2011e). At this point, the quantity of secreted insulin becomes insufficient to normalize the progressively increasing hyperglycemia. Although T2DM might be asymptomatic as the case of IGT but severe hyperglycemia is sufficient to trigger the development of micro vascular complications (Lee, 2003). Early intervention is therefore crucial to counteract the development of T2DM and to prevent cardiovascular complications. At this stage lifestyle intervention involving physical activity could play a crucial role in slowing disease progression.

Investigating the effects of glucose tolerance on the cardiovascular benefits of physical activity is a key area covered in this thesis.

1.3.1 Interfering with the natural history of T2DM

1.3.1.1 Physical activity, glucose and lipid metabolism

Physical activity has a beneficial role in glucose and lipid metabolism. Insulin-independent effects on glucose transport in isolated skeletal muscle have been reviewed elsewhere (Fontana et al., 2007; Holloszy, 2005; Krook et al., 2003). This key finding provides evidence for the existence of functional exercise/contraction-mediated glucose uptake pathways, despite the impaired insulin-mediated pathway in T2DM (Chibalin et al., 2000). Direct evidence for exercise-specific effects on glucose and lipid metabolism is available; however, additional studies are still needed for a comprehensive understanding of the effects of exercise on human health. Clinical studies are therefore necessary to translate basic/experimental research observations to public health.

Physical exercise offers both preventive and curative benefits on T2DM, making it an important component of an early control strategy. Physical activity, coupled with a reduction of caloric intake, may drastically slow or even prevent the development of T2DM in people with IGT (Corpeleijn et al., 2009;). Reduced mortality has been reported in physically active T2DM patients compared to those leading a more sedentary lifestyle (Leibiger et al., 2001). Furthermore, regular physical exercise has beneficial effects on cardiovascular risk factors in people with IGT and T2DM (Tsuruzoe et al., 2001). However, disease progression is associated with problems such as musculoskeletal complications and low motivation, which may affect compliance to a training program (Saltiel and Kahn, 2001). This suggests that a more effective exercise intervention should be initiated during the early stages of T2DM.

1.3.1.2 Physical activity and weight loss

Obesity and physical inactivity are both independent risk factors for T2DM. Elevated levels of FFA, resulting from dysregulated metabolism in adipose tissue, leads to impaired insulin sensitivity in liver and skeletal muscle, as well as adipose tissue, which triggers the pathogenesis of T2DM in obesity (Feldstein et al., 2008; Henquin, 2009; Henquin et al., 2009; Longo et al., 2008). Physical activity may counteract the diabetogenic effect of obesity by reducing fat mass, or through other biological pathways. Weight loss, as little as 5% to 10%, can reduce hyperglycemia and improve other cardiovascular risk factors in T2DM (Andres and Zierler, 1956; Obici et al., 2002b; Ravier et al., 2009). Improvements in insulin sensitivity in obese individuals have also been reported following a similar reduction in body weight (Andres and Zierler, 1956). Indeed, weight loss can reduce the fatty acid supply and thereby reduce the amount of lipid contained within liver (Campbell et al., 1994b) and skeletal muscle (Jensen, 1998b; Levine et al., 1998b). Together, these observations underscore the importance of a negative energy balance to prevent insulin resistance and hyperglycemia, as well as other cardiovascular risk factors in T2DM. However whether a similar exercise modality (low-moderate intensity exercise) will have comparable effects on weight loss and other cardiovascular risk factors across different stages (NGT, IGT, and T2DM) of T2DM pathogenesis is unclear.

1.3.1.3 Physical activity and insulin sensitivity

Positive effects of physical activity on insulin sensitivity in both normal and insulin resistant states are well documented. As early as the 5th century, physical activity was already advocated in the treatment of diseases, reviewed by Levine et al

(Levine et al., 1998a). Today, the robust effect of exercise on insulin sensitivity has become evident. Cross sectional studies have reported increased insulin sensitivity in trained compared to non-trained subjects (Abel et al., 2001; Arcaro et al., 1999; Shankar et al., 2004; Shankar and Steinberg, 2005). Likewise, regular endurance training can prevent age-induced insulin resistance in the elderly (Paradisi et al., 2001). Interestingly, older but active individuals were found to be more insulin sensitive compared to young sedentary subjects (Paradisi et al., 2001). Additionally, a growing body of evidence from epidemiological studies has linked physical inactivity with the increasing prevalence of T2DM (Jensen, 1998a; Jensen and Levine, 1998; Jensen et al., 1998). Physical activity is therefore an effective way to improve insulin sensitivity or/and prevent the development of insulin resistance.

Over the last two decades, the physiological and molecular mechanisms through which physical activity improves skeletal muscle glucose uptake have been extensively investigated (Outlined in Figure 2). The positive effects of exercise in skeletal muscle, together with the effects in other organs, help to maintain glucose homeostasis, even in states of insulin resistance. Aerobic exercise has long been considered the most effective mode of physical activity for improving insulin sensitivity. However, greater consideration of a minimal and more realistic exercise intervention that is practically applicable in a normal primary care setting is warranted. Investigating the effect of a minimal unsupervised exercise intervention on insulin sensitivity (Study 3) will therefore provide scientific evidence for therapeutic effects of this exercise modality in obesity and T2DM.

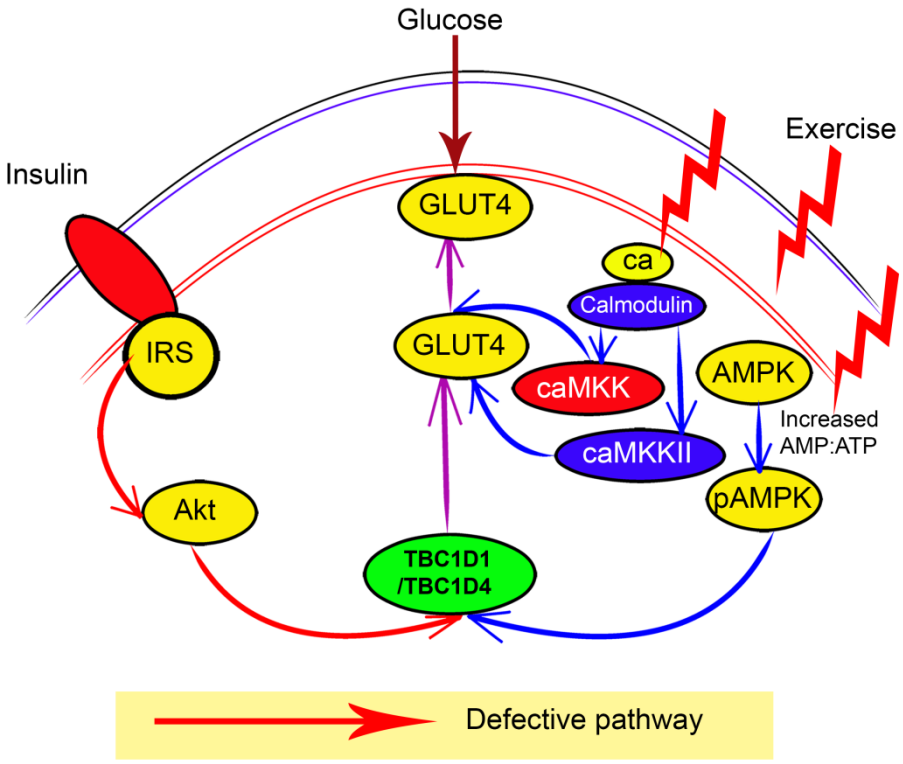

Figure 2: **Exercise-dependent glucose uptake in skeletal muscle.** In insulin resistance, insulin-dependent pathways for glucose uptake are impaired while contraction-dependent pathways remain intact. Exercise facilitates the translocation of GLUT4 to the plasma membrane through molecular mechanisms other than the insulin receptor, subsequently inducing glucose uptake into skeletal muscle.

1.3.1.4 Exercise capacity and T2DM

Impairments in cardiopulmonary fitness have been observed in obesity and T2DM (Gupta et al., 1998; Steinberg et al., 1997). In most clinical exercise studies, cardiopulmonary fitness is often estimated by measuring the maximum oxygen uptake during an exercise test, hence expressed as maximum oxygen uptake (VO_2 max)(Ong and Ong, 2000; Pi-Sunyer et al., 1998; Seyoum et al., 2006).The existence of T2DM confers an additional reduction in cardiopulmonary fitness beyond that seen with obesity (Kristen J. Nadeau et al., 2009). This suggests that progression from obesity to overt T2DM is accompanied by worsening cardiopulmonary fitness, emphasizing the importance of early lifestyle interventions to prevent T2DM. Cardiopulmonary fitness is a good predictor of cardiovascular events both in obese and T2DM subjects. Thus, improvement in fitness offers a protective advantage against cardiovascular risk factors in T2DM (Seyoum et al., 2006; Wei et al., 2000). Physical exercise is the main treatment modality with a positive impact on cardiopulmonary fitness. Evidence from both epidemiological and clinical studies suggests that regular exercise improves cardiopulmonary fitness in normal, obese and T2DM subjects, and lowers cardiovascular events and mortality in subjects with T2DM (Gupta et al., 1998; Kristen J. Nadeau et al., 2009; Seyoum et al., 2006; Steinberg et al., 1997; Wei et al., 2000).

1.4 METABOLISM AND T2DM

1.4.1 Regulation of glucose metabolism

Glucose is the endpoint breakdown product of carbohydrate digestion that is used by all living organisms as an important energy substrate and metabolic intermediate in many pathways. Uptake and metabolism of glucose is crucial for cellular functioning and is tightly regulated by the hormone insulin (Corpeleijn et al., 2009; Longo et al., 2008; Saltiel and Kahn, 2001). In a normal physiological state, pancreatic ß-cells secrete insulin in response to a meal (Feldstein et al., 2008; Henquin, 2009). The postprandial presence of insulin in the circulation diminishes hepatic glucose production and facilitates glucose uptake into peripheral tissues. Skeletal muscle accounts for ~75% of whole body insulin-stimulated glucose uptake, rendering it a critical tissue for glucose metabolism (Wannamethee et al., 2011b; Wannamethee et al., 2011d). Moreover, resting skeletal muscle has a low rate of glucose utilization in overnight fasted humans (Andres et al., 1956), reflecting a conservation of fuel for tissues with a compulsory glucose requirement (e.g. brain).

1.4.2 Glucose uptake

Glucose uptake in skeletal muscle occurs by facilitated diffusion, a process that is stimulated by insulin and mediated by glucose transporters. GLUT4 is the primary glucose transporter in skeletal muscle, but GLUT1 may also facilitate glucose uptake to a lesser extent (Gerrits et al., 1993; Klip and Paquet, 1990; Olson and Pessin, 1996). Once glucose enters the cell, it can either be transported back outside or, in the presence of hexokinase, irreversibly phosphorylated to glucose-6-phosphate, which can be channeled into different metabolic pathways including glycogen synthesis, as summarized in Figure 3.

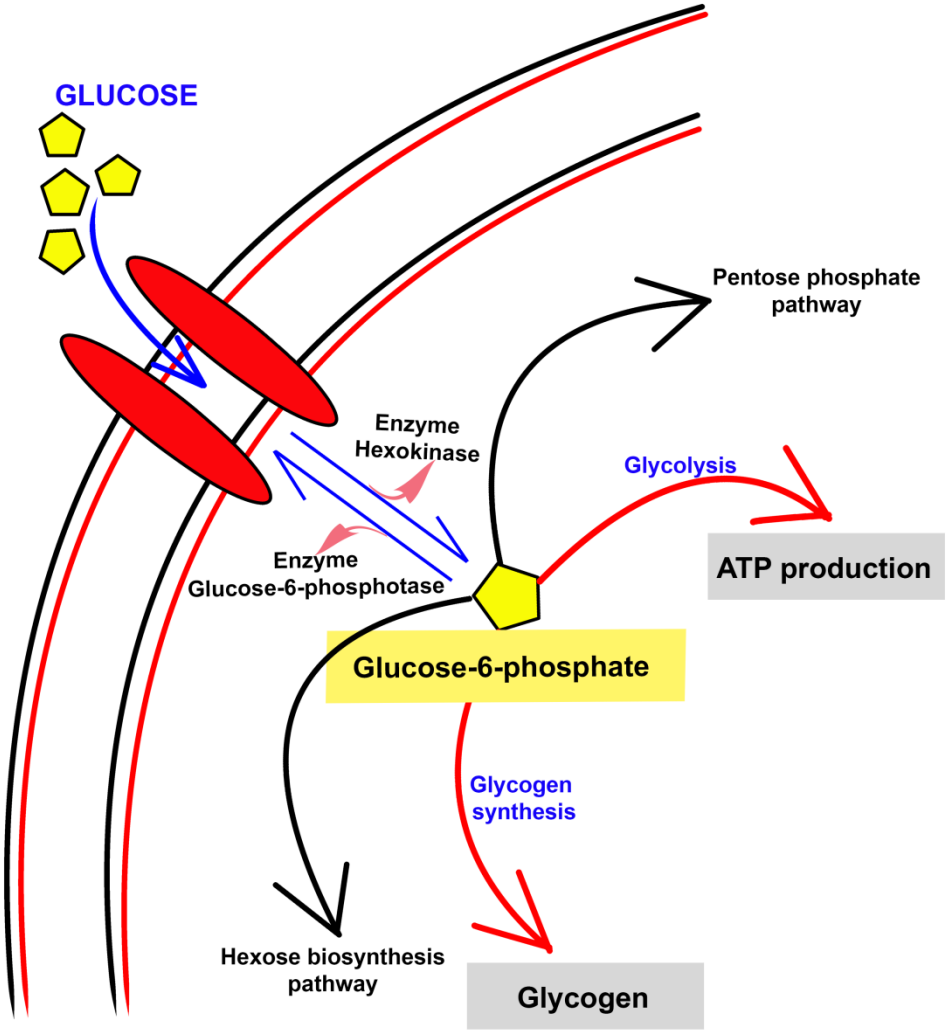

Figure 3: **Fate of cellular glucose.** Glucose enters skeletal muscle cells and is phosphorylated by Heokinase into glucose-6-phosphate. After phosphorylation, glucose can either be used for energy production or stored in the form of glycogen. A small percentage of glucose can either be channeled to hexose biosynthesis or pentose phosphate pathways for a variety of metabolic functions.

1.4.3 Glucose uptake as a target for intervention

A primary defect in T2DM is insulin resistance, which result in perturbations in glucose and lipid metabolism. Hyperglycemia, as a result of impaired glucose uptake in skeletal muscle and unregulated hepatic glucose production, is the main trigger for clinical diabetes. Interventions directed towards improving skeletal muscle glucose uptake are, therefore, important in the ongoing battle against the rise of T2DM. A number of novel pharmaceutical agents for treatment of T2DM have been proposed and widely investigated. This include among others, sulfonylureas, biguanides, and thiazolidinediones. Most of these agents have shown a positive effect on glucose uptake. However, low efficacy and mechanism-based side effects favor the continued development of new avenues of safe and potentially effective agents.

1.5 LIPID METABOLISM AND GLUCOSE HOMEOSTASIS

Free fatty acids are elevated in obesity (Boden, 2002; Boden and Shulman, 2002), providing evidence that availability of excess fat in the form of FFA may lead to impairments in muscle glucose metabolism and storage, and consequently to glucose intolerance and T2DM (Boden and Shulman, 2002). Defects such as increased hepatic gluconeogenesis and decreased glucose oxidation in skeletal muscle have been linked to excess availability of FFA. In obesity, the excess FFAs are readily available for oxidation in skeletal muscle at the expense of glucose (Randle et al., 1963). Furthermore elevated serum FFA can interfere with glucose utilization both *in vitro* and *in vivo* (Boden and Jadali, 1991; Boden et al., 1991; Randle et al., 1963). Together this evidence implicate disturbed lipid metabolism in the pathogenesis of T2DM.

Quantitatively, insulin-stimulated glucose disposal in skeletal muscle is of major importance compared to that of adipose tissue. However, insulin is a potent inhibitor of lipolysis in adipose tissue, a physiological process with major implications for glucose homeostasis. Elevated levels and increased oxidation of FFAs contribute to the development of insulin resistance in skeletal muscle (Boden, 1997; Randle et al., 1994a; Randle et al., 1994b). FFAs blunt the effect of insulin on glucose metabolism, resulting in increased hepatic glucose production, both by stimulating key enzymes and providing energy for gluconeogenesis (Foley et al., 1992; Toft et al., 1992). Moreover, uncontrolled lipolysis as a result of insulin resistance increases the production of glycerol that acts as a substrate for gluconeogenesis (Campbell et al., 1992; Nurjhan et al., 1992a; Nurjhan et al., 1992b; Toft et al., 1992). Consequently, increased production of FFAs and glycerol, resulting from blunted insulin effects on lipolysis, may result in deleterious effects on glucose homeostasis. Dysregulated metabolic cross-talk between skeletal muscle and adipose tissue that occurs in obesity plays a role in the development of skeletal muscle insulin resistance as illustrated in Figure 4. According to Randle, a competition for oxidation between glucose and FFA occurs in muscles (Randle et al., 1963), such that high flux of FFA favors its oxidation at the expense of glucose, consequently inhibiting muscle glucose uptake. Generally, increased plasma FFA levels could lead to impaired skeletal muscle insulin sensitivity and as a result affect whole body glucose metabolism.

Recent observations have however challenged the Randle hypothesis (Shulman, 2004; Wolfe, 1998). Contrary to what Randle *et al.* observed in rat heart and hemidiaphragm, the decrease in glucose uptake was not due to increased fatty acid oxidation, rather a primary defect in glucose uptake resulted in secondary defects in glucose oxidation (Wolfe, 1998). In addition, intracellular glucose-6-phosphate decreased in response to increased FFA availability (Roden, 2004; Shulman, 2004). Using the euglycemic/hyperinsulinemic clamp technique together with C-NMR, a substantial decrease in intracellular glucose-6-phosphate in obese and T2DM patients was observed, rather than an increase as predicted by Randle (Shulman, 2004). Moreover, a defect in glycogen synthesis without concomitant increase in glucose-6-phosphate in lipid-infused humans has also been reported (Roden, 2004; Shulman, 2004). This has led to the proposal of alternative mechanisms involving intrinsic defects in skeletal muscle insulin signaling (Griffin et al., 1999; Roden et al., 1996; Shulman, 2004; Yu et al., 2002). Mechanistic studies have uncovered potential mechanisms through which different lipid derivatives including FFAs can cause insulin resistance in skeletal muscle. A number of pathways related to these mechanisms have also been proposed. Targeting FFA-related pathways in skeletal muscle to reverse insulin resistance may have clinical implications in T2DM treatment.

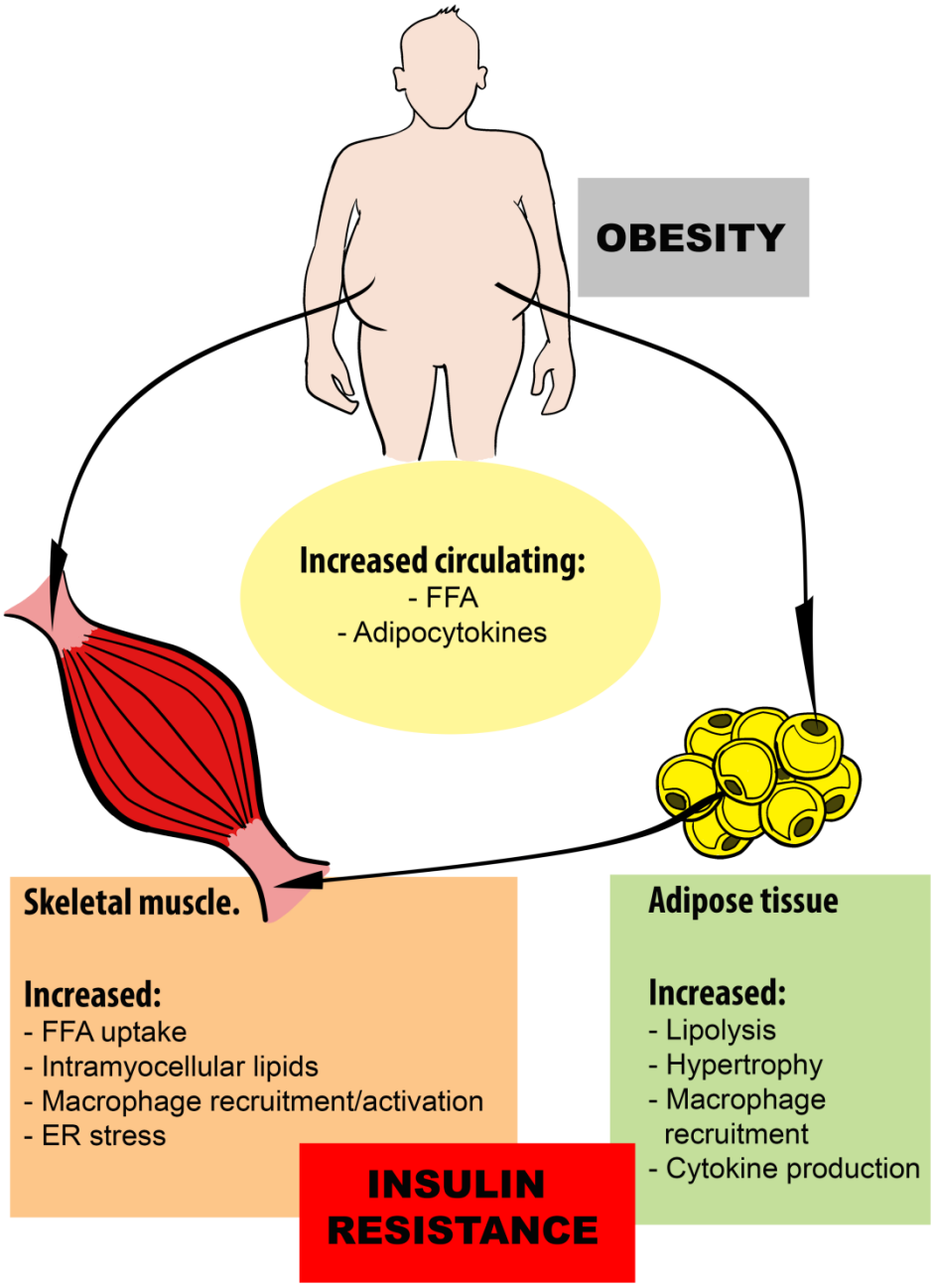

Figure 4: Role of obesity and dysregulated lipid metabolism in the development of skeletal muscle insulin resistance. Dysregulated metabolism in adipose tissue as a result of obesity causes an increase in the levels of pro-inflammatory cytokines and free fatty acid in the circulation. These metabolites impact skeletal muscle and cause insulin resistance. Other factors related to obesity can directly target skeletal muscle also causing insulin resistance.

1.6 INSULIN RESISTANCE

1.6.1 Insulin resistance in different target organs

Insulin resistance is an insufficiency in insulin action, which results in an increase in endogenous hepatic glucose production, and impaired stimulatory effects of insulin on peripheral organs. In skeletal muscle, failure of insulin action is manifested as impaired glucose uptake and glycogen synthesis. In adipose tissue, insulin resistance is characterized by increased lipolysis as a result of impaired insulin action. Increased lipolysis is the cause of elevated levels of free fatty acids in the circulation. The suppressive effect of insulin on free fatty acids is indeed impaired in obesity (Campbell et al., 1994a; Levine et al., 1998a) and T2DM (Groop et al., 1991). Furthermore, impaired glucose uptake into adipose tissue contributes to both hepatic and skeletal muscle insulin resistance (Abel et al., 2001), providing evidence for metabolic cross-talk in the pathogenesis of insulin resistance. This metabolic communication between adipose tissue and skeletal muscle suggests that excessive metabolites such as free fatty acids target skeletal muscle and could subsequently trigger or worsen the already existing insulin resistance in skeletal muscle (see Figure 4 above). The quantitative importance of skeletal muscle in insulin-stimulated glucose disposal, and its role as a target for many metabolically active molecules in the course of insulin resistance, brings skeletal muscle into the forefront of this thesis.

Insulin-dependent signaling pathways are present in virtually all tissues, including for example the vascular endothelium (Arcaro et al., 1999; Steinberg et al., 1996) . Since the metabolic cross-talk between tissues coordinates the whole-body response to insulin in obesity and T2DM, a brief understanding of insulin resistance in the brain and blood vessels, is necessary to understand pathogenesis from a systemic perspective. In obesity, an impaired insulin-mediated vasodilatation, which is a recognized precursor of atherosclerosis, is noted (Arcaro et al., 1999; Steinberg et al., 1996). Whether glucose metabolism in skeletal muscle is affected by insulin-mediated vasodilatation of blood vessels supplying skeletal muscle remains disputed (Clark, 2008; Steinberg and Baron, 1999; Yki-Jarvinen and Utriainen, 1998) . However, it has been shown that access of insulin to target tissues is inhibited by a high fat diet, potentially by inhibiting capillary recruitment (Ellmerer et al., 2006).

Central inhibition of insulin action such as resistance in the central appetite-suppressing and metabolic action of insulin plays an important role in the development of skeletal muscle insulin resistance. Recently it has become clear that physiological glucose homeostasis in the brain requires insulin action (Obici et al., 2002a; Obici et al., 2002b; Okamoto et al., 2004), contrary to the traditional understanding of the brain as an insulin-independent organ (Seaquist et al., 2001). Although basal levels of insulin can stimulate brain glucose uptake (Bingham et al., 2002), this effect is significantly reduced in insulin resistance (Anthony et al., 2006). Furthermore neurons in the hypothalamus express the insulin-responsive insulin transporter GLUT4 (Leloup et al., 1996). Impairments in insulin receptors in the hypothalamus consequently cause hyperphagia that lead to diet-induced obesity (Leloup et al., 1996).

1.6.2 Obesity, insulin resistance and metabolic cross-talk

The following section will review the available evidence related to the interaction between different tissues and organs (metabolic cross-talk) in the development of skeletal muscle insulin resistance in obesity. Emphasis will be placed on the adipose

tissue/skeletal muscle cross-talk, especially the role of free fatty acids and circulating adipokines in the development of insulin resistance. Novel *tissuekines,* produced by liver and adipocytes that hold potential therapeutic effects in skeletal muscle, will also be discussed. Finally, evidence for potential drug targets to modulate the negative effects of excessive metabolites on insulin signaling in skeletal muscle will be reviewed and discussed. Perturbations in FFA metabolism play a crucial role in the pathogenesis of insulin resistance in obesity. Plasma FFA turnover is related to whole-body lipolysis. The increased lipolysis occurring in obesity leads to a chronic elevation of FFA in skeletal muscle and other tissues. Circulating FFA mediate the metabolic cross-talk between adipose tissue and skeletal muscle.

1.7 NOVEL CIRCULATING FACTORS, GLUCOSE AND LIPID METABOLISM.

Accumulating evidence suggests that secreted factors from adipocytes and skeletal muscle participate in the physiological regulation of glucose and lipid metabolism in energy homeostasis (Guilherme et al., 2008; Pedersen and Febbraio, 2008). The identification of adipocyte- and skeletal muscle-derived molecules, which interact with insulin-sensitive tissues, has expanded the understanding of glucose metabolism. There is a growing appreciation that Fibroblast Growth Factor (FGF)-21, a novel member of the FGF family, participates in a number of endocrine functions including the regulation of glucose and lipid metabolism (Coskun et al., 2008; Kharitonenkov et al., 2005; Ryden, 2009; Wente et al., 2006). FGF-21 is a potent regulator of insulin-dependent glucose uptake in both murine 3T3-L1 adipocytes and primary human adipocytes (Kharitonenkov et al., 2005). Transgenic overexpression of FGF-21 improves insulin sensitivity and lowers blood glucose and triglycerides levels in animal models of obesity (Kharitonenkov et al., 2005). Together, these observations emphasize the role of FGF-21 in modulating glucose and lipid metabolism.

1.7.1 Clinical importance of FGF-21 in human disease

Current therapeutic options for treatment of T2DM are suboptimal, since the majority of patients on oral agents fail to achieve the targeted clinical outcomes (1995; Scheen, 2003). Limited efficacy, tolerability and reported side effects are common to all the available therapies (e.g. insulin, metformin, peroxisome proliferator-activated receptor-gamma agonists, alpha glucosidase inhibitors), necessitating a intensive search for new agents. Furthermore, a continuous search for a potential biomarker that will aid in early prediction or/and detection of T2DM is crucial. Fibroblast Growth Factor 21 (FGF-21) is an emerging novel circulating *tissuekine* with possible diagnostic and therapeutic potentials.

1.7.1.1 FGF-21 as a potential drug target

Evidence arising primarily from *in vitro* and *in vivo* studies in animals suggests that FGF-21 exerts beneficial metabolic effects on both carbohydrate and lipid metabolism (Coskun et al., 2008; Kharitonenkov et al., 2005; Ryden, 2009; Wente et al., 2006). In diabetic animal models, FGF-21 treatment improved glucose and lipid homeostasis and preserves β-cell function (Coskun et al., 2008; Hotta et al., 2009; Kharitonenkov et al., 2005; Kharitonenkov et al., 2007; Kralisch and Fasshauer, 2011; Sarruf et al., 2010; Wente et al., 2006). Systemic administration of recombinant FGF-21 decreases plasma triglycerides, FFA, and cholesterol in genetically-modified obese and diabetic rodents (Kharitonenkov et al., 2005; Xu et al., 2009). Moreover, long-term

FGF-21 therapy in diabetic rhesus monkeys improves lipid profiles (Kharitonenkov et al., 2007). Interestingly, unlike classic FGFs, proliferation or mitosis is unaffected by FGF-21 (Huang et al., 2006; Kharitonenkov et al., 2005; Wente et al., 2006), offering a promising and potentially safe treatment option for T2DM. Whether FGF-21 has direct effects on glucose metabolism in skeletal muscle, is unknown. Furthermore the relationship between FGF-21 serum levels and other metabolic parameters of clinical importance needs further clarification.

Several adipokines (*tissuekines* secreted from adipose tissue) have been identified and shown to influence insulin action in skeletal muscle (Pittas et al., 2004). These include TNFα, IL-6, and adiponectin that, together with other possibly unknown factors, might constitute the missing link(s) between adipose tissue and skeletal muscle insulin resistance (Greenberg and McDaniel, 2002). The involvement of the cytokine-responsive JAK/STAT pathway in the cross-talk between adipocyte/liver and skeletal muscle has been reported (Emanuelli et al., 2001; Rui et al., 2002; Ueki et al., 2004). However, knowledge regarding the role of STAT3 in the development of skeletal muscle insulin resistance in humans is sparse and inconclusive. Further, detailed investigations on the role of STAT3 in the development of skeletal muscle insulin resistance may identify targets of therapeutic potential for the treatment of T2DM.

1.7.2 Insulin signaling in skeletal muscle

Insulin stimulates glucose uptake in skeletal muscle by increasing the abundance of glucose transport proteins, mainly GLUT4 (Czech and Corvera, 1999), at the plasma membrane. This process is initiated upon binding of insulin to insulin receptors at the cell surface. Insulin binding triggers a cascade of intracellular signaling events, including the consecutive phosphorylation of several cytosolic proteins, such as the insulin receptor substrate molecules (IRS), phosphatidyl inositol 3 kinase (PI3K), and protein kinase B (PKB/Akt) (Czech and Corvera, 1999). Akt/PKB and members of the protein kinase C (PKC) family are key molecules in the canonical insulin signaling cascade that ultimately lead to increased intracellular glucose transport. Total GLUT4 protein content is unaltered in skeletal muscle from T2DM patients (Handberg et al., 1990; Pedersen et al., 1990; Shepherd and Kahn, 1999). Thus, impaired glucose uptake in insulin-resistant skeletal muscle cannot be explained by a decrease in the biosynthesis of GLUT 4. Rather, impairments in insulin signaling or GLUT4 trafficking are likely to play a role (Cusi et al., 2000; Krook et al., 2000; Krook et al., 1998) in T2DM-related defects in glucose metabolism.

Both *in vitro* and *in vivo* studies in humans and laboratory animals provide evidence to support a role for a selective insulin signaling defect in skeletal muscle (Kim et al., 2003; Krook et al., 2000; Leng et al., 2004). In T2DM patients, insulin-mediated glucose uptake in skeletal muscle is reduced by approximately 50%. A molecular explanation for the development of insulin resistance in skeletal muscle points to specific alterations in the insulin signaling pathways (Bjornholm et al., 1997; Bouzakri et al., 2003; Cusi et al., 2000; Krook et al., 1998). Alterations in the expression or translocation of GLUT4 to plasma membrane have also been implicated as a potential cause of skeletal muscle insulin resistance.

Furthermore, the impairment in glucose uptake that causes skeletal muscle insulin resistance arises from an aberrant insulin response, but also involves influence of growth factors and locally acting hormones (sometimes referred to as *tissuekines*) secreted by different organs. In humans and animal models of T2DM, factors secreted from liver, adipose tissues and other organs, play a crucial role in the development of skeletal muscle insulin resistance. This provides a mechanism for metabolic crosstalk between different tissues and organs.

1.7.2.1 The JAK-STAT pathway

Various biological processes, such as the cellular response to cytokines and growth factors, are mediated by the evolutionary conserved Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathways (Figure 5). Depending on the signal, tissue, and cellular milieu, activation of this pathway results in a wide range of responses. These responses include apoptosis, cell survival, differentiation, proliferation and migration, highlighting the essential role for JAK/STAT signaling in homeostatic processes like glucose and lipid dynamics (Manea et al., 2010; Marrero et al., 2006).

The JAK family contains four tyrosine kinase cytosolic proteins known as JAK1, JAK2, JAK3 and TYK 2, all of which are coupled to different receptors including those of cytokines (Darnell et al., 1994; Persico et al., 1995). In response to ligand binding to cytokine receptors, JAKs tyrosine-phosphorylate and activates these receptors. Activated JAKs may also tyrosine phoshorylate and activate other signaling molecules including the signal transducer and activators of transcription (STAT) factor family (Darnell et al., 1994; Persico et al., 1995). Seven STAT isoforms are known, i.e. STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6, and are differentially expressed in a tissue-specific manner (Aaronson and Horvath, 2002; Levy and Darnell, 2002; Pellegrini and Dusanter-Fourt, 1997).

The JAK/STAT pathway is therefore a link between cell surface receptor activation, nuclear transcriptional events, and various physiological outcomes. A growing body of evidence implicates this pathway in the adipokine-mediated cross-talk between adipocytes and liver or skeletal muscle (Emanuelli et al., 2001; Rui et al., 2002; Ueki et al., 2004). However, the specific role of STAT3 in the development of skeletal muscle insulin resistance in humans is inconclusive (Carey et al., 2006; Kim et al., 2011; Weigert et al., 2004). Further investigations to uncover STAT3-dependent mechanisms contributing to the development of skeletal muscle insulin resistance is therefore warranted.

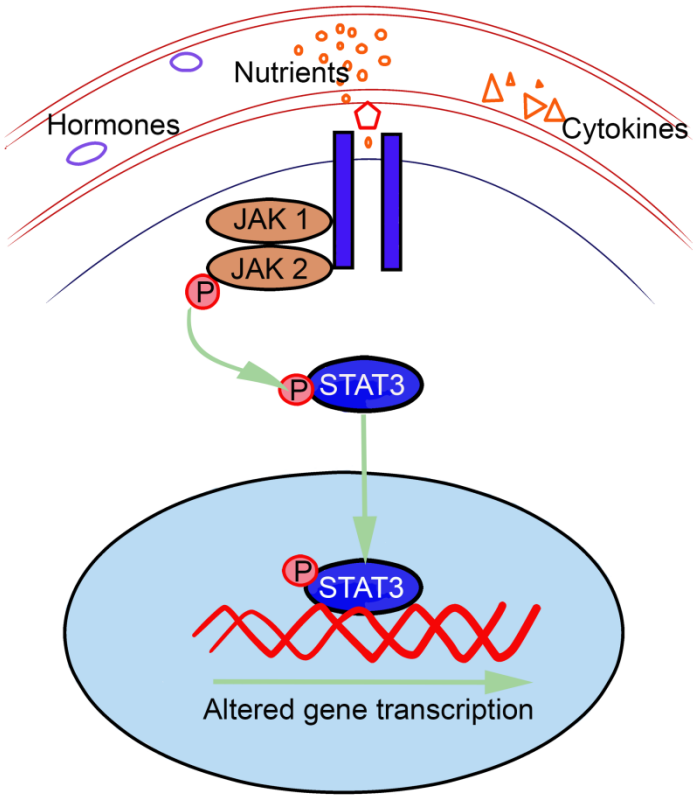

Figure 5: The JAK/STAT pathway. Various factors such as nutrients and cytokines signal through the JAK/STAT pathway. Activation of this pathway results in a number of biological processes mainly by transcriptional regulation. Activation of STAT3, a key member of the STAT family, has been linked to insulin resistance in liver and adipose tissue.

1.7.2.2 STAT3 and insulin sensitivity

STAT3 is a transcription factor expressed in multiple metabolic tissues that is activated through phosphorylation of Tyr^{705} and Tyr^{727} in response to cytokines, growth factors and nutrients. Targeted disruption of STAT3 leads to embryonic lethality in mice (Takeda et al., 1997), an effect not observed following disruption of other STAT family members. Moreover, accumulating evidence suggests that STAT3 responds to a wide array of physiological stimuli, implying a fundamental and novel biological importance of STAT3 versus other related family members. For that reason, the role of STAT3 in the development of insulin resistance in T2DM was explored. Studies involving STAT3 are therefore covered in this thesis (Study 3).

Signaling pathways involving STAT3 play a role in regulating both the hepatic and peripheral insulin sensitivity. Activation of STAT3 may have a dual effect on insulin signaling depending on the stimulus, physiological context and/or duration of activation (Carey et al., 2006; Kim et al., 2011; Weigert et al., 2004). In liver hepatocarcinoma cell lines, STAT3 knockdown prevents amino acid-induced insulin resistance (Kim et al., 2009a). Activation of STAT3 in adipocytes is linked to growth hormone-induced insulin resistance in rats chronically treated with arginine (de Castro Barbosa et al., 2009). In contrast, acute IL-6-induced activation of STAT3 in rat liver inhibits hepatic glucose production (Inoue et al., 2006). These findings underscore the

importance of understanding the varying effects of acute and chronic activation of this signaling molecule on insulin signaling.

1.7.2.3 Circulating factors and STAT3 activation

Various circulating metabolites such as cytokines, hormones and FFA activate STAT3 (Kim et al., 2008; Kim et al., 2011; Oberbach et al., 2010). In human smooth muscle cells, short-term palmitate exposure up-regulates STAT3 phosphorylation (p-STAT3) whereas long-term exposure down-regulates p-STAT3 and concomitantly increases SOCS3 protein abundance, implying negative feedback regulation of this signaling cascade (Oberbach et al., 2010). In mouse primary hepatocytes chronically treated with IL-6, mTOR upregulated phosphorylation of STAT3 and incresed SOCS3 expression, causing an impairment of insulin signaling (Kim et al., 2008). Furthermore, IL-6 induced insulin resistance in cultured myotubes derived from people with IGT (Kim et al., 2011). Collectively, these studies provide evidence to suggest circulating factors and hormones indirectly signal through STAT3 and differentially regulate insulin signaling in a variety of tissues. Thus, excessive STAT3 signaling may impose negative feedback regulation on canonical insulin signaling pathways controlling metabolic action in T2DM.

Given the complex regulation of whole body metabolism, several questions regarding the role of circulatory factors including glucose, lipids and cytokines are worthy of further penetration. Molecular mechanisms involved in skeletal muscle glucose and lipid metabolism are not completely resolved. Furthermore the collective role of physical activity in the clinical management of obesity and T2DM needs further evaluation. Investigation of the regulation of glucose and lipid metabolism integrated with description of the corresponding clinical implication, from a whole body physiology context to cellular mechanisms is therefore important.

2 AIMS

The overall aim of this thesis is to investigate the regulation of glucose and lipid metabolism in states of normal and impaired insulin sensitivity. The studies are designed to investigate the molecular mechanisms involved in the development of skeletal muscle insulin resistance. An overall evaluation of study findings further relates to the clinical significance in obesity and T2DM.

Specifically the following questions were posed:

1. Does low moderate-intensity exercise have similar effects on cardiovascular risk factors in overweight individuals with varying degrees of insulin sensitivity?

2. Is FGF-21 differentially regulated in normal and impaired glucose homeostasis, and does FGF-21 exert direct effects on glucose metabolism in human skeletal muscle?

3. Is STAT3 differentially regulated in normal and T2DM, and does STAT3 plays a role in the development of lipid-induced skeletal muscle insulin resistance?

3 MATERIALS AND METHODS

3.1 SUBJECTS

The subjects examined in Study *I*, *II* and *III* were recruited from Gustavsberg, a suburban area proximal to Stockholm, Sweden. Recruitment was achieved through newspaper advertisement and letters of invitation to former participants in the Stockholm Diabetes Prevention Program (SDPP) who lived within the catchment area (Eriksson et al., 2008). Clinical evaluations of the participants were performed at the Gustavsberg Vårdcentral primary health care center and at the Department of Clinical Physiology, Karolinska University Hospital, Stockholm.

Individuals aged 45 to 69 years, with BMI >25 kg/m^2, were included in the study. Study participants were qualified as T2DM if HbA1c values were between 7.4 and 9.3% National Glycohemoglobin Standardization Program standard (57 to 78 mmol/mol International Federation of Clinical Chemistry (IFCC) standards). Exclusion criteria were as follows: physical impairments, symptoms of angina pectoris, atrial fibrillation as determined by electrocardiogram, systolic or diastolic blood pressure of >160 or >100 mmHg respectively, and insulin treatment. Insulin treatment was an exclusion criterion since it would interfere with the calculation of HOMA-IR.

Upon inclusion into the study, the participants were classified into T2DM, IGT, or NGT by an oral glucose tolerance test (OGTT). The duration of diabetes was 5.1±3.7 years for people with T2DM (mean ±SD). Participants were stratified based on glucose tolerance state (NGT, IGT, or T2DM). In Study *I*, a total of 213 subjects [NGT (n=128), IGT (n=35), or T2D (n=50)] were analyzed before and after the study timeframe of 4 months. A single-blind randomization procedure was used to assign individuals to either a control or intervention group. For Study *II* and *III*, only T2DM patients (40 in Study *II* and 20 in Study *III*) and BMI- and age-matched NGT subjects were analyzed. A skeletal muscle biopsy was obtained from T2DM (10 in Study *II* and 20 in Study *III*) patients as well as an equivalent number of BMI- and age-matched NGT subjects. The clinical characteristics of the study subjects are presented in the respective articles. Primary human muscle cells used in Study *II* were obtained from a separate cohort of subjects who were scheduled for abdominal surgery at the Karolinska University Hospital, Huddinge, Sweden. These subjects were free from metabolic disorders and presented normal fasting glycemia.

3.2 CLINICAL CHARACTERISATION OF THE STUDY SUBJECTS

Exercise study protocol

For Study *I*, the participants in each category (NGT, IGT or T2DM) were randomized to the exercise or control group (only 10 T2DM patients on exercise intervention were analyzed in Study *II*). Subjects were asked to maintain their usual dietary habits. The participants in the exercise group were instructed to increase their weekly level of physical activity by 5 hours of walking with poles (Nordic walking) for 4 months. Instructions for Nordic walking were given by an exercise physiologist. Walking intensity was prescribed as a pace that caused slight shortness of breath and perspiration. Written informed consent was obtained from all participants. The study was approved by the Ethics Committee of Karolinska Institutet, Stockholm.

3.2.1 General clinical characteristics

Body weight was measured with a calibrated electronic scale (Hugin, Mustelia, EB5011). Systolic (SBP) and diastolic (DBP) blood pressures were determined to the nearest 5 mm Hg, in the seated position (Speidell & Keller Tonometer). At the time of inclusion, an OGTT was performed. A mean fasting plasma glucose was determined prior to and 2 hours after the ingestion of 75g of glucose solution. Glucose was assessed with a HemoCue B-Glucose analyzer. Fasting venous blood samples were analyzed for total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, and HbA1c at the Laboratory of Clinical Chemistry at Karolinska University Hospital, Stockholm. Fasting serum insulin was determined using an adipokine (HADK2-61K-B09) assay kit from Linco Research (Electra-Box Diagnostica AB, Tyresö, Sweden), according to the manufacturer's instructions. Plasma samples from each subject were extracted from whole blood and analyzed in duplicate. Results were quantified using the Luminex Bio-Plex 200 system (Bio-Rad, Stockholm, Sweden). Insulin resistance was calculated using the HOMA IR model (15), as fasting insulin (μU/ml) x fasting glucose (mM)/22.5. These measurements were repeated after four months of intervention (Figure 6). Tables with clinical characteristics of the study subjects are presented in respective articles.

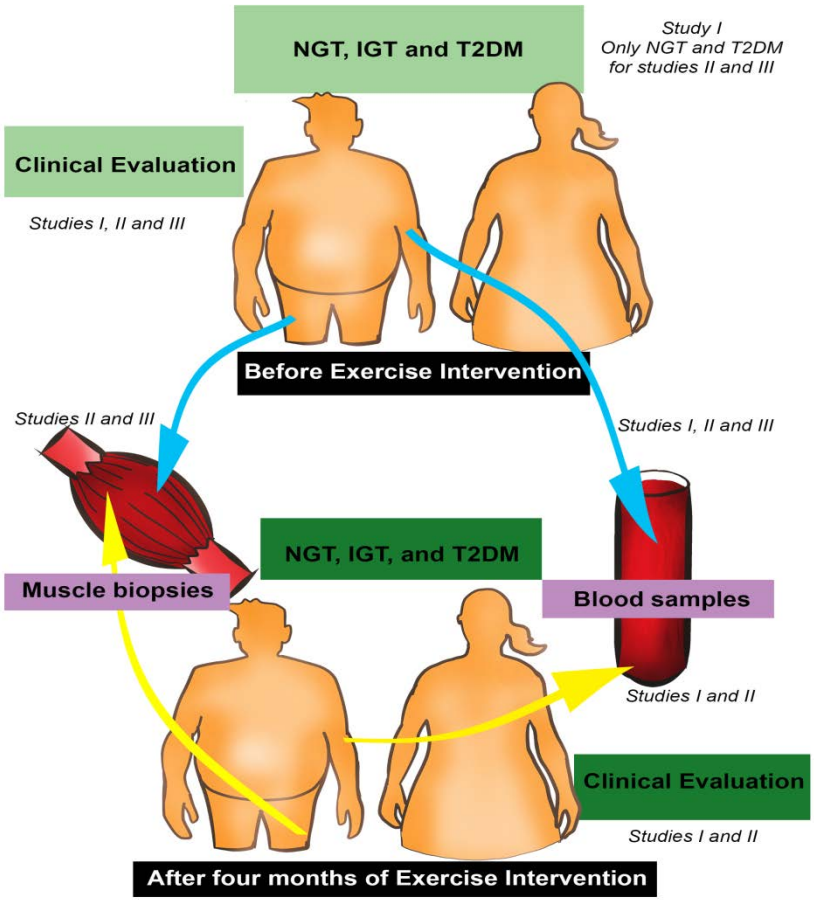

Figure 6: **The overall human study protocol**. Clinical material included in the three different studies. Clinical evaluation, blood samples and muscle biopsies from individuals with NGT, IGT and T2DM before and after intervention in Study I, II and III.

3.2.2 Exercise testing

Bicycle exercise tests were performed using a Rodby ergometer RE 820/830. A steady baseline was obtained prior to the initiation of the test. The initial work load was set at 50 W and continuously increased by 10 W/min. A Sensor Medics ergo spirometer Vmax Encore (Sensor Medics Corporation; Yorba Linda, USA) was used to measure gas exchange. The gas analyzers were calibrated with two calibrated gases containing 16.0% O_2, 4.0% CO_2 and 26.0% O_2, 0.0% CO_2. Oxygen uptake (VO_2) was calculated breath by breath, and the mean value for the latest 30 seconds was used. The VO_2 at peak exercise (peak VO_2) was determined at the point of subjective exhaustion.

3.2.3 Muscle biopsy procedure

Muscle biopsies in Study *II* and *III* were obtained from individuals with NGT (10 in Study *II* and 20 in Study *III*) and T2DM patients (10 in Study *II* and 20 in Study *III*). A local anesthesia (Lidocaine hydrochloride 5mg/ml) was injected at the site of incision. An incision (5 mm long/10 mm deep) was made to expose the *quadriceps femoris* muscle. A biopsy (20-100 mg) was taken from the *vastus lateralis* portion of the *quadriceps femoris* using a Weil-Blakesley contochome. Biopsies were immediately frozen in liquid nitrogen and stored at -80°C to await further analysis.

3.2.4 Physical activity estimation

3.2.4.1 Self-reported physical activity

In Study *I*, self-reported exercise was obtained from both the control and intervention participants at baseline and after four months. Participants were asked to complete a questionnaire to report their physical activity and indicate their average weekly amount of low, medium and high intensity exercise. The frequency and duration of activities were also recorded. Exercise diaries were used by participants in the exercise group to record details such as date and duration of each Nordic walking bout.

3.2.4.2 Accelerometer

To validate the self-reported physical activity assessment method, several individuals also used personal accelerometers for seven days shortly after randomization (Haffner et al., 1997), in order to generate a second, objective assessment of activity. Of the **214** individuals in entire cohort, 25 participants (n=11 from the control group and n=14 from the intervention group) wore a belt accelerometer during operative hours (ActiGraph model GT1M; ActiGraph, Pensacola, Florida, USA). Physical activity was recorded as total activity counts per minute and minutes per day of inactivity, low, moderate, or vigorous activity.

3.3 SERUM ANALYSIS

3.3.1 FGF-21 serum analysis

In Study *II*, circulating FGF21 was measured in serum using a commercially available enzyme-linked immunosorbent assay kit (Human FGF21, Bio vendor, Czech Republic), following the manufacturer's instructions. Serum samples were diluted 1:2 with the dilution buffer prior to assay. Positive and negative controls were included. All samples were measured in duplicate. All values were within the standard curve range.

3.3.2 Plasma FFA analysis

In Study *III*, circulating FFA was measured in plasma using the plasma Human Free Fatty Acids detection kit (Zenbio), according to the manufacturer's instructions. Samples were diluted 10 times with the dilution buffer before dispersal into a 96-well plate in duplicate. A standard curve using standards of known concentration was used to calculate the concentration of FFA within the samples. All measurements fell within the acceptable range of the standard curve. Plasma concentration of IL-6 and TNF-a was measured using a Novex multiplex Luminex assay for quantitation and detection of Cytokines (Life Technologies, Carlsbad, CA).

3.4 MATERIALS

Dulbecco's modified Eagle's medium, fetal bovine serum, penicillin, streptomycin, and Fungizone were obtained from Invitrogen (Stockholm, Sweden). Recombinant human FGF21 used in Study *II* was provided by Lilly Research Laboratories (Lilly Corporate Center, Indianapolis, IN) or purchased from ProSpec-Tany TechnoGene Ltd (Rehovot, Israel). General laboratory reagents, including palmitic acid for Study *II*, were obtained from Sigma (St. Louis, MO). Radioactive reagents were purchased from Amersham (Uppsala, Sweden). Oligonucleotide primers used in Study *II* and *III* were purchased from Oregene (Oregene) and SYBR Green probes were from Invitrogen (Invitrogen, Carlsbad, CA). Total and phospho-specific protein antibodies were from Cell Signaling Technology (Danvers, MA, USA).

3.5 CELL CULTURE PROCEDURES

3.5.1 Primary human skeletal muscle cell culture

Rectus abdominus biopsies were obtained from four males and four females who underwent abdominal surgery. The mean age and BMI of the subjects were 54±6 years and 25.6±1.5 kg/m^2, respectively. The subjects had no known metabolic disorders and they presented with normal plasma glucose. Trypsin digestion was used to separate muscle satellite cells from the muscle biopsies. Isolated myoblasts were propagated in growth media [DMEM (1 g/L glucose) with 20% FBS, 1% Penicillin/streptomycin, 1% fungizone] and grown to >80% confluence prior to exposure to differentiation media (DMEM with 2% FBS, 1% Penicillin/streptomycin, 1% fungizone), which stimulates differentiate and formation of myotubes (Al-Khalili et al., 2004a) . Differentiated myotubes were exposed to dimethyl sulfoxide (DMSO, vehicle) or FGF21 (1 μg/mL) for 2, 6, and 24 h. Cells were serum-starved with or without FGF21, as appropriate, for 4 hours prior to a specific metabolic experiment (glycogen synthesis or glucose uptake).

3.5.2 L6 cell culture

Rat L6 muscle cells (received as a gift from Professor Amira Klip, Hospital for Sick Children, Toronto, ON, Canada) were grown in MEM-α media (10% FBS, 1% penicillin/streptomycin, and 1% Fungizone) until confluent (>80% confluence) and then cultured with differentiating media (MEM-a with 2% FBS, 1% penicillin/streptomycin, and 1% Fungizone) for 4 days.

3.6 METABOLIC STUDIES IN CELL CULTURE

3.6.1 Glycogen synthesis

Differentiated skeletal muscle myotubes were serum-starved for 4-18 hours prior to the experiment. Myotubes were then incubated in the absence or presence of 60 or 120nM insulin for 30 min followed by a 90 min incubation with 5mM of glucose containing insulin and D-glucose [U-^{14}C] (1 μCi/mL; Amersham, Uppsala, Sweden). Myotubes were washed in ice-cold PBS and then lysed in 0.03% of sodium dodecyl sulphate (SDS). Carrier glycogen was added to the lysate, heated at 95°C and incubated for 30 min. Subsequently, 95% ethanol was added and the samples were incubated overnight in -20°C to precipitate glycogen. Following centrifugation, the precipitating glycogen pellets were washed with 70% ethanol and resuspended in distilled water. Liquid scintillation counting was used to measure radioactivity (WinSpectral 1414 liquid scintillation counter; Wallac/PerkinElmer, Waltham, MA, USA). Protein concentration was estimated using the lysate from the same experiment.

3.6.2 Glucose uptake

Following approximately 18 hours of serum starvation, differentiated myotubes were incubated in glucose-free DMEM in the absence or presence of 120 nM insulin at 37°C for 30 min. Myotubes were subsequently incubated for 10 min in 5 mM 2-[G-H^{3}] deoxy-D-glucose. After washing cells 3 times in ice-cold PBS, cells were lysed in 0.5 M NaOH. Scintillation fluid was added to 500 μl of lysate and radioactivity was measured by liquid scintillation counting (WinSpectral 1414 liquid scintillation counter; Wallac/PerkinElmer, Waltham, MA, USA).

3.6.3 Media lactate determination

Lactate concentration in the media was determined using a lactate kit from Biomedical Research Service Centre, University at Buffalo (Buffalo, NY). Media samples were diluted 1:20 and then 20 μL of the dilution was added to a 96-well microplate. The enzymatic reaction was initiated with the addition of 50 μL Lactate Assay Solution to each well. The solutions were mixed by gentle agitation and the microplate was covered and incubated at 37°C for 1 h. The reaction was terminated by adding 50 μL of 3% (0.5 M) acetic acid per well and the samples were mixed by brief agitation. Absorbance was measured at 492 nM.

3.6.4 siRNA transfection and fatty acid treatment

siRNA oligos for STAT3, SOCS3, or scrambled sequences (OnTargetplus) were purchased from Dharmacon (Chicago, IL). On day 3 of differentiation at ~70% confluence, myotubes were cultured in antibiotic-free MEM-α media and transfected with the specific siRNA (1 mg/mL) by calcium phosphate precipitation (Cell Phect Transfection kit, Amersham Pharmacia). On day 6 of differentiation, transfected

myotubes were exposed to BSA (control) or BSA-conjugated fatty acid (0.25 mmol/L palmitate) for 24 hour. During the last 4 h of the incubation procedure, cells were cultured in serum-free media in the presence or absence of palmitate. Thereafter, myotubes were incubated in the presence or absence of 60 or 120 nmol/L insulin for determination of glucose incorporation into glycogen and protein phosphorylation. For time-course experiments, differentiated L6 myotubes were exposed to BSA (control) or BSA-conjugated fatty acids (0.25 mmol/L palmitate) or mouse recombinant IL-6 (20 ng/mL) for 0, 2, 6, 12, 24, or 36 h. Cells were then harvested and lysates were prepared for Western blot analysis.

3.6.5 Cell surface GLUT1 and GLUT4 determination

In order to determine the abundance of GLUT1 and GLUT4 at the cell surface, myotubes were treated with either FGF-21 or vehicle (DMSO). The incubation protocol previously described for glucose uptake in myotubes was used, followed by an additional incubation for 5 min at 18°C. Myotubes were then washed and biotinlyated with Krebs- Henseleit bircabonate buffer (KHB) supplemented with 5 mM HEPES and 0.1 % BSA with 100 µM Bio-LC-ATB-BGPA {4, 4 - O - [2 - [2 - [2 - [2 - [2 - [6 - (biotinylamino) hexanoyl] amino] ethoxy]ethoxy]ethoxy] -4 - (1-azi - 2, 2, 2, rifluoro-ethyl) benzoyl] amino-1, 3-propanedyl bis-D-glucose} for 8 min, followed by 3 min irradiation. Myotubes were then washed with PBS before being solubilized and scraped into 1 ml PBS with 2 % thesit ($C_{12}E_9$) and protease inhibitors. Cell extracts were rotated for 60 min in microtubes at 4°C, followed by 10 min centrifugation at 20,000 g. The supernatant was removed for protein measurement. Equal amounts of protein were mixed with 50 µl of PBS-washed streptavidin agarose beads (50% slurry; Pierce, Inc., Rockford, IL, USA). The streptavidin-biotin complex was incubated > 16 hours at 4°C with end-to-end rotation. The streptavidin agarose beads were then washed several times in PBS with varying concentration of thesit. Photolabeled glucose transporters were eluted from the streptavidin agarose beads by heating in 4 x Laemmli buffer for 20 min at 56°C. Proteins were separated by electrophoresis on pre-cast gels (biorad) and Western immunoblot analysis was performed to determine GLUT1 and GLUT4 cell surface abundance.

3.7 ANIMAL STUDIES

3.7.1 Animal models

For Study *II*, male wild-type C57BL/6 mice were maintained on a 12-h light-dark cycle and allowed free access to standard rodent chow. Mice were fasted 4 h prior to study. Mice were anaesthetized via an intraperitoneal injection of 2.5% avertin (0.02 mL/g of body weight), and the extensor digitorum longus (EDL) and soleus muscles were rapidly removed for *in vitro* studies, as described below. Mice were euthanized by cervical dislocation immediately after muscle dissection. The Ethics Committee on Animal Research in Northern Stockholm approved all experimental procedures.

3.7.2 Isolated skeletal muscle procedures

Following dissection, EDL and soleus muscles were incubated in oxygenated Krebs-Henseleit prebuffer at 30°C under a constant gas phase (95% O_2/5% CO_2) in separate vials. Muscles were exposed to either DMSO (vehicle control) or FGF21 (1 µg/mL) for 6 h. The final concentration of DMSO in all vials was adjusted to 2.25 mM. Media was refreshed every hour. During the last hour of the incubation, the muscles were also incubated in the absence or presence of insulin (0.36 nM Actrapid;

Novo Nordisk, Bagsværd, Denmark). Thus, the muscles have been subjected to four different conditions: basal (DMSO vehicle control), insulin, FGF21, or FGF21 and insulin. Finally, for measurement of glucose uptake, the muscles were incubated under the same conditions as described earlier. Thereafter, the muscles were incubated in a glucose-free pre-buffer for 10 min. They were then transferred to new vials containing pre-oxygenized Krebs–Henseleit buffer supplemented with 1 mM 2-deoxy-[1,2,3H]glucose (2.5 μCi/mL) and 19 mM mannitol and incubated for 20 min. After the incubation period, the muscles were washed in ice-cold Krebs-Henseleit buffer, blotted on filter paper, and quickly frozen with aluminium tongs pre-cooled in liquid nitrogen and stored at -80°C. Scintillation fluid containing 2-deoxyglucose was used to assess glucose uptake for 20 min at 30°C. The muscles were pulverized in microcentrifuge tubes over liquid nitrogen. Powdered muscle was homogenized in 0.4 mL of ice-cold lysis buffer [20 mM Tris (pH 8.0), 137 mM NaCl, 2.7 mM KCl, 10 mM NaF, 1 mM MgCl,1 mMNa3VO4, 0.2 mM PMSF, 10% glycerol, 1% Triton X-100, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin A]. Homogenates were rotated for 60 min at 4°C and subjected to centrifugation (20 000 g for 10 min at 4°C), and the supernatant was transferred to a second microcentrifuge tube for protein determination. Protein concentration was assessed using the Pierce method. Glucose transport activity is expressed as nM 2-deoxyglucose × mg/(protein*20 min).

3.8 EXPERIMENTAL ASSAYS AND ANALYSIS

3.8.1 Protein concentration assay and western blot analysis

Protein concentration for Western blot and other analyses was measured in homogenate prepared from skeletal muscle cells or biopsies by the Braford (Bio-rad, Richmond CA, USA) or Pierce (Rockford, IL, USA) methods. The assays were performed according to the respective manufacturer's instructions.

Aliquots of cell lysates were mixed with 4x Laemmli buffer and heated at 56°C for 30 min. Proteins were resolved by SDS-PAGE using pre-casted gels, and transferred to nitrocellulose membranes. Following protein transfer, membranes were blocked in 7.5% low fat milk in TBST at room temperature for 1 hour in order to prevent non-specific binding. Membranes were washed to remove milk solution and then incubated overnight at 4°C with antigen-specific antibodies. Membranes were washed 5 times with TBST and incubated with appropriate secondary antibodies (horseradish peroxidase-conjugated) for 1 hour at room temperature. Proteins were visualized by enhanced chemiluminescence and quantified by densitometry. Results were normalized to a respective total protein or GAPDH, a housekeeping protein.

3.8.2 Gene expression studies

Gene expression studies using real time polymerase chain reaction (RT-PCR) were used in Study *II* and *III*. mRNA expression was measured in *vastus lateralis* skeletal muscle, human and L6 myotubes using quantitative RT-PCR (ABIPRISM 7000 Sequence Detection System, Applied Biosystems). Total RNA was purified from skeletal muscle specimens using Trizol reagent (Invitrogen, Carlsbad, CA) and from human and L6 myotubes using RNeasy Mini Kit (Invitrogen). Purified RNA was treated with DNase using a DNA-free kit (Ambion), and cDNA synthesis was performed with SuperScript First Strand Synthesis system (Invitrogen). A SYBR green-based gene expression assay was used to assess mRNA (Origene). All samples were assayed in duplicate and values were compared against the housekeeping genes β-actin and 18S as internal controls. Standard curve and relative expression methods were used to quantify mRNA expression (Applied Biosystems).

3.9 STATISTICAL ANALYSES

In the Studies included in this thesis, data is presented as Mean ± SEM. Paired and unpaired Student *t*-tests were used to compare differences within and between groups, respectively. Data was examined for normality before performing any further statistical analysis and appropriate statistical tests were assigned for each set of data. Log transformation was performed on data sets that did not display normal distribution. Pearson's correlation analysis was used to establish relationships between two variables. Further details of statistical analysis are explained specifically in each separate study.

4 RESULTS AND DISCUSSION

The biological processes regulating glucose and lipid metabolism are critical to the maintenance of whole-body energy homeostasis. Appropriate intracellular glucose and lipid breakdown satisfies the intrinsic fuel requirement and thereby translates into systemic physiological health. Conversely, perturbations in this delicate substrate balance leads to impaired fuel utilization, which can ultimately manifest as ill health. Obesity and T2DM represent a culmination of imbalances in energy homeostasis that persist over time as a result of impaired glucose and lipid metabolism. Understanding both the cellular and whole-body regulation of substrate homeostasis in normal and insulin resistant states is key to the management of T2DM. A combination of experimental and clinical approaches to directly translate basic mechanistic discoveries to the treatment and prevention of T2DM is therefore warranted. To this end, the studies presented in this thesis were conducted in humans, animal models and *in vitro* cell systems to ensure a proper translation of basic scientific enquiries to clinical application.

4.1 CLINICAL IMPLICATIONS FOR EXERCISE-INDUCED BENEFITS IN T2DM

The exercise-induced benefits on glucose and lipid metabolism are of clinical relevance in the prevention and treatment of T2DM (Chibalin et al., 2000). Additionally, accumulating evidence stresses the importance of regular exercise as effective intervention against cardiovascular risk factors (Tsuruzoe et al., 2001), obesity (Feldstein et al., 2008; Henquin, 2009; Henquin et al., 2009; Longo et al., 2008), and impaired insulin metabolism in T2DM (Corpeleijn et al., 2009;). Aerobic exercise is regarded as a highly effective method of physical activity to improve insulin sensitivity. However, greater consideration of a minimal and more realistic exercise intervention that is practically applicable in a normal primary care setting is warranted. In Study *I*, the effects of a moderate, unsupervised exercise intervention (Nordic walking) has been investigated on cardiovascular risk factors in overweight individuals with NGT, IGT and T2DM.

4.1.1 Pronounced beneficial effects of Nordic walking in normal as compared to impaired glucose metabolism.

The vast majority of exercise intervention studies involve relatively intense, expensive, and closely supervised exercise programs that demand a large number of personnel and considerable resources. Often, these exercise programs do not reflect lifestyle interventions that are achievable in a normal primary care setting. Studies aimed at defining a physical activity level that is more realistic in terms of time and effort for older sedentary individuals, yet still confers vital health benefits, are therefore warranted. Nordic walking is a validated moderate exercise modality, which is easy to perform. Importantly, Nordic walking is associated with improved adherence (Figard-Fabre et al., 2011) and is associated with low risk for injury or other complications. The focus of Study *I* was to determine the effects of a low-moderate, unsupervised exercise intervention on cardiovascular risk factors and metabolic control in a traditional clinical setting of a local primary health care center. The hypothesis that Nordic walking (Church et al., 2002), will reduce cardiovascular risk and improve metabolic control was tested in overweight people with NGT, IGT and T2DM.

4.1.1.1 Clinical characteristics of the study volunteers

A total of 213 subjects were included and classified on the basis of an oral glucose tolerance test (OGTT) into either NGT (n=128), IGT (n=35) or T2DM (n=50). The participants in each category (NGT, IGT and T2DM) were randomized to the exercise or control group and asked to maintain their usual eating habits. Baseline clinical characteristics for the study participants are presented in Table 1 of article number 1 at the articles section. Self-reported high-intensity physical activity was more frequent in the NGT control group than in the NGT intervention group. Subjects in the three cohorts were age and BMI-matched, but some slight differences across intervention groups in several clinical parameters were present. The total cholesterol level was lower and lipid-lowering statin medication was more frequent in the NGT intervention group, compared with the NGT control group. The triglyceride level was lower in the T2DM control group, but statin medication did not differ in comparison with the T2DM intervention group. There were no other significant differences between the control and intervention groups.

4.1.1.2 Effect of regular low-moderate intensity exercise (Nordic walking).

The aim of the study was to achieve cardio-protective benefits and better metabolic control in a cohort of sedentary subjects by implementing a relatively moderate-intensity exercise modality in a normal primary health care setting. A previous study examining a cohort of individuals with similar clinical characteristics to the present cohort provided evidence that four months of Nordic walking intervention, performed at intervals of 45-60 min x 3 times per week, was insufficient to improve cardiovascular risk factors in T2DM (Fritz et al., 2006). Therefore, increasing the frequency and intensity of exercise may be necessary to induce health benefits in T2DM. Compared to the previous study (Fritz et al., 2006), the current study participants increased not only the exercise intensity, but also number of individual exercise sessions.

Self-reported physical activity and anthropometric parameters (BMI and waist circumference) were improved after 4 months of regular walking in the NGT-exercise group compared to NGT-control group. Exercise capacity was improved in the IGT-exercise group compared to the IGT-control group. Four months of Nordic walking did not result in any significant difference between the T2DM-exercise and T2DM-control group. However, improvements in HbA1c, 2 hour glucose and exercise power output was noted in NGT, IGT and T2DM individuals who reported ≥80% of the prescribed recommended amount of Nordic walking in their exercise diaries. Together, these findings provide evidence for a varying response to a similar exercise modality in NGT, IGT and T2DM individuals, and stress the importance of adherence to achieving better cardio-protective benefits.

Overall, the clinical exercise intervention study (Paper II) highlights that a 4-month low-moderate intensity Nordic walking program improves body weight, BMI, and waist circumference in overweight people with NGT. Improvements in weight, BMI and waist circumference constitute cardio-protective benefits. Moreover, in participants who reported ≥80% of the prescribed exercise, HbA1c, 2 h glucose, and exercise capacity improved, underscoring the importance of adherence to achieving favorable metabolic control (Saltiel and Kahn, 2001).

4.1.1.3 Clinical implications

Visceral fat is a predictor of mortality and waist circumference may be one simple measure of cardiovascular risk to monitor improvement following a period of lifestyle intervention. The findings presented here suggest that exercise intervention has a more pronounced effect on the anthropometric risk factors in people who do not have derangements in glucose metabolism.

Although Nordic walking exercise improves cardiovascular risk-factors in overweight T2DM participants, participants with NGT achieved greater improvements (*paper II*). A three month Nordic walking program reduces fat mass and blood pressure in obese people with NGT (Figard-Fabre et al., 2011). Moreover, previous study has reported improvements in fat mass, but not HbA1c in T2DM patients after completing a four month Nordic walking program (Gram et al., 2010). In paper *II* improvements in metabolic control were reported in obese participants after four months of Nordic walking in exercise responders but not in non-responders. Although varying frequency, intensity and adherence to exercise protocols may explain the inconsistencies in exercise effects, intrinsic factors governing exercise-response/non-response need further investigation.

The results presented here may indicate that the ability to respond to lifestyle intervention, such as exercise, is more effective during the early stages of the T2DM pathogenesis. Moreover low motivation and musculoskeletal complications that may occur in T2DM result in lack of adherence to exercise programs (Saltiel and Kahn, 2001). Together these observations highlight the importance of early exercise intervention in the prevention and treatment of T2DM.

4.1.1.4 Study limitation

A low-cost moderate intensity exercise was investigated in the current study. Higher intensity exercise protocols such as aerobics and/or resistance training can result in a more beneficial outcome (Roumen et al., 2008; Sigal et al., 2006; Snowling and Hopkins, 2006); however positive effects on cardiovascular risk factors could still be achieved with this relatively low level of physical activity. Even though more pronounced effects were noted in the NGT group. However, IGT and T2DM participants who reported good compliance achieved a better metabolic control in a number of clinical parameters. Of clinical relevance, this study could be achieved without occurrence of musculoskeletal complications which is more prevalent in T2DM (Arkkila and Gautier, 2003). Thus, Nordic walking if performed regularly, might offer a safe mode of introductory exercise, even in T2DM.

While the current study highlights Nordic walking improves cardiovascular risk factors in people with varying degrees of glucose tolerance, the following study limitations need to be considered when interpreting the data. First individual participants reported a considerably varying physical activity levels at baseline. This might have affected the outcome hence explaining part of the variation in Nordic walking effects. Furthermore this study involved unsupervised, self-reported exercise and whether the participants in the intervention group added Nordic walking in their pre-existing daily activity or replaced their daily activities with Nordic walking is not known.

Secondly, the relatively small number of study participants might have affected the statistical power. A greater number of participants would have strengthened the statistical power, hence increasing the chances of attaining significance in many tested parameters. Lastly while food intake can affect the study outcomes, individual food intake was not addressed in this study, despite the possibility of increased food intake

with increased physical activity levels. Of note, this study aimed at investigating physical activity *per se*, and not lifestyle modification.

4.2 SYSTEMIC REGULATORS OF GLUCOSE AND LIPID METABOLISM

Various hormones, cytokines, and growth factors circulating in serum play a role in the regulation of glucose and lipid metabolism (Pittas et al., 2004). These factors exert their effects in an autocrine, paracrine and/or endocrine manner; hence, their effects are typically systemic. Fibroblast growth factor 21 (FGF-21) is an endocrine metabolic regulator with systemic effects on both glucose and lipid metabolism. Reports from studies conducted in animal models and *in vitro* systems have provided evidence for the role of FGF-21 in the regulation of glucose and lipid metabolism in liver, adipose tissue and pancreas (Coskun et al., 2008; Hotta et al., 2009; Kharitonenkov et al., 2005; Kharitonenkov et al., 2007; Kralisch and Fasshauer, 2011; Sarruf et al., 2010; Wente et al., 2006). Whether FGF-21 has a direct effect on glucose metabolism in skeletal muscle is still unknown. The focus of Study *II* was therefore to investigate the direct effects of FGF-21 on glucose metabolism in skeletal muscle. Furthermore, using serum from NGT and T2DM subjects, the aim was to establish the relationship between circulating FGF-21 levels and clinical parameters related to glucose and lipid metabolism. The findings in this study provide further support for the clinical significance of this novel *tissuekine* in the diagnosis and treatment of obesity and T2DM.

4.2.1 FGF-21 as a metabolic regulator of glucose metabolism in skeletal muscle

4.2.1.1 FGF-21 has a direct effect on glucose uptake in human skeletal muscle

Owing to its endocrine properties, FGF-21 circulates in the serum and exerts its effects in a variety of tissues and organs such as liver, pancreas, and skeletal muscle. The beneficial effects of this endocrine regulator on glucose metabolism in liver and adipose tissue are well established (Coskun et al., 2008; Hotta et al., 2009; Kharitonenkov et al., 2005; Kharitonenkov et al., 2007; Kralisch and Fasshauer, 2011; Sarruf et al., 2010; Wente et al., 2006). Skeletal muscle is quantitatively an important tissue in glucose metabolism (Wannamethee et al., 2011d). However, evidence related to the the metabolic effects of FGF-21 on glucose uptake in skeletal muscle is lacking.

Cultured human skeletal muscle and isolated rodent muscle was used to determine whether FGF-21 could directly regulate glucose uptake. Differentiated myotubes were exposed to 1 μg/ml of recombinant FGF-21 and glucose uptake was measured after 6 and 24 hours. FGF-21 increased basal glucose uptake in a time-course dependent manner. Glucose uptake tended to increase after 6 hours and was increased after 24 hours of exposure with FGF-21. In cultured murine adipocytes, FGF-21 enhances glucose uptake as a result of an increased GLUT1 mRNA and protein expression (Kharitonenkov et al., 2005). To determine whether a similar effect occurs in skeletal muscle cells, GLUT1 mRNA was measured in cultured myotubes and cell surface photolabelling was assessed to determine the relative abundance of GLUT1 and GLUT4 proteins at the plasma membrane. GLUT1 mRNA expression was increased following 24 hours exposure to FGF-21. Moreover, exposure to FGF-21 increased the relative abundance of GLUT1 protein on the cell surface. To determine whether FGF-21 has an additive effect with insulin on glucose uptake, myotubes were exposed to FGF-21 or DMSO as control for 6 or 24 hours and measured glucose uptake under basal and insulin-stimulated states. Indeed, both 6 and 24 hour FGF-21 exposure had an

additive effect with insulin on glucose uptake. These results provide evidence for a synergistic action between insulin and FGF-21 on glucose uptake.

Based on the observations from human cell culture system, these findings were validated in whole skeletal muscle. Using an *in vitro* muscle glucose uptake assay, isolated mouse EDL muscle was pre-exposed to FGF-21 for 6 hours, followed by 20 min incubation with insulin. Consistent with the findings in cultured human myotubes, insulin-stimulated glucose uptake was increased in EDL muscle pre-exposed with FGF-21. Conversely, FGF-21 was without effect on the basal glucose uptake in EDL muscle.

Taken together, FGF-21 increased glucose uptake in both the absence and presence of insulin in primary human myotubes,. However, in isolated mouse skeletal muscle, FGF-21 only increased glucose uptake under insulin-stimulated conditions. These disparities may be related to the FGF-21 exposure time or intrinsic differences between cultured and whole skeletal muscle. In cultured human muscle cells, FGF-21 promotes increased protein abundance of cell surface GLUT1, but not GLUT4 protein. As cultured muscle has a higher GLUT1: GLUT4 ratio as compared to whole muscle, this may explain the enhanced sensitivity to FGF-21 on glucose uptake in cultured muscle. Furthermore, in cultured human myotubes, the most pronounced effect of FGF-21 on basal glucose uptake was noted after 24 hour, a time point that is challenging to establish in *in vitro* muscle incubation experiments. Alternatively, primary muscle cultures are derived from muscle satellite cells, which do not fully recapitulate whole muscle (Al-Khalili et al., 2004b); consequently, this could also explain the discrepancies between the model systems.

4.2.1.2 Therapeutic implications in treatment of T2DM.

The results presented in Study *II* are consistent with the reported metabolic effects of FGF-21 in adipose tissue (Kharitonenkov et al., 2005). FGF-21 increases glucose uptake in mouse 3T3-L1 cells, and in primary human adipocytes (Kharitonenkov et al., 2005). Here, these findings are extended to skeletal muscle, a quantitatively important tissue in glucose homeostasis. Previous studies reported improvements in metabolic profiles in obese animal models following treatment with FGF-21. Enhanced β cell function in the pancreas and regulation of lipid and glucose metabolism in liver are among other reported beneficial effects of FGF-21(Coskun et al., 2008; Hotta et al., 2009; Kharitonenkov et al., 2005; Kharitonenkov et al., 2007; Kralisch and Fasshauer, 2011; Sarruf et al., 2010; Wente et al., 2006). Collectively, these findings suggest that FGF-21 could act as a potential therapeutic option for the treatment of T2DM.

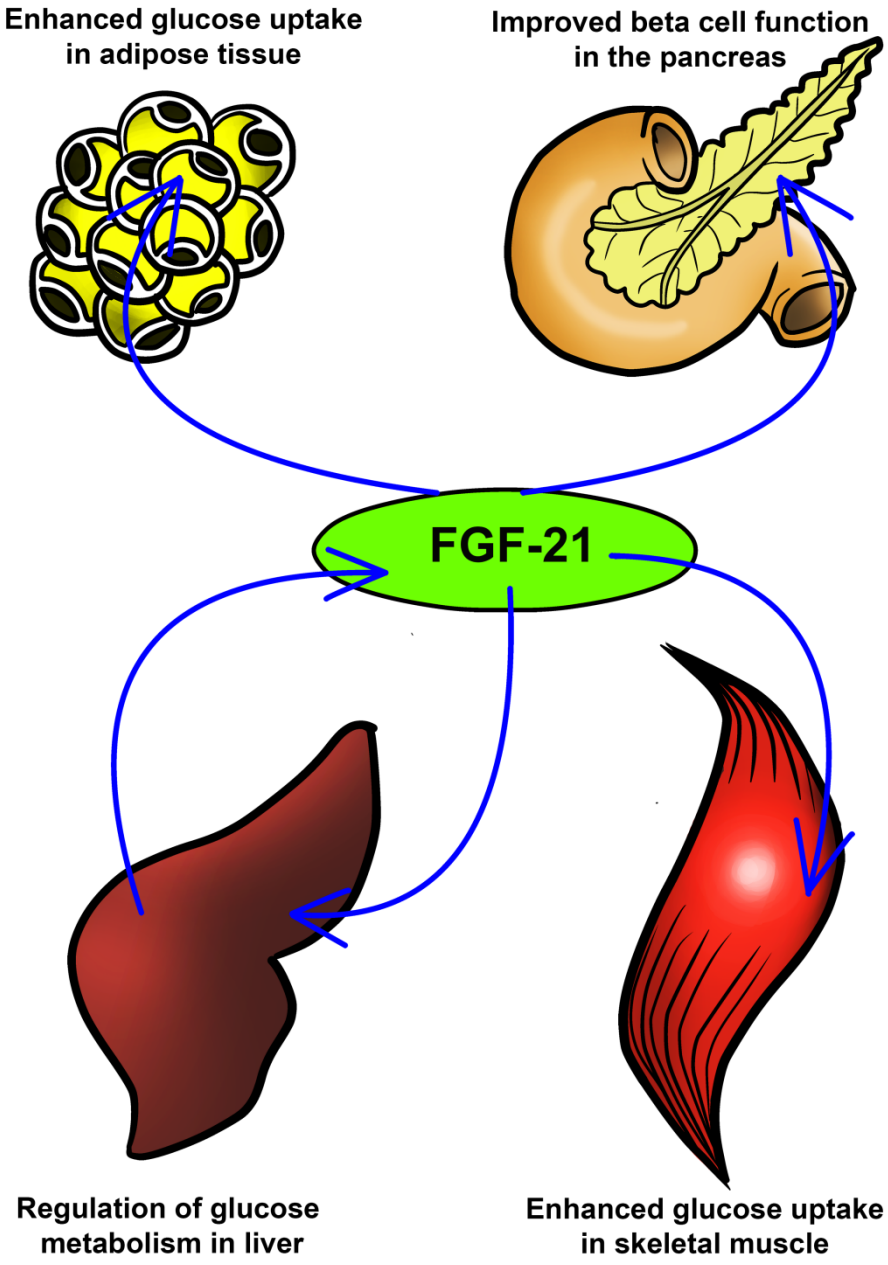

Figure 7: Effects of FGF-21 on glucose metabolism. Systemic FGF-21 circulation promotes enhanced glucose uptake in skeletal muscle and adipose tissue, improved β cell function in pancreas, and greater regulation of glucose and lipid metabolism in liver.

4.2.2 FGF-21 as a potential biomarker

4.2.2.1 FGF-21 serum levels are increased in T2DM.

Liver and adipose tissue are the main source of FGF-21, but skeletal muscle and thymus (Hojman et al., 2009; Mraz et al., 2009; Nishimura et al., 2000) have also been reported to secrete FGF-21. The relative tissue-specific contribution to serum FGF-21 levels is unknown. A number of clinical and epidemiological studies have analyzed circulating FGF-21 and described the relationship to diabetes and other pathologies related to glucose homeostasis. Serum levels of FGF-21 are higher in states of

abnormal glucose metabolism as compared to normal states (Hojman et al., 2009; Mraz et al., 2009; Semba et al., 2012). In Study *II*, levels of FGF-21 were found in T2DM patients compared to age and BMI-matched normal glucose tolerant subjects, confirming the previously observed paradox. Higher FGF-21 levels have also been found in insulin resistant adults (Semba et al., 2012). Furthermore, a positive association between FGF-21 serum levels and markers of diabetes complications has been reported in clinical and epidemiological studies (An et al., 2012; Jian et al., 2012). FGF-21 is an independent marker for the presence of the metabolic syndrome in obesity in adults (Tyynismaa et al., 2011; Zhang et al., 2010; Zhang et al., 2008). In the cohort examined in Study *II*, serum FGF-21 levels were significantly greater in T2DM patients in the tertile of subjects presenting the highest fasting insulin and BMI. Moreover, BMI was identified as an independent predictor of serum FGF-21 levels. Recent studies reported an increase in levels of FGF-21 in serum and a positive correlation with intra-hepatic lipid content in NAFLD, reflecting the ability of FGF-21 to independently predict liver steatosis (Dushay et al., 2010; Li et al., 2010; Yan et al., 2011; Yilmaz et al., 2010). Collectively these findings suggest that FGF-21 could act as a potential biomarker for metabolic diseases.

4.2.2.2 Clinical implications in obesity and T2DM.

Based on the positive effects of FGF-21 on glucose and lipid metabolism, the paradoxical higher levels of FGF-21 in serum from people with disturbed glucose homeostasis may reflect a compensatory mechanism as a physiological defense against dysregulated state. A state of FGF-21 resistance may also account for its higher levels in obesity and T2DM. Obesity is an "FGF-21-resistant" state (Fisher et al., 2010). In Study *II*, parallel reduction in BMI and FGF-21 serum levels was noted following a four month lifestyle intervention that involved regular walking in T2DM patients (figure 8). This was accompanied by concomitant improvements in insulin sensitivity. Changes in FGF-21 serum levels following lifestyle and pharmacological interventions have been reported elsewhere (Cuevas-Ramos et al., 2012; Dutchak et al., 2012; Fletcher et al., 2012; Wei et al., 2012). Thus, because FGF-21 levels are dynamically influenced by various intervention modalities, it could be used as a marker to monitor clinical progress following an intervention. Furthermore, the continuously increasing levels of FGF-21 with increase in the severity of obesity and insulin resistance observed in Study *II* and other published reports suggests that FGF-21 may be used to monitor the progression of obesity and T2DM. However the validity of this notion requires further investigations in prospective follow-up studies.

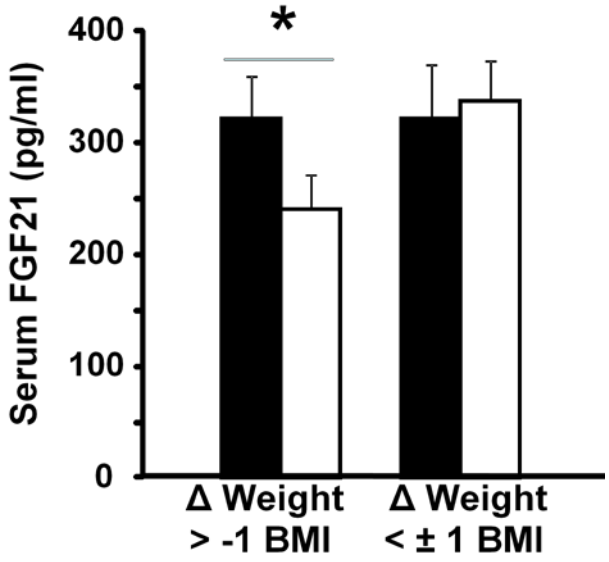

Figure 8: Serum FGF21 concentrations before and after four months of participation in an adult fitness program (low-moderate intensity exercise). The T2DM subjects were divided into two groups based on whether or not they achieved an improvement in BMI of at least one unit (n=10 in each group). Solid and empty bars represent before and after four months of exercise intervention respectively. Results are presented as mean ± SEM *p-value <0.05, ** <0.01, *** <0.001.

4.2.2.3 Study limitation

The cross-sectional nature of Study *II*, including the correlation analysis used to establish associations between different parameters might not explain causality. Furthermore the relatively small sample size used to analyze FGF-21 serum levels might limit the translation of this result to situations beyond the scope of this study. However, the findings in this study and others provide important information on the metabolic effects of FGF-21, including its clinical significance. This information will aid in the design and execution of more comprehensive prospective follow-up studies in order to provide a better understanding of the novel metabolic regulator, FGF-21.

4.3 MOLECULAR REGULATORS OF GLUCOSE AND LIPID METABOLISM IN SKELETAL MUSCLE.

High levels of circulating metabolites such as FFAs and cytokines significantly contribute to the development of insulin resistance in liver and skeletal muscle. These metabolites signal through STAT3 and regulate a variety of biological processes. The involvement of STAT3 in the development of insulin resistance in liver and adipose tissue has previously been reported (Inoue et al., 2004; Kim et al., 2007). Whether this transcription factor plays a role in the development of skeletal muscle insulin resistance and T2DM is incompletely understood.

4.3.1 STAT3 is constitutively phosphorylated in skeletal muscle from T2DM patients

4.3.1.1 Clinical characteristics of the study subjects

In Study *III*, 20 overweight, but otherwise healthy participants with normal glucose tolerance (NGT) and 20 T2DM patients were selected from a primary health care clinic. The NGT and T2DM participants were matched for age and BMI. Individuals on insulin or with symptomatic coronary heart disease were excluded. Venous blood was collected for standard clinical chemistry analysis and *vastus lateralis* skeletal muscle biopsies was obtained from the participants following an overnight fast, as described earlier (Al-Khalili et al., 2003).

4.3.1.2 Increased STAT3 phosphorylation in T2DM

Protein abundance and phosphorylation of STAT3 was measured in skeletal muscle biopsies from NGT and T2DM subjects. Phosphorylated STAT3 was increased in skeletal muscle biopsy from T2DM patients, compared to age- and BMI-matched NGT subjects (Figure 9A). Protein phosphorylation of JAK2, an upstream regulator of STAT3, was also increased in skeletal muscle from T2DM patients (Fig. 9*B*). To further investigate the JAK/STAT pathway downstream of STAT3, SOCS3 mRNA and protein abundance was measured in muscle from similar cohort. Increased SOCS3 mRNA and protein abundance was observed in biopsies from T2DM patients. Interestingly, p-STAT3 protein positively correlated with SOCS3 protein and mRNA expression in individuals with NGT and T2DM. Since STAT3 is involved in adipogenesis (Zhang et al., 2011), obesity may directly influence STAT3 signaling. However, these findings are consistent with an observed increase in skeletal muscle p-STAT3 abundance in non-obese people with IGT (Kim et al., 2011). Furthermore, to avoid misinterpretation of the effect of obesity, the subjects were matched for BMI. Thus, aberrant skeletal muscle STAT3 signaling appears to be an early marker of insulin resistance that precedes clinical diagnosis of T2DM.

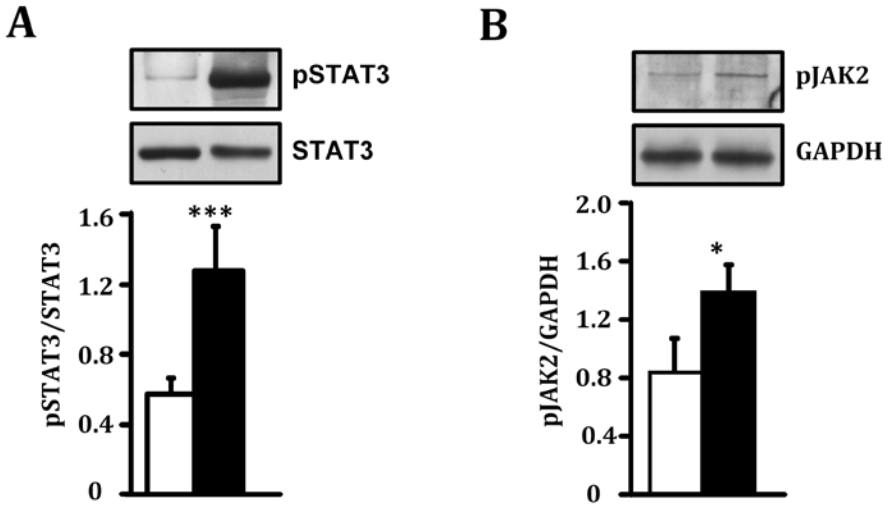

Fig 9: Protein phosphorylation in skeletal muscle from people with normal glucose tolerance or T2DM. Phosphorylation of **(A)** STAT3 **(B)** JAK2. Normal glucose tolerance (*Open Bar*) and T2DM (*Closed Bar*), n = 20 subjects. Results are mean ± SEM. ***P < 0.001, **P< 0.01 and *P < 0.05 vs. NGT, respectively.

4.3.1.3 A link between increased JAK/STAT signaling and insulin resistance

SOCS3, a key player linking the JAK/STAT pathway to insulin signaling, is implicated in the development of insulin resistance in obesity and T2DM (Howard and Flier, 2006). SOCS3 protein and mRNA are increased in skeletal muscle from severely obese or T2DM patients, compared to lean people with normal glucose tolerance (Rieusset et al., 2004). Consistently, an upregulation of SOCS3 protein abundance and mRNA expression was observed in skeletal muscle from T2DM patients, supporting a link between aberrant signal transduction and reduced insulin sensitivity. A positive association between p-STAT3 and SOCS3 protein and mRNA levels in normal glucose tolerance and T2DM was also observed, suggesting a physiological link between phosphorylation of STAT3 and SOCS3 induction. In liver, STAT3 phosphorylation upregulates SOCS3 protein and subsequently causes insulin resistance (Kim et al., 2009a; Kim et al., 2008). In Study *III*, we extend these findings to skeletal muscle and to states of T2DM.

4.3.2 STAT3 phosphorylation and skeletal muscle insulin resistance

4.3.2.1 Association between circulating FFA and STAT3 phosphorylation in skeletal muscle.

Elevated FFA serum levels are associated with insulin resistance and T2DM. Several other molecules circulating in serum such as Tumor necrotic factor alpha (TNFα), negatively regulate insulin signaling in skeletal muscle and cause insulin resistance. To investigate the possible cause of the increased STAT3 phosphorylation in T2DM at the whole body level, various clinical parameters were measured and correlation analysis was performed. Plasma FFA level was positively correlated with skeletal muscle p-STAT3 abundance, and inversely correlated with measures of insulin sensitivity in normal glucose tolerance individuals. However, despite the finding of elevated FFA levels and insulin resistance in T2DM, the correlation between FFA and p-STAT3 observed in NGT subjects was lost with T2DM. Plasma FFA level accounted

for greatest variation in skeletal muscle p-STAT3 abundance, highlighting a relationship between circulating FFAs, STAT3 phosphorylation, and measures of insulin sensitivity. The positive association between circulating FFA levels and p-STAT3 might indicate that FFAs are indeed the cause of increased STAT3 phosphorylation in T2DM. Several nutrients and circulating metabolites have been linked to STAT3 activation in different tissues (He et al., 2006; Kim et al., 2008; Rieusset et al., 2004; Senn et al., 2003). Given the clinical evidence that elevated FFA levels are a biomarker for the conversion from IGT to T2D (Charles et al., 1997; Paolisso et al., 1995), interventions that lower FFA may prevent excessive p-STAT3 and maintain appropriate insulin signaling responses in skeletal muscle to control glucose and lipid metabolism.

4.3.2.2 Palmitate induces insulin resistance via STAT3 phosphorylation

Phosphorylation and subsequent activation of STAT3 has been reported in cultured myotubes exposed to either FFAs (Weigert et al., 2004) or IL-6 (Kim et al., 2011; Weigert et al., 2004). To determine whether these systemic factors cause skeletal muscle insulin resistance via a STAT3-mediated mechanism,the direct effect on L6 cultured myotubes was studied. Exposure of cultured myotubes to palmitate resulted in a slow, but persistent phosphorylation of STAT3 and reduced insulin-stimulated Akt phosphorylation. However, IL-6 exposure resulted in a rapid, but transient phosphorylation of STAT3, without altering insulin action on p-Akt abundance. This time-related difference in STAT3 phosphorylation between IL-6 and palmitate exposure may explain the divergent effects between these two stimuli on insulin signaling. Interestingly, the slow but persistent phosphorylation of STAT3 resulted into impairments in insulin signaling as opposed to the rapid, acute STAT3 phosphorylation. This finding may suggest that chronic but not acute STAT3 phosphorylation is a culprit in the pathogenesis of insulin resistance in skeletal muscle.

4.3.3 STAT3 as a potential therapeutic target

Studies performed in tissue-specific knockout mice reveal that STAT3 plays a role in the development of insulin resistance in liver (Inoue et al., 2004). Using siRNA, the direct role of STAT3 on lipid-induced insulin resistance in skeletal muscle was determined. A parallel down-regulation of SOCS3 protein abundance was observed following STAT3 silencing. Palmitate exposure triggered STAT3 phosphorylation, consequently causing a reduction in insulin-stimulated Akt phosphorylation and glucose incorporation into glycogen. Importantly, STAT3 silencing prevented the palmitate-induced increase in SOCS3 protein abundance, as well as the lipid-induced reduction in insulin-stimulated Akt phosphorylation and glucose incorporation into glycogen. This finding that STAT3 silencing improves insulin sensitivity is consistent with earlier parallel findings in liver hepatocarcinoma cells and human myotubes following treatment with amino acids or IL-6, respectively (Kim et al., 2009a; Kim et al., 2008; Kim et al., 2009b; Kim et al., 2011). (Kim et al., 2009a; Kim et al., 2008; Kim et al., 2009b; Kim et al., 2011). Collectively these findings suggest that STAT3 could act as a potential therapeutic target for T2DM. However, tissue-specific effects of STAT3 silencing require further clarifications.

STAT3 is constitutively phosphorylated in skeletal muscle from T2DM patients. Chronic elevation in circulating metabolites like FFAs is likely to cause a persistent phosphorylation of STAT3 and negatively impact skeletal muscle insulin signaling and glucose uptake. siRNA-mediated silencing of STAT3 protein prevents the development of lipid-induced insulin resistance in skeletal muscle. Therefore, interventions targeting STAT3 directly or focused towards normalizing elevated

circulating metabolites might prevent the development of skeletal muscle insulin resistance early in the pathogenesis of T2DM.

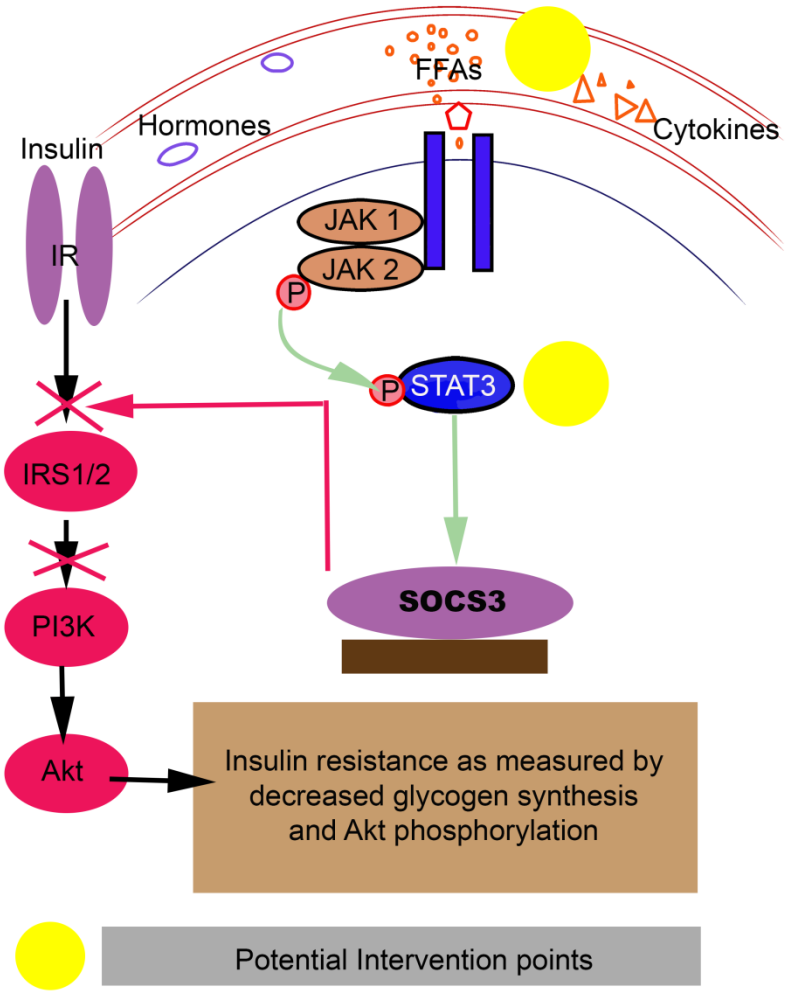

Fig 10: Lipid-induced skeletal muscle insulin resistance. Elevated levels of FFA cause constitutive phosphorylation of STAT3, resulting in the induction of SOCS3 protein and consequently causing insulin resistance through negative regulation of insulin signaling. Interventions targeting STAT3 or lowering FFAs levels in serum may have significant effects in T2DM prevention and treatment.

4.4 SUMMARY OF FINDINGS

Obesity and T2DM are metabolic disorders characterized by impaired glucose and lipid homeostasis. Impairments in the normal physiological regulation of glucose and lipid metabolism, resulting from both genetic and environmental factors, cause hyperglycemia which triggers clinical diabetes. Studies presented in this thesis, which are aimed at investigating the regulation of glucose and lipid metabolism in skeletal muscle and serum, describe molecular interactions in a whole-body physiology context and evaluate the corresponding clinical implications in obesity and T2DM.

- In Study *I*, the effects of a moderate-intensity exercise on cardiovascular risk factors in overweight individuals with T2DM are reported. The beneficial effects of moderate physical activity on cardiovascular risk factors were more pronounced in normal glucose tolerant individuals as compared to people with impaired glucose tolerance. This highlights the importance of early lifestyle intervention (exercise) in the treatment of T2DM.
- In Study *II*, a paradoxically higher level of FGF-21 in obesity and T2DM was reported. Evidence for the role of this novel endocrine regulator on glucose metabolism in skeletal muscle was provided. The findings in Study *II* extend the metabolic effects of FGF-21 to skeletal muscle, a qualitatively important tissue in glucose homeostasis.
- In Study *III*, a differential regulation of STAT3 in normal and T2DM states was reported. Study *III* provides evidence that constitutive STAT3 phosphorylation plays a role in the development of lipid-induced insulin resistance in skeletal muscle. Indeed, silencing of STAT3 in L6 myotubes prevents palmitate-induced insulin resistance, as measured by glycogen synthesis and p-Akt. Targeting STAT3 in skeletal muscle could therefore present therapeutic benefits in T2DM.

Collectively, substrate (glucose and lipid) regulation and its clinical significance in obesity and T2DM has been described. Metabolic characteristics from the whole body physiology perspective into specific systemic modulators in serum has been analyzed. The studies presented in this thesis dissect basic molecular mechanisms that fuel the pathogenesis of T2DM in skeletal muscle, as summarized in Figure 11.

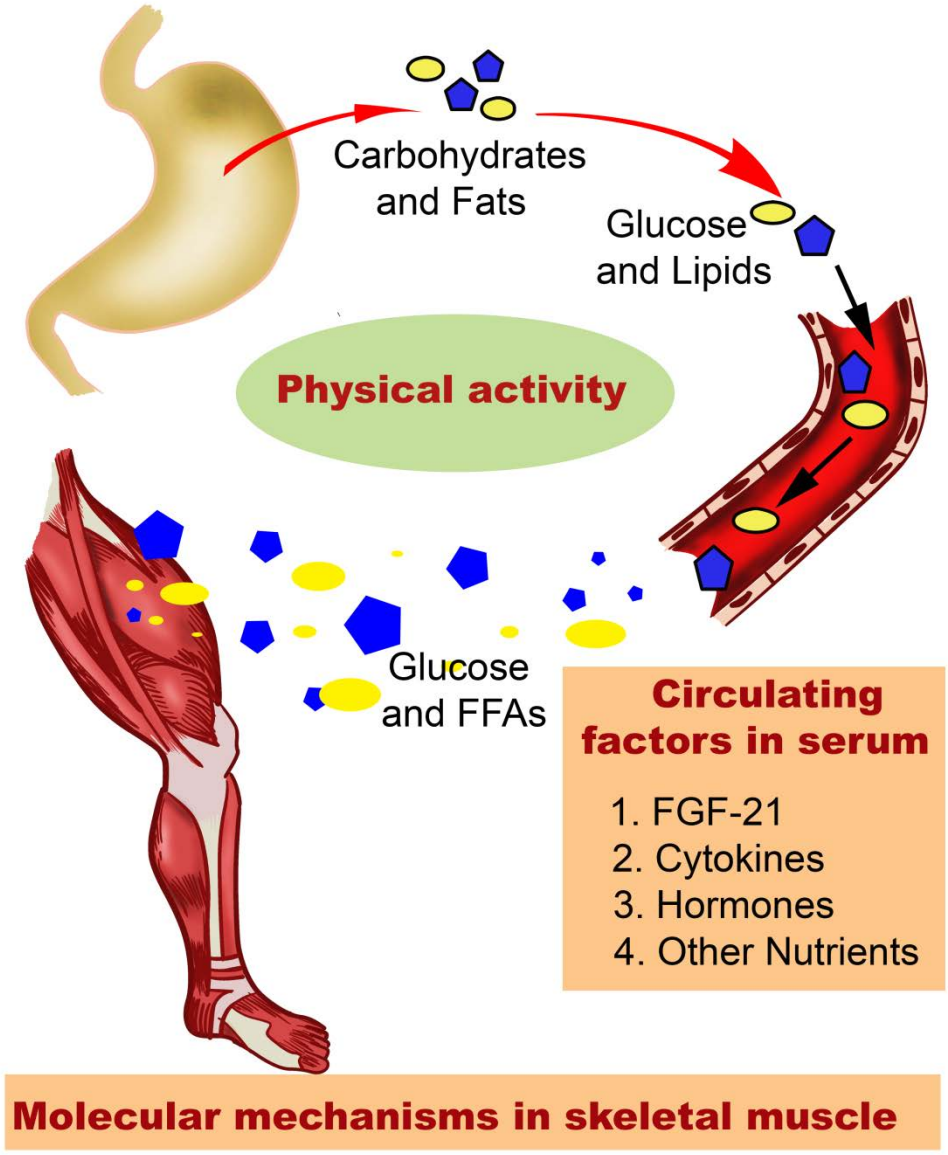

Figure 11. Regulators of glucose and lipid metabolism investigated in this thesis. Physical activity has positive effects on glucose and lipid metabolism. These effects are noted at the systemic level. Circulating factors like FGF-21 target different organs and tissues and also regulate glucose and lipid metabolism. Skeletal muscle is an important consumer of both glucose and lipids, and thus crucial for the regulation of these two key substrates.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

The overall objective of work presented in this thesis was to investigate the regulators of glucose and lipid metabolism in skeletal muscle and serum, describe their interactions and finally, evaluate their clinical implications in obesity and T2DM. The approach taken involved investigating the interaction between physical exercise and glucose metabolism at the whole-body level. The studies were advanced to undertake a more specific investigation of a novel regulator in serum, and finally to dissect the molecular mechanisms involved in the development of insulin resistance and T2DM in skeletal muscle.

Studies from this thesis provide evidence for differential effects of low-moderate exercise (Nordic walking), in subjects with normal and impaired glucose tolerance. Anthropometric variables related to cardiovascular risk factors improved in NGT individuals, but not in those with impaired glucose tolerance. This underscores the importance of early lifestyle intervention in the prevention of cardiovascular complications in overweight individuals. Nordic walking offers a safe mode of exercise that can easily be tolerated with T2DM patients. The findings reported in Study *I* show that, with high compliance, individuals with T2DM can also achieve significant metabolic improvements with Nordic walking. Indeed, in Study *II*, T2DM patients who underwent exercise intervention and responded by lowering their BMI, were able to improve metabolic control, as well as trigger a decrease in serum FGF-21 levels. While differences in compliance could explain the varying effects of exercise in different individuals, other extrinsic and intrinsic factors could play a paramount role in this biological phenomenon. Future research should therefore focus on identifying exogenous and endogenous regulators of exercise response and non-response.

Treatment of T2DM is relatively challenging. The available pharmacological agents have limited efficacy and mechanism-based side effects. An urgent need for safe and more effective agents has stimulated research in the field, and a number of novel molecules with therapeutic potential are continuously being identified. Current evidence points to FGF-21 as a novel metabolic regulator with therapeutic potential in the treatment of T2DM. Earlier studies investigating FGF-21 support its role in glucose and lipid metabolism in liver, adipose tissue and pancreas. Study *II* extends the findings to skeletal muscle. Mechanisms governing FGF-21-dependent glucose uptake previously described in adipose tissue were shown to also occur in skeletal muscle. Results from the analysis of FGF-21 in serum confirmed the earlier reported paradox of higher FGF-21 levels in obesity and T2DM. This phenomenon is hypothesized to arise from FGF-21 resistance that occurs in obesity and T2DM. Indeed moderate intensity exercise, which resulted in a minimal weight loss, lowered the levels of FGF-21 in serum of T2DM patients who participated in Study *II*. Whether the decreased FGF-21 serum levels was a result of a decrease in its production due to decreased fat mass, or improvements in FGF-21 resistance *per se*, is a question for further research.

Future research on the metabolic regulator FGF-21 should address the reported paradox on its serum levels in obesity and T2DM. Even though the available evidence implicates FGF-21 resistance, the possibility of increased FGF-21 serum levels as a compensatory mechanism against impaired metabolism, should not be overlooked. However, in both cases, serum FGF-21 levels could reflect a state of impaired glucose and lipid metabolism, a phenomenon that can be harnessed as a biomarker. Investigating the role of FGF-21 as a potential biomarker should constitute future research opportunities.

The mechanisms involved in the pathogenesis of skeletal muscle insulin resistance in T2DM remain incompletely resolved. A wide array of nutrients and

hormones interact with insulin signaling via complex pathways and cause insulin resistance in skeletal muscle. Until recently, the involvement of STAT3 in the development of insulin resistance was known to involve only the liver and adipose tissue. A recent study showed that STAT3 is involved in the development of cytokine-induced insulin resistance in skeletal muscle. Indeed, the findings in Study *II* confirmed the involvement of STAT3 in the development of skeletal muscle insulin resistance. This finding was further extended to T2DM pathogenesis. Constitutive STAT3 phosphorylation appears to be involved in lipid-induced skeletal muscle insulin resistance since silencing STAT3 in cultured rat myotubes could prevent palmitate-induced insulin resistance. STAT3 could therefore present a potential drug target for treatment of T2DM. Furthermore, these findings provide evidence that early intervention aimed at normalizing FFA levels in serum could prevent the development of insulin resistance.

Collectively, the work presented in this thesis emphasizes the importance of understanding various regulators of glucose and lipid metabolism from the whole body physiology context to molecular mechanisms in skeletal muscle. Metabolic alterations result from the interplay between biological processes within the cells, tissues and organs. These alterations may translate into ill health such as T2DM. Translational studies involving both molecular and clinical studies will help to identify molecules with both clinical significance and therapeutic potential. Identification of these molecules is crucial for the fight against obesity and T2DM.

6 ACKNOWLEDGEMENTS

The whole process towards the production of this thesis required dedication, team work, supervision, financial support and, above all, the right motivation. This would not have been possible without the support of different people from within and outside the scientific community. I wish to express my immense gratitude and thanks to people whose helping hands were always there for me.

I wish to convey my deepest gratitude to **Professor Anna Krook**, my main supervisor, for constant motivation, advice and endless support that were of great help for my projects. Thank you, **Anna,** for being kind and always positive towards my work. Your positivity gave me a reason to keep going, and your kindness fueled my motivation. You have shared so much knowledge on science and communication that helped me to significantly boost my scientific standard. This will always guide my scientific journey. You are such a wonderful supervisor.

My sincere gratitude and thanks to my co-supervisor **Professor Juleen Zierath**. Thank you, **Juleen,** for trusting me and giving me the opportunity to join Integrative Physiology, and above all, for giving me valuable advice on how to communicate science. Your advice, both scientific and general, has been a cornerstone to my carrier growth. You have been a wonderful leader and a great source of inspiration.

Special thanks to my external mentor, **Professor Jan Lindsten,** for always being there. Your wisdom has always helped me to face and overcome challenges. Our constant meetings translated into energy and motivation that helped me to move forward. Thank you, Jan, for making my scientific journey fine-tuned .You have been a true and wonderful mentor.

I would also like to acknowledge **Docent Alexander Chibalin**, for helping me with technical and core scientific methods. Your broad scientific and general knowledge has really been instrumental towards accomplishing this work. I really enjoyed working with you.

I would like to thank **Dr. Megan Osler** for introducing me to the world of scientific research and organization. Thank you, **Megan,** for guiding me through material organization and processing. I learned a lot from your high organizing skills, including the proper use of excel spreadsheet that helped a lot with data handling and analysis. Special thanks to **Dr. Boubacar Benziane** for teaching me the science and art of Western blotting, **Dr. Marie Björnholm** for guiding me through journal clubs and literature discussion in general, and Docent **Lubna Al-Khalili** for your profound expertise in cell culture. My work wouldn't have been complete without your support.

My humble appreciation to **Dr. Tomas Fritz** for sharing his expertise in muscle biopsy and exercise test techniques. Thank you, Tomas, for giving me the opportunity to activate and extend my clinical knowledge and skills. Many thanks to Professor **Kenneth Caidahl**, Department of Clinical Physiology, for allowing me to run the ergometer cycle exercise test under your supervision.

I would also like to express my immerse gratitude and thanks to our previous administrator at Integrative physiology, **Mrs. Margareta Svedlund**. Thank you so much, **Margareta,** for always helping me with the visa, in addition to so many other administrative works. You have really helped me getting along with the Swedish administrative system. Special thanks to **Arja Kantz,** our current administrator, for so much help, especially towards the end of my studies. Your support means a lot to this work. Special thanks to **Docent Dana Galuska** for your help with ethical clearance, especially so for helping me with translation and processing the ethical clearance for my Tanzania study. Thank you, **Professor Marc Gilbert,** for sharing your in depth knowledge in biochemistry. My deepest gratitude to **Dr. Stefan Nobel** for organizing

wonderful SRP-diabetes seminars that helped to improve my knowledge in diabetes research.

Special thanks to my present colleagues in the lab, Dr. **Håkan Karlsson**, Dr. **Mutsumi Katayama**, Dr. **Ulrika Widegren**, **Rasmus Sjögren**, Dr. **Thais De Castro Barbosa**, **Leonidas Lundell**, Dr**. Jonathan Mudry**, Dr**. Qunfeng Jiang**, **Eva Palmer, Torbjörn Morein, Katrin Bergdahl**, Dr. **Carolina Nylen**, **Ann-Marie Pettersson**, **Milena Schönke**, Dr. **Laurene Vetterli** and my previous colleagues, **Dr. Ferenc Szekeres** and Dr. **Jie Yang** for your kind support. Thank you, Dr. **Julie Massart,** for words of encouragement when I was writing this thesis.

My deepest gratitude to my office mates at integrative physiology, **David Lassiter**, **Isabelle Riedel**, **Maria Holmström**, Dr. **Emmani Nascimento**, Dr. **Hanneke Boon**, Dr. **Henriette Kirchner**, and Dr. **Louise Mannerås Holm**. Thank you for valuable discussions and your energy that made our office a better place. You guys are wonderful.

I wish to extend my deep gratitude to my friends, Dr. **Sameer Kulkarni**, Dr. **Reginald Austin**, Dr. **Sydney Carter**, **Stephen Ochaya** and **Robby Tom**, for the valuable discussions that we shared. Thank you very much for your friendship.

I am deeply grateful to our family friends Mr. and Mrs. **Ubena John**, and their daughter **Janelle** for their friendly support. All my Tanzanian friends in Stockholm, for bringing the Tanzanian taste in Sweden. My friend **Ngesa Ezekiel,** for your deep scientific insight and critical thinking. My life in Stockholm wouldn't have been complete without you.

My special thanks to colleagues at the department of physiology at MUHAS in Tanzania, Dr. **Benjamin Mtinangi**, Dr. **Josiah Ntogwisangu**, Dr. **Emmanuel Ballandya**, Dr. **Omary Chillo**, Dr. **Davis Ngarashi** and Dr. **Mwanamkuu Maghembe** for your kind support during my absence.

Thank you so much Professor **Kisali Pallangyo** for inspiring me into academia and giving me the necessary support whenever I needed. Special thanks to my local supervisor in Tanzania, Professor **Janeth Lutale** for your professional support. Sincere gratitude to Dr**. Marina Njelekela**, for your kind support. Thank you Dr. **Julie Makani** for ever ending motivation. Special thanks to my colleagues and close friends, Drs. **Abel Makubi** and **Francis Dida**, for acting on my behalf during my absence at MUHAS. Special thanks to my friends, Dr. **Joel Msafiri** and Dr. **Goodluck Tesha** for your assistance during my absence in Tanzania.

My deepest appreciation to my dear wife, **Juliana Masaulwa**, and my family**.** Thank you Juliana for taking care of the kids and always making sure I am healthy and happy. Many thanks to **Ethan** and **Kenedy**. I wish to thanks my mother Mrs. **Pulkeria Mashili** for her everlasting love and support and my father Dr. **Lazaro Mashili** for inspiring me into the medical field. To my sister, **Joyce Mashili** and my brothers, **Jerry** and **Jamal,** I say Thank you so much.

My sincere gratitude to my beloved neighbors in Dar es Salaam, Mr. and Mrs. **Gudluck Mosha**. Your kindness and wisdom guided my family during my absence. Thank you very much for your true friendship and wisdom.

Finally I would like to thank the Novo Nordisk Foundation for financial support, without which, anything towards accomplishing this work would have been impossible.

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