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TRANSCRIPTIONAL, EPIGENETIC AND LIPIDOMIC RESPONSES TO METABOLIC INTERVENTIONS - IMPLICATIONS FOR HUMAN OBESITY

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Transcriptional, Epigenetic and Lipidomic Responses to Metabolic Interventions - Implications for Human Obesity

av

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To the memory of Bertil Sjödin

ABSTRACT

Obesity is increasing worldwide in an epidemic manner and better prevention and treatments are urgently needed. Obesity is strongly associated with insulin resistance and predisposes for type 2 diabetes. A deeper understanding of the underlying mechanisms behind obesity-driven insulin resistance is needed to identify new potential treatment targets.

Skeletal muscle is the main organ for glucose and fat metabolism. It is characterized by the ability to switch between glucose and fat as an energy source depending on the requirements and nutrient availability. This ability is referred to as metabolic flexibility. In the obese state, the skeletal muscle has a surplus of fatty acids and glucose, and this energy overload leads to stress and inflammation. This metabolic stress and inflammation, as well as the presence of excess fatty acids *per se*, impairs insulin signalling and metabolic flexibility in skeletal muscle. The overall aims of this thesis were to study the effects of energy alterations on gene expression, epigenetic marks, and lipid profile in blood and skeletal muscle to elucidate the underlying mechanisms leading to perturbations in skeletal muscle metabolism.

The interplay between our genes and the environment to which we are exposed, can explain some of the obesity and insulin resistance epidemic. Different environmental factors may alter DNA methylation, which is an epigenetic modification that can alter the function of genes. In **Paper I,** we obtained peripheral blood from obese subjects before and after weight loss through a diet intervention and subsequent gastric bypass surgery. In the peripheral blood, we assessed the level of DNA promoter methylation on selected genes important for metabolism. Promoter DNA methylation of *PGC1α* was altered after diet-induced weight loss, while gastric bypass surgery, inducing a substantial weight loss, was associated with methylation changes of *PDK4, TNF* and *IL6* promoters. In **Paper II**, we studied the effects of a short insulin exposure on skeletal muscle DNA methylation, and identified broad methylation changes in genes regulating metabolic pathways. One gene, previously not implicated in metabolic regulation, *DAPK3*, exhibited differential methylation status in response to insulin. DNA methylation of *DAPK3* also differed between normal glucose tolerant and type 2 diabetic subjects, and changed *in vivo* in response to glucose ingestion.

In **Paper III** we found that when skeletal muscle was exposed to AICAR, an activator of AMP-activated protein kinase, and a mimic of low cellular energy state, there was reduced expression of several cytokines, involved in metabolic inflammation. Using an unbiased bioinformatics approach, we identified the Sp1 transcription factor to be involved in the down-regulation of *IL6* mRNA and the relatively unknown transcription factor Zbtb14 to alter *LIF* mRNA.

In **Paper IV**, we again studied the effects of diet-induced weight loss in skeletal muscle of obese subjects. The three-week diet intervention reduced body weight and insulin levels. Gene expression of *FATP1, MLYCD, PDK4, UCP3* and *SCD1* was altered, all in favour to promote fatty acid oxidation in skeletal muscle. We also found an altered lipid profile, with specific lipid species associated with the improvement of skeletal muscle insulin sensitivity.

In conclusion, we elucidate potential regulatory mechanisms of skeletal muscle metabolic health, which is of relevance in the overall understanding of the metabolic perturbations of obesity and insulin resistance. We show that DNA methylation is dynamic and a potential regulator of "immunometabolic" gene expression. We provide insight in how weight loss improves skeletal muscle metabolism and identify specific lipid species that may play a role in improving skeletal muscle insulin sensitivity.

LIST OF SCIENTIFIC PAPERS

Articles included in this thesis

I. Kirchner H, **Nylén C**, Laber S, Barrès R, Yan J, Krook A, Zierath JR and Näslund E. Altered promoter methylation of PDK4, IL1B, IL6 and TNF after Roux-en Y gastric bypass. *Surg Obes Relat Dis* 2014; 10:671-678.

II. Mudry JM, Lassiter DG, **Nylén C**, García-Calzón S, Näslund E, Krook A, Zierath JR. Insulin and glucose alter death-associated protein kinase 3 (DAPK3) DNA methylation in human skeletal muscle. *Diabetes* 2017; 66:651-662.

III. **Nylén C**, Aoi W, Lassiter DG, Lundell, LS, Wallberg-Henriksson H, Näslund E, Pillon NJ and Krook A. AICAR-induced reduction of baseline IL-6 and LIF mRNA in skeletal muscle involves the transcription factor Sp1. *Manuscript Submitted.*

IV. **Nylén C**, Lundell LS, Massart J, Zierath JR and Näslund E. Short-term low calorie diet remodels skeletal muscle lipid profile and metabolic gene expression in obsess adults. *Manuscript Submitted.*

Articles not included in this thesis

Al-Khalili L, Chibalin AV, Yu M, Sjödin B, **Nylén C**, Zierath JR, Krook A. MEF2 activation in differentiated primary human skeletal muscle cultures requires coordinated involvement of parallel pathways. *Am J Physiol Cell Physiol* 2004 Jun;286(6):C1410-6.

Chibalin AV, Leng Y, Vieira E, Krook A, Björnholm M, Long YC, Kotova O, Zhong Z, Skane F, Steiler T, **Nylén C**, Wang J, Laakso M, Topham MK, Gilbert M, Wallberg-Henriksson H, Zierath JR. Downregulation of diacylglycerol kinase delta contributes to hyperglycemia-induced insulin resistance. *Cell* 2008 Feb 8:132(3):375-86.

Lassiter DG, **Nylén C**, Sjögren R, Chibalin AV, Wallberg-Henriksson H, Näslund E, Krook A, Zierath JR. FAK tyrosine phosphorylation is regulated by AMPK and controls metabolism in human skeletal muscle. *Diabetologia*. *In Press*.

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LIST OF ABBREVIATIONS

Act D	Actinomycin D
AICAR	5-Aminoimidazole-4-carboxamide ribonucleotide
AMP	Adenosine monophosphate
AMPK	5´AMP-activated protein kinase
ANOVA	Analysis of variance
AP-1	Activator protein 1
ATP	Adenosine triphosphate
BMI	Body mass index
C/EBP	CCAAT/Enhancer binding protein a
cDNA	Complimentary DNA
Cer	Ceramide
CpG	Cytosine nucleotide preceding guanine nucleotide
CREB	Cyclic AMP-responsive element binding protein
CRISPR	Clustered regulatory interspaced short palindromic repeats
CRP	C-reactive protein
CT	Computer tomography
DAG	Diacylglycerol
DAPK3	Death-associated protein kinase 3
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
FAC	Fluorescence activated cell sorting
FAT/CD36	Fatty acid translocase
F-glucose	Fasting-glucose
F-insulin	Fasting-insulin
FTO	Fat mass and obesity associated gene
GEO	Gene expression omnibus
GLP-1	Glucagon-like peptide 1
GSEA	Gene set enrichment analysis
HbA1c	Haemoglobin A1c
HDL	High density lipoprotein
HOMA-IR	Homeostatic model assessment-insulin resistance
IL-1β	Interleukin 1-beta
IL-6	Interleukin 6
IL-8	Interleukin 8
JNK	c-Jun N-terminal kinase
KEGG	Kyoto encyclopedia of genes and genomes
LC-MS	Liquid chromatography-mass spectrometry
LDL	Low density lipoprotein
LIF	Leukemia inhibitory factor
MBD	Methyl-CpG-binding domain
miRNA	Micro RNA
MLYCD	Mitochondrial malonyl-CoA decarboxylase
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
NFκB	Nuclear factor kappa B
Nfyc	Nuclear transcription factor y subunit c
NGT	Normal glucose tolerant
NIH	National Institute of Health
ns	Non significant

OGTT	Oral glucose tolerance test
p38/MAPK	Mitogen-activated protein kinase p38
p65/RELA	Nuclear factor NFκB p65 subunit
PC	Phosphatidylcholine
PDK4	Pyruvate dehydrogenase kinase isozyme 4, mitochondrial
PE	Phosphatidylethanolamine
PGC1α	Proliferator-activated receptor gamma coactivator 1-alpha
PPARγ	Peroxisome proliferator activated receptor gamma
qPCR	Quantitative polymerase chain reaction
RBPJ	Recombination signal binding protein immunoglobulin kappa J
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RYGB	Roux-en-Y gastric bypass
SBU	Statens beredning for social och medicinsk utvärdering; Swedish state agency for social and medical assessment
SCD1	Stearoyl-CoA desaturase 1
siRNA	Small interfering RNA
SLC27A1/FATP1	Fatty acid transport protein 1
SM	Sphingomyelin
SOreg	Scandinavian Obesity surgery registry
SOS	Swedish Obese Subjects study
Sp1	Specificity protein 1
T2DM	Type 2 diabetes mellitus
TFAM	Transcription factor A, mitochondrial
TG	Triglyceride
TNFα	Tumour necrosis factor alpha
UCP3	Uncoupling protein 3
WHO	World Health Organisation
Zbtb14	Zinc finger and BTB domain containing 14

1 INTRODUCTION

1.1 Implications of obesity on world health

Globally 1.9 billion adults are estimated to be overweight, which means that there are more people in the world who are overweight than undernourished (WHO, 2016b). In most countries worldwide, overweight and obesity kills more people than underweight. The worldwide prevalence of obesity in 2014 was estimated to 600 million individuals, a number that have more than doubled since 1980 (WHO, 2016b). Type 2 diabetes, ischemic heart disease and several types of cancer are strongly associated with obesity and cause premature morbidity and mortality, and constitute a major economic, medical and social burden in most countries. Recently, the World Health Organization (WHO) reported that 12.4 % of children under 5 years are overweight in the European region (WHO, 2016a), indicating that we have only seen the beginning of this epidemic. Despite this potential catastrophic scenario, it is important to keep in mind that obesity is preventable. The work of this thesis focuses on obesity and the associated insulin resistance, a metabolic state that precedes type 2 diabetes.

1.2 Definitions and classifications

Overweight and obesity are defined as an excess fat of weight in relation to height. Fat normally constitutes about 10-15 kg in middle-aged men, and about 50% more in middle-aged women (SBU, 2002). The most commonly used measure of obesity is body mass index (BMI) (**Table 1**). BMI is calculated as kg of body weight per square meter of height (kg/m^2).

The different classifications of BMI reflect the associated risk of mortality and were developed by the WHO based on large epidemiological studies in North America (WHO, 1997). However, BMI does not discriminate between men and women and does not take into account the fact that the increased body weight can be due to increased skeletal muscle mass and not increased fat content.

Table 1. *Adult BMI classifications according to WHO (1997).*

BMI (kg/m^2)	Classification
<18.5	Underweight
18.5-24.9	Normal weight
25-29.9	Overweight
30-34.99	Obese class I
35-39.99	Obese class II
≥ 40	Obese class III

BMI, but also the localization of the adipose tissue, is associated with morbidity and mortality. When the adipose tissue is centred around the waist as in increased visceral, intra-abdominal fat, the risk of developing cardiovascular disease as well as type 2 diabetes is increased to a higher extent (Despres, Lemieux, & Prud'homme, 2001). The waist-to-hip ratio is a measure that complements BMI, by taking into account the central, visceral fat. A person with a waist circumference of 102 and 88 cm for men and women respectively, is regarded as obesity (SBU, 2002).

Other measures of fat mass include skin fold measurements and dual energy X-ray absorptiometry, as well as computer tomography (CT) and magnetic resonance imaging (MRI) scanning. MRI-scanning gives a very accurate readout of visceral and subcutaneous fat depots (Gray et al., 1991). However it is a very expensive technique that also requires the access to an MRI-scan, making it suitable exclusively for smaller research cohorts rather than population based measures. We have used BMI to describe our cohorts, as this is the most comparable means of measure in the literature.

1.3 Mechanisms behind the development of obesity

1.3.1 Energy homeostasis

Obesity is a result of excessive energy intake or insufficient energy expenditure, or both (**Figure 1**). The energy content of food and drinks comes from fats, carbohydrates and proteins. Carbohydrates and protein contain less energy per unit than fat. Our body can regulate and limit the levels of carbohydrates and proteins stored in the body, but fat can be stored to a great extent. Energy expenditure includes basal metabolism, the thermic effect of food and physical activity. Easily accessible energy-dense food, in combination with reduced physical activity, is commonly accepted to be responsible for the fast development of obesity (WHO, 1997).

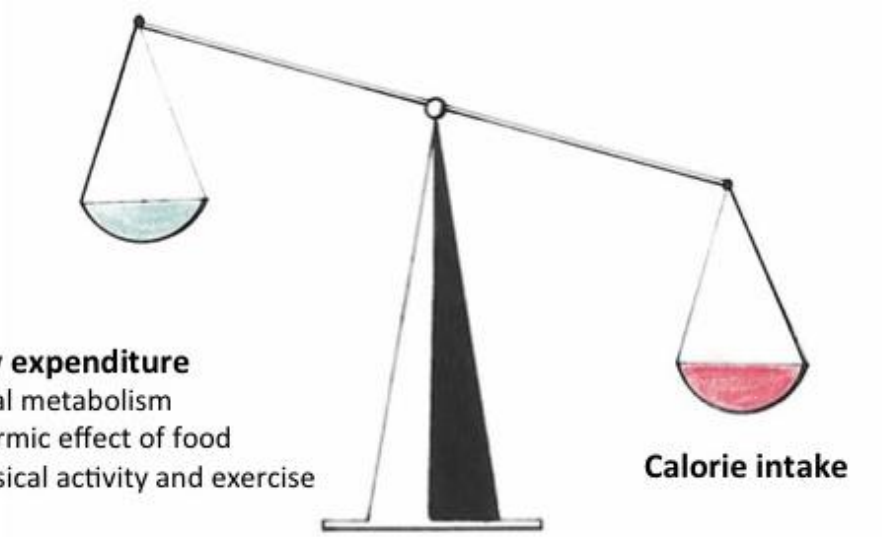

Figure 1. *Scale illustrates calorie intake/expenditure homeostasis.*

1.3.2 Genetics

One idea that accounts for the current epidemic of obesity and type 2 diabetes centres around the "thrifty genes hypothesis". The "thrifty genes hypothesis" suggests that certain genes, important for storing fuels as fat so that people can survive long periods of starvation, important historically in times of scares food availability. Now in modern time, in states of constant nutrient surplus, these genes important for storing fuel instead promote obesity, (Neel, 1962).

Indeed our genetic heritage is important for the risk of developing obesity. Studies of twins have revealed that despite growing up in different environments, monozygotic twins still often share the same phenotype regarding body weight and BMI. Adoptive children also to a

large extent develop the same phenotype as their original parents, regardless of the food habits of the adoptive parents. (Stunkard, Foch, & Hrubec, 1986; Stunkard, Sorensen, et al., 1986). Thus, there is a strong genetic component to body weight and BMI.

More than 30 exceptionally rare monogenic obesity syndromes, including Prader-Willi syndrome, are identified as well as, 11 rare non-syndromic monogenic conditions related to obesity (Pigeyre, Yazdi, Kaur, & Meyre, 2016). One of the non-syndrome monogenic conditions is leptin deficiency (Zhang et al., 1994). Leptin is a protein that is released from adipose tissue that sends signals of satiety to the brain. Deficiency of the melanocortin-4 receptor that relays the leptin signal in the central nervous system, is another monogentic obesity condition (Huszar et al., 1997). Thus, the deficiencies in the production or resistance to the action of secreted protein may also contribute to obesity.

In addition, about 227 gene loci associated with the risk to develop obesity have currently been identified through genome wide association studies (reviewed in (Pigeyre et al., 2016)). One particular gene that has gained attention is the FTO gene (Frayling et al., 2007) and it has been shown that a person with one or two copies of the FTO allele have 1.2 or 3 kg extra weight respectively in comparison to a person who lacks the allele (Loos & Yeo, 2014). Although there is clear evidence that genes are important for the variation of body weight across a population, genetic variants only account for about 5% of the variation in body weight (Pigeyre et al., 2016) and thus cannot explain the pandemic of obesity. There are still missing links in the search for the aetiology of obesity. New research areas have emerged and gained focus the last centuries, such as the influence of our gut microbiota (Torres-Fuentes, Schellekens, Dinan, & Cryan, 2017), as well as the field of epigenetics (Kirchner, Osler, Krook, & Zierath, 2013), a regulatory mechanism investigated in this thesis.

1.4 Epigenetic regulation

The exact definition of epigenetics has been debated over the years. A definition commonly accepted is "*the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in the genetic sequence*" (Riggs, 1996). The term *"Epigenetics"* was coined by the developmental biologist C.H. Waddington (1905-1975), in his classical description of the undefined cell type that rolls through the epigenetic landscape to become a highly defined, specialized cell (Waddington, 1957). "Epi" comes from the Greek prefix meaning "on top of", which refers to a regulative mechanism "on top of the DNA sequence". Epigenetic modifications of the genome include histone modifications, DNA methylation and microRNAs. These epigenetic marks influence the central dogma of DNA transcription, RNA translation and protein synthesis in the cell (**Figure 2**).

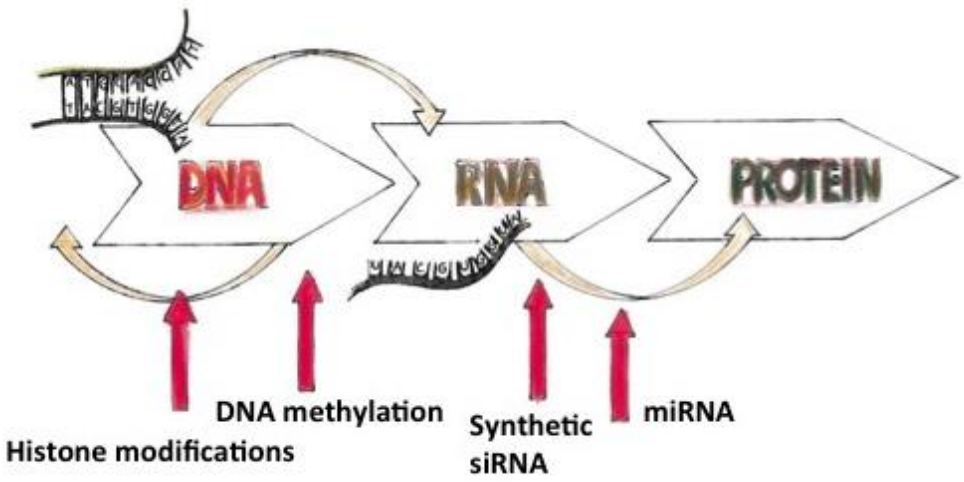

Figure 2. *The "central dogma of biology". DNA is transcribed to RNA, and RNA is translated to protein: Red arrows showing the points of interaction with the epigenetic regulatory mechanisms. Synthetic siRNA can be used experimentally to silence genes as described in the Methods section.*

DNA methylation of a promoter region of a gene often results in silencing and reduced mRNA transcription of that gene. Mechanistically, a methyl group (CH_3) is attached to the 5´carbon of the nucleotide side chain (**Figure 3**). This mechanism can modify the binding sites for the transcriptional complex and thus inhibit transcription. However, DNA methylation can also change the configuration of the DNA so that transcription is enhanced. Classically, a C nucleotide that precedes a G nucleotide in the DNA sequence is most commonly methylated, called CpGs. However, non-CpG methylation also exists, not only in plants where it was first discovered, but also in humans (Yan, Zierath, & Barres, 2011).

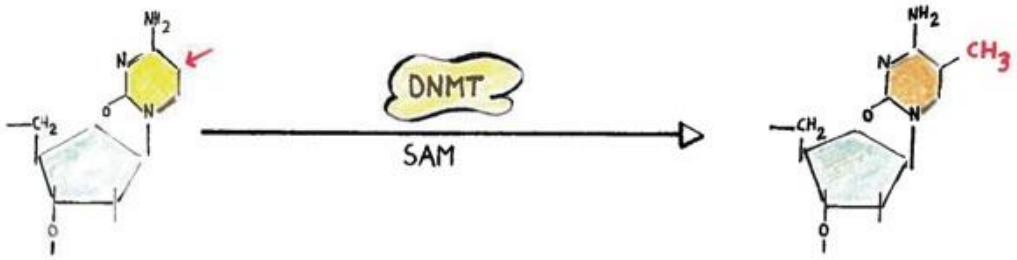

Figure 3. *The process of DNA methylation. A methyl (CH_3) group is attached to the 5´carbon of the nucleotide side chain.*

DNA methylation is a well described regulatory mechanism in imprinting of genes, where during embryogenesis one allele of a gene is silenced to allow the other to be expressed (Reik & Walter, 2001). DNA methylation is also involved in oncogenic processes, where the genes involved in the repair mechanism of mutated genes are silenced through hyper-methylation, resulting in uncontrolled and heterogenic growth (Kulis & Esteller, 2010). DNA methylation may provide a potential molecular mechanism for the interaction between genetic and environmental factors also in metabolic homeostasis (Kirchner et al., 2013). David Barker was the first who recognized that nutritional status "*in utero*" could affect health later in life (Barker, 1990). Indeed, the children of mothers who experienced the Dutch famine while they were pregnant, had an altered DNA methylation of the insulin like growth factor 2 gene (Heijmans et al., 2008) and experienced greater risk of obesity and cardiovascular disease

later in life (Roseboom, Painter, van Abeelen, Veenendaal, & de Rooij, 2011). In the county of Överkalix in the north of Sweden, grandchildren of men that experienced scarce availability of food just before puberty, conversely exhibited increased longevity (Bygren, Kaati, & Edvinsson, 2001). Collectively, this indicates that epigenetic regulation is powerful in affecting lifetime and trans-generational risk for metabolic disease.

Twin studies have shown that the blood epigenetic profile of 3-year old twins is very similar, but this profile changes substantially with age and exposure to environmental factors (Fraga et al., 2005). Exercise and weight loss through bariatric surgery are powerful external stimuli for epigenetic alterations (Barres et al., 2013; Barres et al., 2012). The epigenetic profile of skeletal muscle differs between people with type 2 diabetes and people with normal glucose tolerance (Barres et al., 2009), suggesting that epigenetic mechanisms can have a regulatory role also in the development of insulin resistance. Thus, changes in the epigenetic profile of type 2 diabetes, obesity and aging can be observed in blood, as well as metabolically active tissues such as skeletal muscle.

1.5 Obesity and insulin resistance

Obesity is responsible for driving the epidemic of insulin resistance and type 2 diabetes in the Western countries (Kahn, Hull, & Utzschneider, 2006). Insulin resistance is defined as the "inability to regulate glucose uptake in peripheral tissues" and is present in many metabolic diseases including type 2 diabetes, cardiovascular disease and hypertension (Reaven, 1988).

Table 2. *Definitions of impaired glucose tolerance and type 2 diabetes (WHO 2006).*

Classification	Fasting plasma glucose		2-h plasma glucose
Impaired fasting glucose	6.1 to 6.9 mmol/L	AND	<7.8 mmol/L
Impaired glucose tolerance	<7.0 mmol/L	AND	≥7.8 to <11.1 mmol/L
Type 2 diabetes	≥7.0 mmol/L	OR	≥11.1 mmol/L

In the clinical setting, there are two main diagnostic tests for type 2 diabetes: the fasting plasma glucose and plasma glucose measured 2 h after ingestion of a 75 g oral glucose drink, using an Oral Glucose Tolerance Test (OGTT) (**Table 2**). In addition, Haemoglobin A1c (HbA1c), a glycosylated form of haemoglobin, is also used as diagnostic criteria for type 2 diabetes (≥6.5%) and reflects the long-term profile of blood glucose levels.

Insulin resistance is rarely diagnosed in the clinical setting since clinical therapy guidance is based upon fasting blood glucose values, but can be measured by an euglycaemic clamp or estimated by the homeostatic model assessment (HOMA-IR) for research purposes. While HOMA-IR, calculated from the fasting glucose and fasting insulin values, mainly reflects the hepatic insulin resistance, the euglycaemic clamp is the "gold standard" method to assess insulin resistance, particularly in skeletal muscle (DeFronzo & Tripathy, 2009).

Insulin is produced by the β-cells of the islets of Langerhans in the pancreas. The main task for insulin is to coordinate energy uptake and storage in the three main target organs skeletal muscle, liver and adipose tissue. Insulin inhibits energy generating processes like

gluconeogenesis in liver and lipolysis in adipose tissue. In the healthy individual, insulin is released by the pancreas after a meal and manages efficiently to clear the nutrients from the blood through a rapid uptake into the peripheral tissues. However, in the insulin resistant individual, peripheral tissues do not respond to insulin adequately and therefore glucose and fatty acid levels remain high in the blood. The pancreas responds with increasing the insulin production and secretion to maintain blood glucose levels within a healthy range. When the β-cells in the pancreas fail to meet the increased need for insulin, blood glucose levels rise and overt type 2 diabetes develops. Insulin sensitivity in the peripheral tissues can be improved by weight loss and exercise and thus type 2 diabetes can be prevented by lifestyle changes of the insulin resistant individual.

1.5.1 Influence of ectopic fat

Free fatty acids are taken up in response to insulin mainly by the adipose tissue, where they are primarily stored. However, when the adipose tissue is saturated as in obesity, fat can also be stored in liver and skeletal muscle, as ectopic lipids (Heilbronn, Smith, & Ravussin, 2004). Increased total lipid content in skeletal muscle is highly associated with obesity and insulin resistance (Krssak et al., 1999). However, with the finding that also highly insulin sensitive athletic individuals can have an increased total skeletal muscle lipid content, the general thought now is that the specific lipid species, as well as the localization, composition and turnover of lipids are more important for the development of insulin resistance (Coen & Goodpaster, 2012; Dube et al., 2008; Kitessa & Abeywardena, 2016).

Fats/lipids do not only provide a source of energy, but also function as structural components in cell membranes. The phospholipid composition can affect the permeability and transport of nutrients across the cell membrane and alterations in the composition are associated with insulin sensitivity (Borkman et al., 1993). In addition, specific lipid species can also function as second messengers, and lipid intermediates such as ceramides and diacylglycerols are identified as inducers of insulin resistance in skeletal muscle (Hotamisligil et al., 1996; Yu et al., 2002). Thus, it is important to study the full lipid profile of the skeletal muscle to understand the underlying mechanisms connecting intramuscular lipids and insulin resistance.

1.5.2 Influence of inflammation

Type 2 diabetes and insulin resistance, with hallmarks like hyperglycaemia, hyperinsulinemia and elevated free fatty acid levels, cause tissue inflammation. The tissue inflammation *per se*, worsens the insulin resistance (Hotamisligil et al., 1996) causing a vicious circle. In people with type 2 diabetes, serum levels of acute-phase proteins (such as C-reactive protein, CRP), as well as cytokines (such as TNFα) are elevated (Spranger et al., 2003). Moreover, infusion of TNFα to healthy humans induces skeletal muscle insulin resistance (Plomgaard et al., 2005). The tissue specific inflammation is however many times greater than the systemic (serum levels) response, suggesting that the tissues are directly affected locally in both an autocrine and paracrine manner (Osborn & Olefsky, 2012).

In obesity, immune cells such as macrophages infiltrate adipose tissue (Weisberg et al., 2003). Macrophages reside in adipose tissue depots and, through the release of chemokines and cytokines, they affect the surrounding insulin responsive tissues, rendering them insulin resistant (Lee & Lee, 2014; Olefsky & Glass, 2010). Additional evidence for the interconnection between metabolism and inflammation are the common signalling pathways (**Figure 4**).

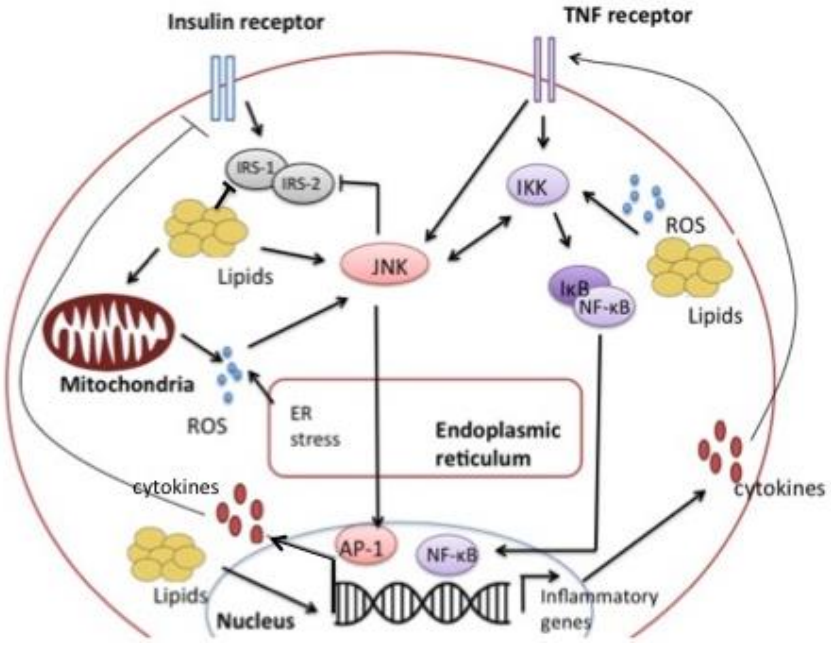

Figure 4. *Metabolic and inflammatory signalling pathways are interconnected through the JNK and IKK- pathways, that when activated, contribute to insulin resistance.*

Chronic low-grade inflammation stimulates the Jun N-terminal kinase (JNK) stress signalling pathway. In addition, elevated levels of free fatty acids, endoplasmic reticulum (ER) stress, as well as reactive oxygen species (ROS) generated from the mitochondria, can activate JNK (Ertunc & Hotamisligil, 2016). In obesity, JNK signalling is activated in liver, skeletal muscle and adipose tissues. Disruption of the JNK pathway prevents the development of insulin resistance and diabetes in obese mice (Hirosumi et al., 2002). Metabolic stressors like hyperglycaemia, hyperinsulinemia and elevated free fatty acid levels *per se* can activate inflammatory signalling pathways that lead to the activation of the transcription factor Nuclear factor κB (NFκB) (Yin, Yamamoto, & Gaynor, 1998). Disruption of this pathway can protect against the development of insulin resistance induced by high fat feeding in animal models (Yuan et al., 2001). Administration of anti-inflammatory salicylates blocks the NFκB signalling pathway and improves insulin sensitivity in humans (Shoelson, Lee, & Yuan, 2003). NFκB and AP-1 are the main transcription factors for many of the cytokines and therefore constitutes major convergent points for metabolic and immune signals. Collectively, inflammation is a cornerstone in metabolic perturbations and in the development of insulin resistance and type 2 diabetes.

1.6 Skeletal muscle metabolism

Skeletal muscle constitutes more than 40% of body mass and is unique when it comes to its ability to rapidly adapt to changing metabolic requirements. Skeletal muscle can increase its energy turnover by 100-fold during strenuous exercise in comparison to its resting state (Richter & Ruderman, 2009). Skeletal muscle is the major organ for insulin-stimulated glucose uptake and an important tissue for studying the mechanisms behind insulin resistance.

1.6.1 AMP-activated protein kinase

AMP-activated protein kinase (AMPK) is the main fuel sensor in the cell coordinating the balance between ATP-consuming and ATP-producing pathways. Thus, AMPK is the key player in the regulation of body weight and whole body energy balance (Hardie, Hawley, & Scott, 2006). AMPK is activated by an increased AMP/ATP ratio and induces metabolic pathways that generates ATP and inhibits processes that require ATP (**Figure 5**).

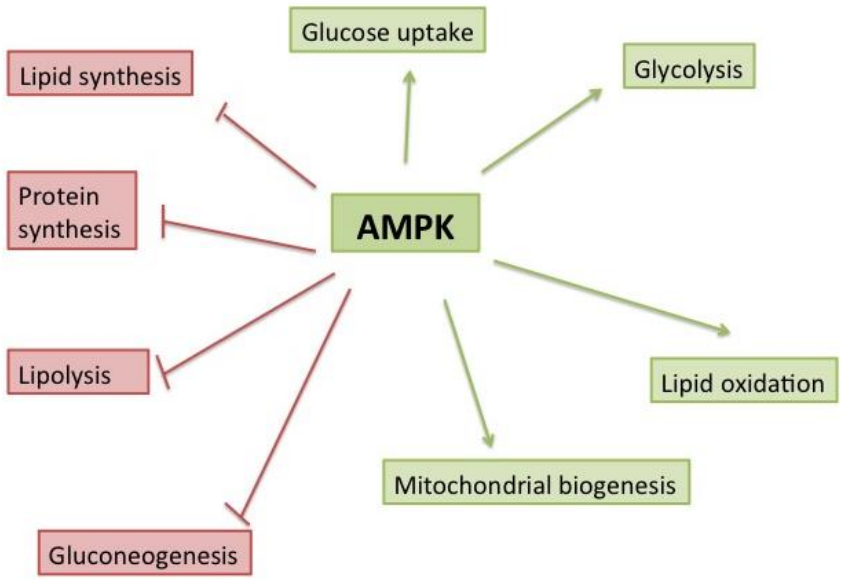

Figure 5. *AMPK activates metabolic pathways resulting in ATP production (green arrows) and inhibits metabolic processes that require ATP (red lines).*

The activity of AMPK is decreased in adipose tissue of insulin-resistant obese individuals compared to obese individual who have normal glucose tolerance (Gauthier et al., 2011). Calorie restriction enhances insulin sensitivity (McCurdy & Cartee, 2005). Interestingly, in a study of mice lacking AMPKα2, calorie restriction did not enhance skeletal muscle insulin sensitivity compared to wild type mice (P. Wang et al., 2012), suggesting that AMPK plays a role in improving insulin sensitivity. Metformin, the first line oral treatment for type 2 diabetes, is an AMPK activator. It also has anti-inflammatory effects (reviewed in (O'Neill & Hardie, 2013)), again connecting metabolic and inflammatory processes. In addition, other known AMPK-activators like salicylates and AICAR share these anti-inflammatory properties, of which the mechanisms are largely unknown. Thus, strategies to activate AMPK may prevent insulin resistance in metabolic diseases such as type 2 diabetes or obesity, with the inflammatory state is heightened.

1.6.2 Mitochondria and metabolic flexibility

Mitochondria are energy producing organelles in the cell that metabolize nutrients and produce ATP and are thus crucial for adequate energy production in skeletal muscle. Pyruvate from glycolysis, and fatty acids are transported to the mitochondrial matrix and oxidized to acetyl-CoA that enters the Krebs cycle where ATP is finally generated. Alterations in mitochondrial function have been implicated in the development of insulin resistance and obesity (Kelley, Goodpaster, Wing, & Simoneau, 1999; Kelley, He, Menshikova, & Ritov, 2002).

The capacity of skeletal muscle "to utilize lipid and carbohydrate fuels and to transition between them" is defined as metabolic flexibility (Kelley et al., 1999; Kelley et al., 2002). Healthy skeletal muscle relies on lipid oxidation in times of fasting and during exercise, in

contrast, with insulin resistance, the fatty acid oxidative capacity of the mitochondria in skeletal muscle is impaired and fuel selection is shifted to glucose oxidation (Kelley et al., 1999). One hypothesis of the association between mitochondrial dysfunction and insulin resistance involves fatty acid overload and incomplete lipid oxidation resulting in accumulation of intracellular lipid metabolites (J. Y. Kim, Hickner, Cortright, Dohm, & Houmard, 2000) that inhibit insulin signalling and subsequently impairs insulin-mediated glucose uptake (Yu et al., 2002).

In this thesis, I focus on the expression of several genes involved in glucose and lipid metabolism that are of importance for mitochondrial function, fuel selection and implicated in the development of insulin resistance. *Peroxisome proliferator-activated receptor γ coactivator 1α* (PGC-1α) is activated by AMPK and stimulates mitochondrial function and thereby fuel disposal (Wu, Inskeep, Bowker-Kinley, Popov, & Harris, 1999; Z. Wu et al., 1999). PGC-1α is down regulated in skeletal muscle from people with type 2 diabetes (Mootha et al., 2003). Another important mitochondrial enzyme is *Pyruvate dehydrogenase kinase isozyme 4, mitochondrial* (PDK4), located in the mitochondrial matrix and suppresses conversion of pyruvate to acetyl-coA, the rate-limiting step in glucose oxidation, favouring lipid oxidation. PDK4 in skeletal muscle is induced by exercise (Hildebrandt, Pilegaard, & Neufer, 2003), starvation and in people with type 2 diabetes (P. Wu et al., 1999). These two players are given high impact as main regulator switches for fuel selection in the mitochondria, essential for metabolic flexibility, making them potential targets for improving metabolic dysfunction and insulin resistance.

1.6.3 Myokines

Skeletal muscle, similarly to adipose tissue, produce and release cytokines to communicate the needs of the myocyte to other organs (Pedersen & Febbraio, 2008). These skeletal muscle specific cytokines are called "*myokines"* (Pedersen & Febbraio, 2008). Several internal and external stressors like exercise, and muscle contraction *per se*, inflammation, hyperthermia, ROS and low glycogen stores stimulate the production and release of myokines *(Welc & Clanton, 2013)*. Currently, several hundred of myokines have been identified (Karstoft & Pedersen, 2016). The prototypic myokine is IL-6, which can be increased 100-folds in response to exercise (Keller et al., 2001), and is thought to be part of the anti-inflammatory and regenerative response of the muscle to exercise (Pedersen & Saltin, 2015). Cytokines seem to have different effects upon cell function depending upon the context in which they are produced, and also by what tissue and cell type. For example, when IL-6 is produced by the immune cells it induces inflammation and when it is produced by the muscle cell in response to acute exercise, it exerts anti-inflammatory effects (Pedersen & Febbraio, 2007). IL-6 is also important for whole-body metabolism. Deletion of Il-6 in mice leads to obesity (Wallenius et al., 2002). In contrast, gene delivery of IL-6 to mice fed a high fat diet induces weight loss and whole-body insulin sensitivity improvements (Ma, Gao, Sun, & Liu, 2015). This illustrates the complexity of IL-6 and underscores the importance of understanding how IL-6 is regulated and the effects on skeletal muscle and whole-body metabolism.

1.7 Interventions and treatments of obesity

Obesity has been recognized as a threat to health since far back in history (Haslam, 2007). Galen, one of history´s greatest physicians gives one of the first descriptions of obesity management around 160A.D.;

"I reduced a huge fat fellow to a moderate size in a short time, by making him run every morning until he fell into a profuse sweat; I then had him rubbed hard, and put into a warm bath; after which I ordered him a small breakfast, and sent him to the warm bath a second time. Some hours after, I permitted him to eat freely of food, which afforded but little nourishment; and lastly, set him to some work which he was accustomed to for the remaining part of the day"(Galen, AD 160)

The first-hand treatment for obesity is lifestyle changes, including caloric restriction and increased energy expenditure. However, for successful weight loss through diet, there is a need for behavioural changes, as the aspect of food intake is highly associated with social habits and preferences.

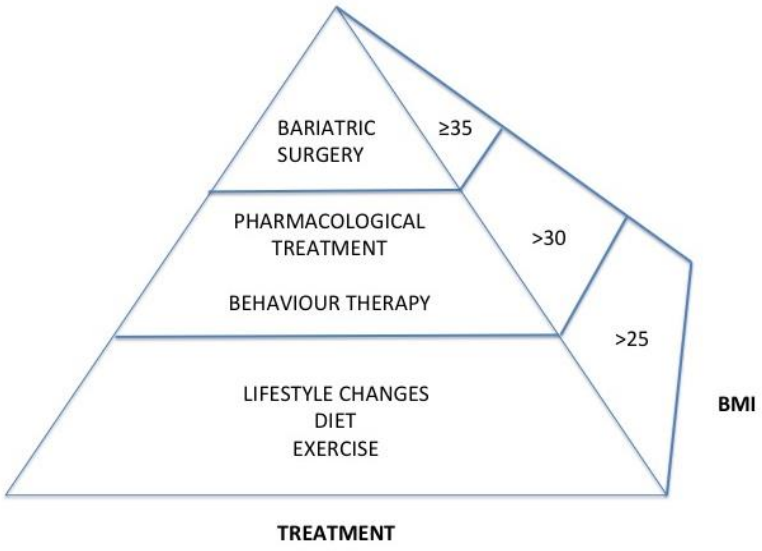

Figure 6*. Recommended treatment of obesity. Redrawn from (SBU 2013).*

1.7.1 Weight loss through diet

Caloric restriction is the first line of treatment for obesity in combination with other lifestyle changes (**Figure 6**). The daily caloric requirement to retain weight for a middle-aged (30-61 years) person with average physical activity level is estimated to around 2,600 kcal for men and 2,100 kcal for women (Livsmedelsverket, 2017). The caloric requirements will, however, vary to a certain extent depending factors such as level of physical activity and skeletal muscle mass. A low calorie diet is defined as <1,000 kcal/day.

Caloric restriction interventions can be successful to reduce weight in obese individuals, especially if combined with behavioural therapy. In the short-term (six months), a low carbohydrate diet has better weight loss effect than a low fat, diet but this difference is lost in the long term (1-2 years) (SBU, 2013). Weight loss through caloric restriction is efficient to improve insulin sensitivity (Lim et al., 2011). In addition, caloric restriction attenuates inflammation and intermittent fasting reduces serum cytokine levels in humans (Faris et al., 2012; Heinonen et al., 2009), although there are some contradictory results that show that some cytokine levels are unaffected (Manco et al., 2007). Although caloric restriction can be successful, especially short term, most obese individuals fail to reduce their weight to normal

weight. Without substantial weight loss the risks of type 2 diabetes and cardiovascular disease remain. By studying caloric restriction and identifying the regulative mechanisms of improved metabolic health, it is possible to provide better dietary recommendations and prescribe treatment regimens.

1.7.2 Pharmacological therapy

Currently, three pharmacological treatments for weight loss are approved in Sweden today, the intestinal lipase inhibitor *Orlistat*, the anti-diabetic drug *Saxenda* (Liraglutid) and *Mysimba* (Naltrexone/Bupropion). Orlistat is a pancreatic lipase inhibitor that inhibits ingested lipids to be digested and taken up in the small intestine, jejunum and ileum, and the fats are instead excreted. Due to the side effects with diarrhoea and increased intestinal gas production many patients stop the treatment early. Saxenda is a GLP-1 (glucagon-like peptide 1) receptor agonist. GLP-1 is a gut hormone released from the small intestine and involved in the regulation of appetite. Mysimba, is a combination drug of naltrexone, an opioid receptor antagonist, and bupropion, a dopamine- and norepinephrine-receptor inhibitor. Both Saxenda and Mysimba are not subsidized by the government in Sweden and are therefore very costly for the patients. The effect on weight loss is modest for all three drugs, a treatment lasting more than 52 weeks resulted in weight loss of 6.1%, 5.4% and 7.4% respectively compared to 2.6%, 1.3% and 3% in the placebo group (Bray, Fruhbeck, Ryan, & Wilding, 2016). Nevertheless, given the world-wide increase in obesity, new treatment regimens are urgently required.

1.7.3 Weight loss through bariatric surgery

In the case of obesity and when weight loss through lifestyle changes and pharmacological treatment fails, there is an indication for bariatric surgery. The Swedish guidelines are adapted from NIH and prescribe surgery at BMI ≥40 or ≥35 with comorbidity (NIH, 1992). In 2016 about 6,000 patients underwent bariatric surgery in Sweden. The most common procedure is Roux-en-Y gastric bypass (RYGB) (64%) but Sleeve Gastrectomy (34%) continues to rise every year, both in Sweden and abroad (SOreg, 2017). In the RYGB procedure, the stomach is divided to create a smaller pouch, about 15-20 ml in volume (**Figure 7**).

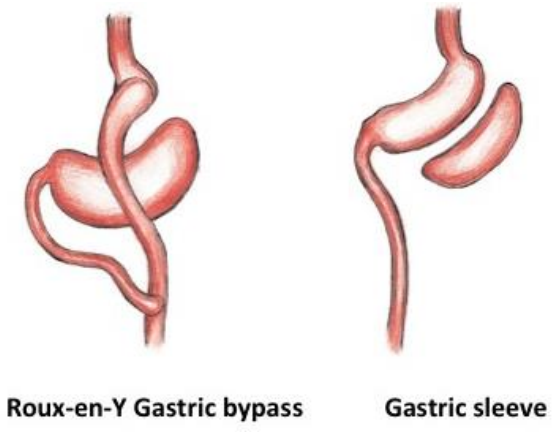

Figure 7. *The current two main bariatric surgery techniques in Sweden.*

The small intestine, jejunum, is also divided and pulled up to make a gastrojejunal anastomosis. The major part of the stomach and the divided jejunum is anastomosed about 1 m distal to the gastrojejunal anastomosis to enable for gastric and pancreatic juices to join with the ingested food. The method is both restrictive and malabsorptive, meaning that the

patient can only eat small portions of food, and fewer nutrients are absorbed due to the bypassing of the jejunum. Another important result of the gastric bypass is the change in gut hormone profile, including increasing GLP-1 (Falken, Hellstrom, Holst, & Naslund, 2011). In the Sleeve gastrectomy procedure, the stomach is reduced into a "gastric sleeve" and the remaining part of the stomach is removed. Currently both procedures are performed by laparoscopy and the complications are few (SOreg, 2017).

The prospective controlled Swedish Obese Subjects (SOS) Study has followed patients that underwent bariatric surgery 15-20 years ago. The study concludes that the maximum body weight loss occurs 1-2 years postoperatively (32±8%) and then stabilizes after 10-15 years (25±11%) (Sjostrom, 2008). The weight loss after bariatric surgery has prominent effects on blood glucose levels and after two years 72% of the diabetic patients improve in their blood glucose and insulin levels to the point that they can stop their diabetic therapy. After 10 years, the type 2 diabetes remission effect is retained for 36% of the patients (Sjostrom, 2008).

RYGB surgery has substantial effects on insulin resistance, lowering blood glucose levels within days after surgery, before weight loss is significant. The mechanism behind this rapid amelioration is not completely stratified, but changes in gastrointestinal peptide release or the peri-surgical fasting itself could be involved (Falken et al., 2011; Lim et al., 2011; Thaler & Cummings, 2009). Cytokines levels are reduced independent of weight loss after RYGB (Miller, Nicklas, & Fernandez, 2011), suggesting that a reduction in inflammation could play a role in the early metabolic benefits after bariatric surgery. The fact that bariatric surgery is the only efficient, long lasting treatment for obesity, makes it an important model to study the mechanism of obesity and insulin resistance.

2 AIMS

Obesity and insulin resistance are a consequence of a many known and unknown factors. The understanding of the underlying mechanisms leading to obesity and insulin resistance is crucial to identify persons at risk, being able to give better lifestyle intervention advice and to identify new approaches for medical treatment. Therefore, the overall aim of this thesis is to study the effects of external factors, such as low energy signals, weight loss, insulin and glucose exposure, on skeletal muscle and blood gene expression of healthy as well as obese and type 2 diabetic subjects. Ultimately, we aim to identify targets for improving skeletal muscle insulin sensitivity and metabolic health.

The specific aims of this thesis are to;

1. Determine if DNA methylation changes are present in human blood after a low calorie diet and after subsequent RYGB surgery.

2. Investigate if insulin and glucose directly alter DNA methylation in skeletal muscle.

3. Investigate the mechanism by which the AMP-mimicking AICAR reduces the mRNA expression of cytokines in skeletal muscle.

4. Determine mRNA expression and lipid profiles of skeletal muscle in obese adults after short-term diet-induced weight loss.

3 EXPERIMENTAL PROCEDURES AND METHODOLOGICAL CONSIDERATIONS

3.1 Human cohorts

Five different human cohorts were included in this thesis. In **paper 1**, 18 (5 males and 13 females) obese, non-diabetic subjects scheduled for RYGB surgery were enrolled. Whole blood was obtained to study clinical chemistry parameters and DNA methylation before and after a two-week low calorie diet. Whole blood from 7 of the subjects (1 male and 6 females) was also studied two days, and 12 months after RYGB surgery. A control group of 6 (6 males) lean subjects undergoing elective cholecystectomy were also enrolled and their blood was obtained in the morning before, and one day after surgery.

In **papers 2, 3 and 4** skeletal muscle biopsies were obtained for DNA methylation, mRNA expression as well as protein analysis. Also samples for clinical chemistry was obtained at all time points. In **paper 2**, two different cohorts of male participants were studied. In the first, 10 non-obese, normal glucose tolerant (NGT) subjects were enrolled for open muscle biopsies and clinical chemistry. In the second cohort, 12 normal glucose tolerant, as well as 12 type 2 diabetic subjects were included. A conchotome muscle biopsy was taken before and after an oral glucose tolerance test (OGTT). In **paper 3**, 13 male, non-obese participants were enrolled for open muscle biopsy and blood chemistry. In **paper 4**, 8 (2 males and 6 females) obese, non-diabetic subjects were enrolled for conchotome muscle biopsies and clinical chemistry before and after a three-week low calorie diet. Written informed consent was obtained from all participants. The Ethics committee of Karolinska Institutet approved all studies included in this thesis. The clinical characteristics and clinical chemistry results of the cohorts are presented in **Table 3.**

Cohort	PAPER 1						PAPER 2			PAPER 3	PAPER 4	
							A	B				
Metabolic state	**Obese**				**Non-obese**		**NGT**	**NGT**	**T2DM**	**NGT**	**Obese**	
	Before diet	**Op-day**	**Discharge**	**Follow-up**	**Op-day**	**Discharge**					**Before diet**	**After diet**
N (Male/Female)	18 (5/13)	18 (5/13)	7 (1/6)	7 (1/6)	6 (6/0)	6 (6/0)	10 (10/0)	12 (12/0)	12 (12/0)	13 (13/0)	8 (2/6)	8 (2/6)
Age (years)	40 ± 11	40 ± 11	33 ± 7	34 ± 6[C]	42 ± 9	42 ± 9	53 ± 6.3	60 ± 9.5	63 ± 3	50 ± 7.0	39 ± 12	39 ± 12
Body weight (kg)	123.9 ± 15.9[D]	118.8 ±14.7[A,D]		81.0 ± 13.8[A,B]	82.2 ± 6.5		82.4 ± 8.5	80.8 ± 7.3	91.5 ± 6.3	80.2 ± 9.2	112. 9	104.9 ± 12.1*
BMI (kg/m^2)	42.3 ± 4.7[D]	40.5 ± 4.6[A,D]		27.3 ± 3.3[A,B]	25.7 ± 2.1		25.2 ± 1.8	25.4 ± 1.6	28.3 ± 1.9*	24.0 ± 1.9	39.7 ± 3.5	36.9 ± 3.6*
F- glucose (mmol/L)	5.7 ± 0.8	5.3 ± 0.9[A]	5.5 ± 0.9	4.7 ± 0.28[A,D,E]	5.3 ± 0.4	6.1 ± 0.5[D]	5.3 ± 0.3	7.4 ± 2.8	8.1 ± 1.3*	5.5 ± 0.3	5.7 ± 0.6	5.2 ± 0.3
2-h glucose (mmol/L)								5.9 ± 0.9	15.8 ± 3.2*			
HbA_{1c} (mmol/mol)							35.9 ± 1.9	37.1 ± 3.2	52.3 ± 7.3*	35 ± 2.5	35.9 ± 6.2	34.7 ± 4.2*
F- insulin (mU/L)	27.0 ± 14.0[D,E]	19.8 ± 11.8[D]	22.2 ± 16.8	7.2 ± 2.18[A,B,C,E]	7.0 ± 2.0	11.6 ± 4.3[D]	5.3 ± 3.8	7.4 ± 2.8	14.5 ± 6.3*	7 ± 3.1	24.6 ± 9.3	10.7 ± 0.3*
2-h insulin (mU/L)								42.3 ± 25.6	52.2 ± 26.6			
Triglycerides (mmol/L)							0.85 ± 0.3	0.98 ± 0.5	1.22 ± 0.5	1.1 ± 0.9	1.6 ± 0.5	1.1 ± 0.6*
Tot cholesterol (mmol/L)							5.3 ± 0.9	5.1 ± 0.6	4.6 ± 0.6	5.9 ± 0.9	5.2 ± 0.7	3.8 ± 0.5*
HDL cholesterol (mmol/L)							1.4 ± 0.3	1.3 ± 0.3	1.3 ± 0.3	1.4 ± 0.3	1.3 ± 0.3	1.1 ± 0.2
LDL cholesterol (mmol/L)							3.7 ± 0.6	3.4 ± 0.3	2.8 ± 0.9*	3.8 ± 0.6	3.2 ± 0.6	2.3 ± 0.6*
HOMA-IR[a]	7.0 ± 4.1[D,E]	5.2 ± 4.5	5.9 ± 5.7	1.2 ± 0.68[A,B,E]	1.6 ± 0.5	3.2 ± 1.3[D]	1.2 ± 0.3	1.6 ± 0.6	5.4 ± 2.8*	1.8 ± 0.9	6.2 ± 2.5	2.5 ± 0.7*

Table 3. *Clinical characteristics. Data are presented as mean±SD. Paired and unpaired t-tests were used to test significance. **α**-level set at 0.05. *= significant in relation to NGT in paper 2 and in relation to before diet in paper 4. In paper 1, significance was marked as following; A=versus obese patients before diet, B= versus obese patients op-day, C= versus obese patients discharge, D=versus non-obese patients op-opday, E=versus non-obese patients discharge. Follow up time was 12.4±2.7 months from day of surgery. NGT=normal glucose tolerant, T2DM=Type 2 diabetes mellitus.*

3.2 Skeletal muscle biopsies

3.2.1 Open muscle biopsy

In **paper 3** and for the *in vitro* studies in **paper 2**, an open muscle biopsy technique was used to collect the muscle biopsies. The procedure has been previously described by our group (Zierath et al., 1992) and involves a larger biopsy with more intact skeletal muscle fibres compared to the more common muscle biopsy procedure. Presumably, the open muscle biopsy is also less "stressed" as it is taken out more carefully, but this has not been scientifically tested. The disadvantage with the open muscle biopsy technique is that it is more invasive and requires a larger skin incision compared to conventional muscle biopsy techniques, which increases the risk of post procedure infections. For the open muscle procedure, the subject is placed in a supine position and the skin of the thigh, overlaying the site of the muscle biopsy is washed with antiseptics. Local anaesthesia and a 4 cm skin incision is applied approximately 15 cm lateral and cranial of the patella. The muscular fascia of the *vastus lateralis* is visualized and carefully divided without disturbing the underlying muscle (**Figure 8**).

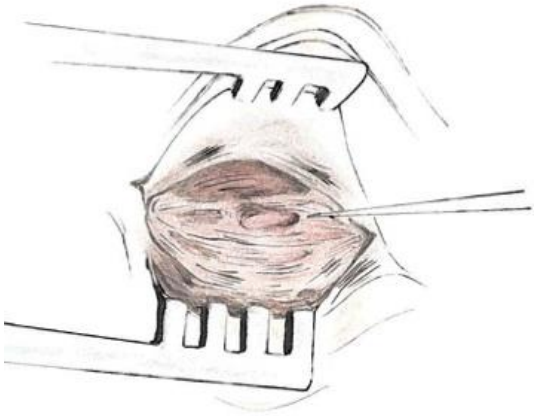

Figure 8*. The open muscle biopsy procedure.*

A muscle biopsy (2 g) is excised and directly placed in room temperature oxygenated Krebs-Henseleit buffer containing 5mM HEPES and 0.1% bovine serum albumin (BSA; RIA Grade; Sigma St Louis, MO) for subsequent *in vitro* experiments. It is important to handle the biopsy carefully, ensuring that the fibres are not stretched, as this can disturb the muscle homeostasis and handling may increase glucose uptake. After haemostasis, the muscle fascia and the skin are sutured with self-dissolving sutures. The open muscle biopsy consists of a long portion of intact muscle fibres that can subsequently be divided into smaller muscle strips (10-20 mg each) and attached to plastic clamps (**Figure 9**).

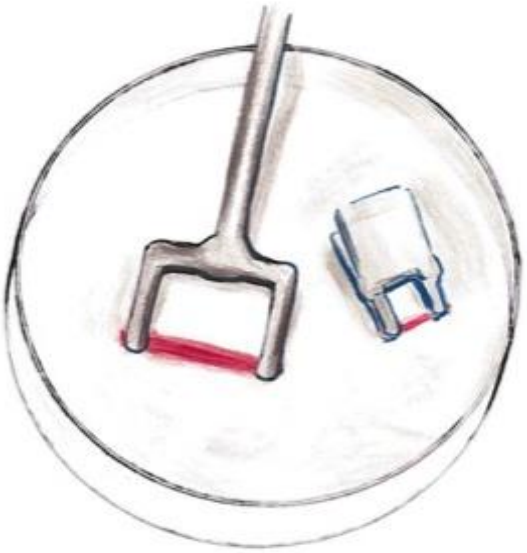

Figure 9*. Muscle strips attached to clamps ready for incubation.*

The muscle strips can then be incubated with different compounds and further studied for DNA methylation and mRNA expression analysis as described in **papers 2 and 3**.

3.2.2 Muscle biopsy technique by conchotome

In **paper 4** and for the *in vivo* studies in **paper 2**, a conchotome was used to collect the muscle biopsies. For this procedure, the skin is washed and local anaesthesia is applied as in the open muscle biopsy procedure. A small skin incision is made overlaying the *vastus lateralis* muscle and a Weil-Blakesley conchotome (**Figure 10**) is introduced through the skin to obtain the muscle biopsy (100 mg).

Figure 10*. Weil-Blakesley conchotome.*

Several samples can be collected through the same incision and the wound does not require suturing. Compared with the open muscle biopsy technique, this technique is technically easier to perform and it is less invasive. As the skin incision is smaller, the risk of post procedure infection is low. The amount of tissue obtained is smaller and because the biopsy is taken out blindly, it is less intact in comparison to the open muscle biopsy.

3.3 Cell cultures

3.3.1 Human primary skeletal muscle cell culture

Cell culture systems are useful tools for validation of results found in human tissues and for mechanistic studies. In **paper 2 and 3,** we used human primary skeletal muscle cell cultures that originate from biopsies obtained during the "open muscle biopsy" from donors described in **Table 3**. Primary skeletal muscle cells or satellite cells are muscle-specific stem cells that are located between the basal lamina and sarcolemma of the muscle fibres. Satellite cells are isolated and purified from the skeletal muscle by trypsin and collagenase digestion, grown as myoblasts, and differentiated into myotubes (Al-Khalili et al., 2003). An advantage with primary skeletal muscle cells is the human origin, and aspects of the metabolic phenotype of the donor is retained also in the culture (Boyle, Zheng, Anderson, Neufer, & Houmard, 2012). Our group has shown that human primary skeletal muscle cells from type 2 diabetic donors also show insulin resistance (Bouzakri & Zierath, 2007). This is a key advantage when studying the mechanisms underlying insulin resistance in skeletal muscle. Primary cell cultures have a limited life span and stop proliferating after a number of cell divisions. The purification process is challenging and often other cell types such as fibroblasts can be difficult to eliminate. Fibroblasts grow faster than myoblasts and can become prominent in the culture, complicating the muscle data analysis. The specific methods for the muscle culturing and different media used are described in detail in the Methods sections in **paper 2** and **paper 3**.

3.3.2 Rat L6 skeletal muscle cell culture

The rat L6 is a commercially available immortalized cell line purchased from ATCC (Wesel, Germany). Cell lines proliferate faster than primary cell cultures and are more resistant to external stress, making cell lines technically easier and faster to grow. In comparison to primary cell cultures that originate from different donors, a cell line stems from a single donor where the culture has been immortalized. Results obtained from a cell line are often more reproducible, but it is important to bear in mind that the results only represent effects noted in one individual. In contrast, validation in primary cultures provides insight into different responses between subjects. In **paper 3**, we used both human primary skeletal cells and rat L6 cells to demonstrate the effects of AICAR on reducing cytokine mRNA. Detailed methods of growth, differentiation and medias used for the rat L6 muscle cells can be found under "Methods and Materials" in **paper 3**.

3.4 Small interfering RNA (siRNA)

When studying the cellular function of a specific gene, it can be useful to selectively modulate the expression of that gene. Inhibition of gene expression can be achieved by using synthetic small or short interfering RNA molecules (siRNA). Synthetic siRNA are small (25 nucleotides) double-stranded RNA molecules that can be transfected into cell cultures to transiently silence a gene of interest. Inside the cell, the siRNA associates with RNA-induced silencing protein complex (RISC), a multi-protein complex that gets activated after an ATP-dependent unwinding of the siRNA. After activation, one of the strands of the siRNA is retained within the RISC, binds the messenger RNA target by perfect pairing. Once siRNA has bound to the target, it is cleaved and degraded (**Figure 11**).

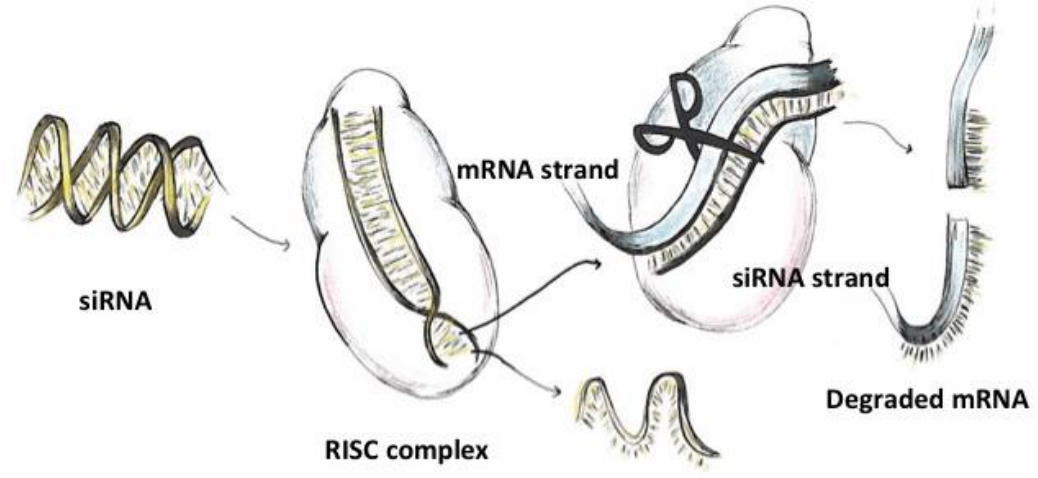

***Figure 11**. siRNA mechanism of silencing mRNA in the cell.*

Although a major portion of the mRNA is degraded, there will usually be some remaining mRNA that avoids the degradation and this may be sufficient in some cases for ensuring cellular function. The siRNA sequences are specific to their target but may have off-target effects, meaning that they may also silence other genes. New techniques such as CRISPR-Cas9 are available to completely silence a gene, however, the siRNA technique still has its advantages of being easy to use, cost efficient and well tolerated by the cells. siRNAs were used in **paper 2** and **3**, the details of the experiments can be found in the "Methods and Materials" section of each respective manuscript.

3.5 Metabolic assays

In vitro systems allow for the determination of the metabolic function of skeletal muscle strips or cells exposed to a certain treatment. In **paper 2**, fatty acid oxidation as well as glucose incorporation into glycogen assays were conducted to see if the silencing of DAPK3 has an impact on metabolism. The methods of these metabolic assays are previously described in detail by our group (Al-Khalili et al., 2006).

3.6 DNA and RNA extraction

DNA and RNA have been extracted using column based extraction kits according to the protocol from the manufacturers. Several different extraction kits (DNeasy, RNeasy, AllPrep kit (all from Qiagen, Hilden, Germany), E.Z.N.A (Omega Bio-tek, Norcross, GA, USA) and mirVana (Thermo Fisher Scientific, Waltman, MA, USA) have been used throughout this thesis depending on the availability in the laboratory at that specific time. DNA and RNA concentrations were measured by Spectrophotometry (NanoDrop1000 by Thermo Fisher Scientific). Details of DNA and RNA extractions can be found in the Methods section of each manuscript.

3.7 mRNA expression analysis

RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific). Quantitative PCR was performed using SYBR Green-based primers in **paper 2** and Taqman probes in **papers 3** and **4**. Primers are listed in the respective manuscripts.

In **paper 4**, RNA samples were subjected to gene microarray analysis using Human Gene 2.1 ST Array (Affymetrix, CA, USA) performed by Bioinformatics and Expression Analysis (BEA) core facility at Karolinska Institutet.

3.8 Immunoblot analysis

Proteins were extracted and immunoblots were performed in **papers 2** and **4** as described in each respective manuscript. The antibodies used are listed in each manuscript and have been validated in our laboratory and used in previous publications from our group.

3.9 Studies of DNA methylation

To date, there are many different ways to measure DNA methylation. The technique used depends on several things, the quality and amount of the DNA, if one is interested in a particular gene or the whole genome, cost of the analysis, equipment and skills for data interpretation (Kurdyukov & Bullock, 2016). We used different techniques in **papers 1** and **2**, and will here be closer described to clarify the differences.

3.9.1 Methylated DNA enrichment by MBD-capture

In **paper 1** we used a methylated DNA enrichment technique, the MethylMiner kit (Thermo Fisher Scientific, Waltman, MA, USA), where fragmented DNA is enriched by MBD-capture (Methyl-CpG-Binding Domain) using magnetic beads. The methylated DNA binds to the beads, while the unbound DNA, lacking methylation, is discarded in the supernatant (**Figure 12**).

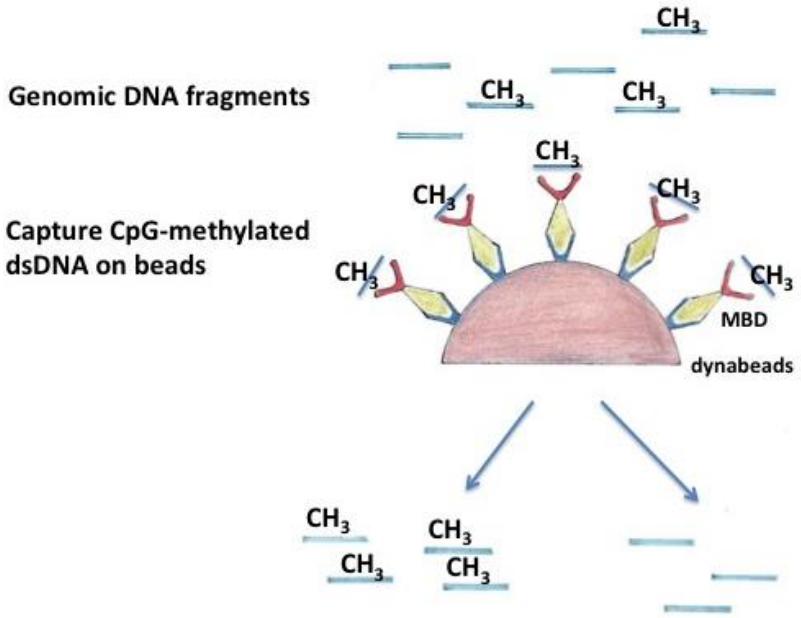

Figure 12*. Graphical presentation of the MethylMiner method.*

Target-specific DNA methylation can then be estimated by qPCR using specially designed primers. The level of DNA methylation is finally represented as the percentage of methylated DNA relative to input (total) DNA of each target measured by qPCR after the MBD-capture. The MBD captured methylated DNA can be used for subsequent sequencing of a large DNA sequence. The technique using qPCR and specially designed primers after MBD-capture does not, in contrast to sequencing, give the exact position of the methylated CpGs. However, when the gene of interest is known, as in **paper 1**, this enables a fast and easy way to estimate the level of DNA methylation. For details see the methods section of **paper 1**.

3.9.2 Bisulfite conversion techniques

In **paper 2** we used two other techniques to measure DNA methylation. Here, in contrast to **paper 1**, we were interested in identifying all genes that were differentially methylated. Both techniques used in **paper 2** are based on bisulfite conversion, which is considered the "gold standard" technique for DNA methylation analysis. The bisulfite treatment of DNA mediates deamination of non-methylated cytosine nucleotides into uracil, but has no effect on 5-methylcytosine (methylated cytosine) residues. This induces a difference in DNA sequence, which can be recognized by hybridization with a microarray or by sequencing (**Figure 13**).

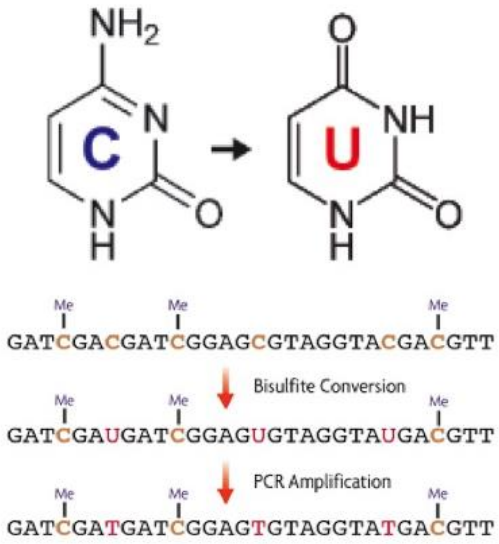

***Figure 13**. Cytosine is converted to Uracil. The bisulfite conversion changes the DNA sequence.*

In **paper 2**, we used bisulfite converted skeletal muscle DNA (Epitect Fast Bisulfite Conversion Kit by Qiagen) from a subset of subjects (n=3) for an Infinium HumanMethylation450 Bead Chip array (llumina, San Diego, CA, USA), to generate a list of differentially methylated genes, induced by insulin treatment. The bead chip microarray uses probes of more than 485 000 individual CpGs in 99% of known genes. Results obtained from microarrays may result in both false positive and false negative results, and thus subsequent technical validation, generally through target-specific sequencing is usually performed.

Pyrosequencing (PyroMark CpG Assays from Qiagen) and individually designed primers were used to validate the results from the microarray. The pyrosequencer can read DNA sequences up to 100bp, and requires that the sequence is known. The advantage of the sequencing technique in comparison to the MethylMiner technique is the precision, giving the exact position of the methylated CpGs. However, the sequencing requires high quality DNA and as pointed out earlier, the sequence must be known. The MethylMiner technique on the other hand, can examine a larger sequence. For details of the microarray and pyrosequencing, including primer sequences, see the methods and results section in **paper 2**.

3.10 Lipidomic analysis

Lipidomic analysis enables the study of all the lipid classes and a majority of the specific lipid species in a tissue. In **paper 4**, we were interested in assessing if the lipid profile in skeletal muscle changed in obese subjects undergoing a diet intervention. Lipids were extracted from skeletal muscle (10 mg) by using the chloroform:MeOH (2:1 vol/vol) phase extraction method and were then analysed by ultrafast liquid chromatography-mass spectrometry- (LC-MS) based lipidomic analysis (Swedish Metabolomics Centre at the Swedish University of Agricultural Sciences in Umeå, Sweden). Lipid spectral count raw data were normalized to input muscle weight before analysis.

3.11 Bioinformatics

We have used bioinformatic approaches in **papers 2, 3 and 4** included in this thesis. In **paper 2**, we used a methylation microarray (Infinium HumanMethylation450 Bead array by Illumina) to identify novel gene targets that were differentially methylated by insulin treatment. The data analysis was performed at Bioinformatics and Expression Analysis

(BEA) core facility at Karolinska Institutet. In **paper 3**, we used a bioinformatic approach to identify transcription factors possibly involved in the basal expression of IL-6 and LIF. The database *dcode.org* (Ovcharenko, Nobrega, Loots, & Stubbs, 2004) was used to identify conserved and putative functional regions of the cytokine promoter. We used open access expression microarrays (for GEO accession number see Supplementary table of **paper 3**) to allow exclusion of transcription factors that were not expressed in human or rat skeletal muscle tissue or cells. In **paper 4**, we performed a lipidomic analysis as well as an expression microarray analysis on human skeletal muscle biopsies before and after a diet intervention. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed using Webgestalt (**paper 2**) (J. Wang, Duncan, Shi, & Zhang, 2013) and the Broad institutes GSEA software (**paper 4**) (Mootha et al., 2003).

3.12 Statistics

In most of the analysis throughout this thesis, we have compared tissues from the same individual or primary skeletal muscle cell donor, allowing for paired statistical testing. After testing the normal distribution with Shapiro-Wilk and D´Agostino-Pearson tests, a paired t-test (normally distributed samples) or Wilcoxon signed rank test (not normally distributed) have been performed when there have been only two conditions to compare, and a two-way ANOVA with post hoc tests (when normally distributed) or Friedman´s test (not normally distributed), when there have been more than two conditions. Correlation analysis in **paper 2** was determined using Spearman correlation coefficient (not normal distribution) and Pearson correlation coefficient (normally distribution) was used in **paper 3** and **4**. Analysis was performed using GraphPad Prism version 7.0 (GraphPad software, Inc., La Jolla, CA, USA) or R (R, 2017). The significance level was set at <0.05. *$p<0.05$, **$p<0.01$ if not stated otherwise. Data is presented as mean±SEM if not stated otherwise.

4 RESULTS AND DISCUSSION

4.1 DNA methylation is associated with metabolic changes

DNA methylation patterns differ in skeletal muscle from people with type 2 diabetes and normal glucose tolerance (Barres et al., 2009). In adipose tissue, DNA methylation differs in people who respond well or poorly to caloric restriction for weight loss (Bouchard et al., 2010). Exercise and weight loss through diet can modify genome wide DNA methylation in skeletal muscle (Brons et al., 2010; Jacobsen et al., 2012; Ronn & Ling, 2013). More specifically, acute exercise as well as cytokines, and free fatty acids can remodel promoter methylation and gene expression of metabolically important genes such as *PPARγ, PDK4* and *PGC1α* (Barres et al., 2009; Barres et al., 2012). Differences in DNA methylation patterns in peripheral blood were identified between people with type 2 diabetes and non-diabetic subjects (Toperoff et al., 2012) and between obese and lean controls (X. Wang et al., 2010). Weight loss by low calorie diet or a specific Mediterranean diet has also been shown to be associated with DNA methylation changes in peripheral blood (Arpon et al., 2016; Milagro et al., 2011). Collectively, this suggests that DNA methylation in skeletal muscle and blood may provide a mechanism for acute and transient as well as more long lasting gene regulation, which may have an impact on metabolic health.

4.1.1 DNA methylation altered by diet-induced weight loss and RYGB

Early metabolic improvements after RYGB surgery are likely to be partly mediated by the calorie restriction that patients undergo before and shortly after the surgery (Jackness et al., 2013; Lim et al., 2011). We have previously shown that DNA methylation could constitute a regulatory mechanism contributing to the metabolic improvements after RYGB (Barres et al., 2013). We were interested in studying if diet-induced weight loss could induce similar promoter DNA methylation changes as RYGB surgery. In addition, we were interested in finding if promoter DNA methylation alterations were present in peripheral blood, as this could enable a valuable proxy marker for improved metabolic health.

In **paper 1,** 18 obese non-diabetic adults were recruited from the waiting list for gastric bypass. The subjects underwent a 14 day diet of 1,000 kcal per day (40-50% carbohydrate, 30-40% protein, and 20% fat as percentage of total calories). On the last day of the diet, the obese patients underwent RYGB bariatric surgery. Peripheral blood was collected before and after the diet intervention and subsequent RYGB surgery and the promoter DNA methylation from the nucleated cells were studied. A subset of subjects (*n=7*) were followed up on the day of discharge (2 days after the surgery) and 12 months later. Six non-obese subjects undergoing elective cholecystectomy were also enrolled in the study to control for the surgical stress and to determine whether promoter DNA methylation in the obese cohort could be reversed by diet or RYGB to the level observed in non-obese subjects. The clinical characteristics of the study participants and the clinical chemistry at the various time points are presented in **Table 3**.

The obese subjects lost significant weight following the diet (4.2 kg±1.4) and following the RYGB surgery (30 kg±11.8), respectively. Glucose levels of the obese subjects after the diet were significantly reduced compared to before, and comparable to glucose levels of the non-obese subjects, while the insulin levels in the obese subjects remained unchanged following the diet intervention. After the RYGB, the fasting plasma insulin levels were reduced compared to both before and after the diet. Collectively this indicates that the diet-induced weight loss had a modest effect on the glucose levels but a more profound weight loss, induced by RYGB, was needed to lower the insulin levels and thus to improve the HOMA-IR.

Type 2-diabetes was one of the exclusion criteria of the study and thus fasting glucose levels in the obese cohort were from the start of the study relatively low, the reason why we did not see a decrease. At discharge, both blood glucose and insulin levels were increased, both in the obese and the lean cohort. Surgery is known to induce transient insulin resistance and hyperglycaemia as a result of the surgical procedure itself that causes stress and inflammation in the body (Thorell, Efendic, Gutniak, Haggmark, & Ljungqvist, 1994) and our results could be a reflection of this process.

Our group has previously demonstrated that the promoter regions of *PDK4* and *PGC1α*, genes, important for substrate selection and mitochondrial function, are differentially methylated in skeletal muscle from obese subjects in comparison to skeletal muscle from non-obese controls (Barres et al., 2013). Furthermore, the methylation levels of the promoter of these genes were restored to non-obese levels after RYGB-induced weight loss (Barres et al., 2013). Using enrichment of genomic DNA with MBD-beads to capture the methylated DNA fraction and subsequent qPCR, the promoters of the genes *PDK4, IL6 or TNF* were not differentially methylated after the diet. Conversely, the promoter methylation of *PGC1α* was decreased after diet-induced weight loss (**Figure 14, left**). The promoter methylation of *PGC1α* also inversely correlated with plasma glucose and insulin levels after the diet, indicating that the promoter DNA methylation of *PGC1α* in peripheral blood may be used as a proxy marker for improved whole-body metabolic health. The decrease in promoter DNA methylation of *PGC1α* in our cohort in blood after diet is similar to the observed promoter DNA methylation decrease after RYGB in skeletal muscle (Barres et al., 2013).

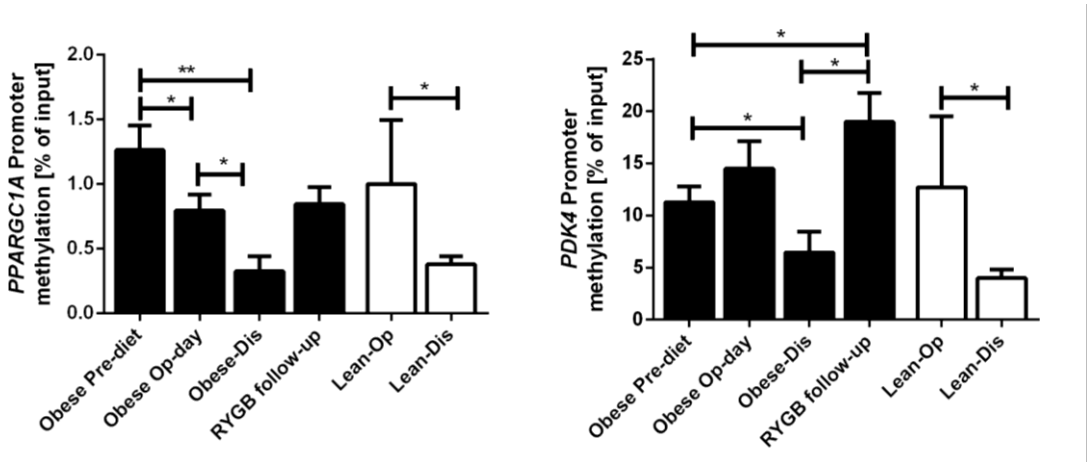

Figure 14. *PGC1α (PPARGC1A) promoter methylation (left) and PDK4 promoter methylation (right), n=6-18.*

A year following the RYGB, the promoter DNA methylation of *PDK4* and *IL6* was increased in comparison to at the start of the study (**Figure 14, right** and **Figure 15, left**), suggesting that a substantial weight loss induced by RYGB, or another specific surgery-related factor, was needed to induce these DNA methylation changes. In contrast, the promoter methylation of *PGC1α* was unchanged in the blood a year after RYGB (**Figure 14, left**), in contrast to the findings in skeletal muscle (Barres et al., 2013).

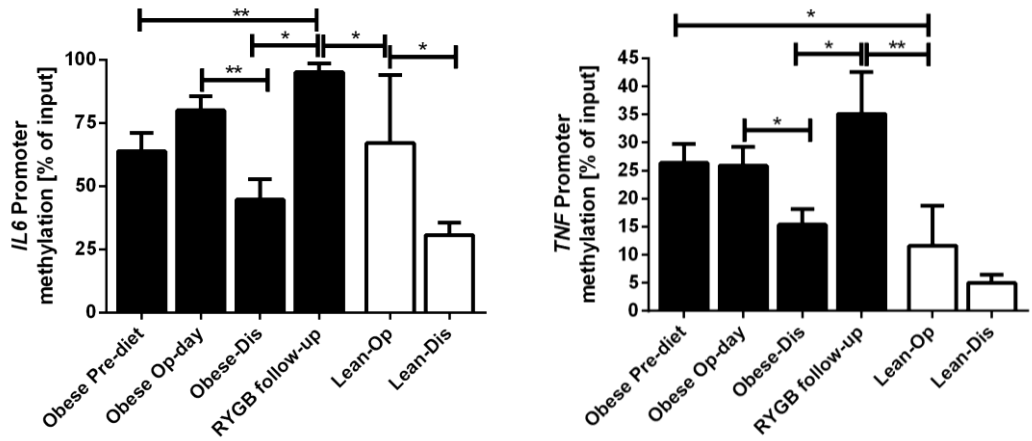

Figure 15. *IL6 (left) and TNF (right) promoter DNA methylation in peripheral blood, n=6-18.*

Interestingly, DNA methylation of all the investigated promoter regions presented here (except for TNF in lean cohort) was decreased early after surgery. This indicates, that factors like including the surgical stress, anaesthesia, and perhaps the total caloric restriction shortly before and after surgery, had a greater effect on DNA methylation than low calorie diet and RYGB respectively. We cannot exclude, that these DNA methylation alterations could be caused by a shift in nucleated blood cell types, and more reflect a general inflammatory state common after surgery (Jakeways et al., 1994) or anaesthesia (Heil, Silva, Pelosi, & Rocco, 2017). This hypothesis is strengthened by the fact that also the non-obese subjects undergoing cholecystectomy demonstrated similar promoter DNA methylation changes.

We measured the plasma cytokine concentrations of IL-6, TNFα and IL-8 before and after the diet and at discharge. We did not find any changes in plasma IL-6 or TNFα levels that correlated with the alterations in *IL6* and *TNFα* promoter DNA methylation, although there was a trend for plasma IL-6 to increase after surgery. Several tissues produce and secrete IL-6 and other cytokines into the blood stream, thus the cytokine levels we measured in plasma in our cohort do not necessarily reflect the promoter DNA methylation levels in nucleated blood cell DNA. A weakness in our study is the inability to measure and interpret mRNA in relation to the DNA methylation in the blood. Only nucleated white blood cells have DNA, while all blood cells including the red blood cells have mRNA (Kabanova et al., 2009). Thus mRNA of peripheral whole blood would not reflect the changes in DNA methylation. Fluorescence activated cell (FAC) sorting of blood cell types prior to the analysis may solve this dilemma for future studies.

In **paper 4**, we studied a different cohort consisting of eight obese, non-diabetic subjects undergoing the same dietary regime of 1,000 kcal/day for a three-week duration. The clinical characteristics are described in **Table 3,** and are comparable to the obese cohort in **paper 1**. A three-week dietary intervention had a more profound effect on weight loss, fasting blood insulin levels and HOMA-IR than a two-week diet as expected. In contrast, the fasting glucose levels were not significantly reduced after the three-week diet although the fasting glucose values were reduced to the same extent. This is likely due to the small size of this cohort (n=8) in comparison to the obese cohort of **paper 1** (n=18).

Muscle biopsies were obtained before and after the diet intervention and promoter DNA methylation of the same targets was measured with the same technique and the same set of primers as **paper 1**. Surprisingly, none of the promoters of *IL6*, *PDK4* or *PGC1α* were significantly differentially methylated in skeletal muscle after diet intervention, although there were trends for decreased level of methylation for all targets (**Figure 16, upper panel**). The

skeletal muscle mRNA levels were increased for *IL6* and *PDK4*, but not for *PGC1α* after the three-week low calorie diet (**Figure 16, lower panel**). Comparing the trends in decreased methylation and subsequent increases in mRNA of IL6 and PDK4, it is tempting to suggest that there could be a regulatory mechanism, although there is no statistical correlation. The promoter DNA methylation levels are very low in this cohort, with the exception of two subjects, and the relevance of small changes is questionable and perhaps present due to technical variation. Our cohort is small, and thus we cannot compare groups within the cohort. However, it is interesting that two of the subjects have an increased promoter DNA methylation in comparison to the other subjects, which is reduced to the level of the other subjects after the diet. Perhaps these two individuals could have a metabolically different response to obesity, although there is no correlation to clinical parameters. To further investigate such a hypothesis, a much larger cohort is needed.

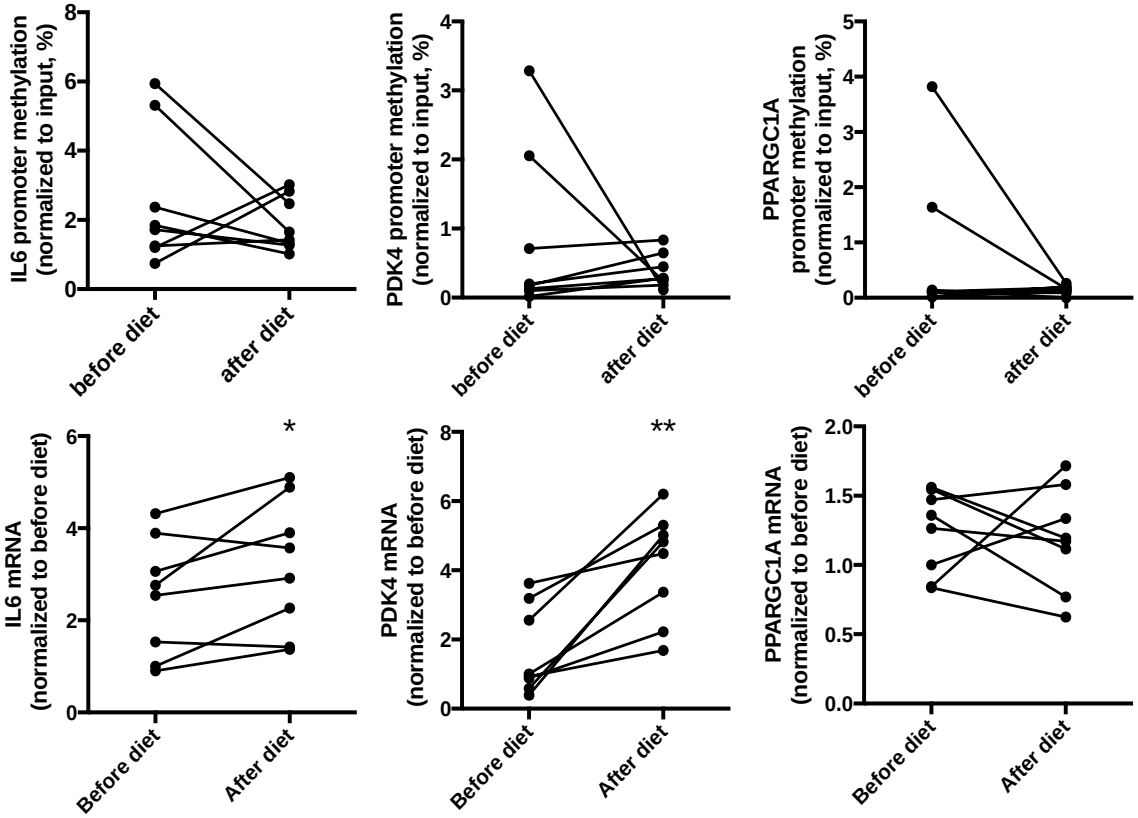

Figure 16. *IL6, PDK4 and PGC1α promoter methylation (upper panel). IL6, PDK4 and PGC1α mRNA (lower panel) in human muscle before and after a three-week diet, n=8.*

The trend for a decrease in *PDK4* promoter methylation and increased *PDK4* mRNA expression gives an inverse relationship similar to previous results in skeletal muscle (Barres et al., 2013; Kulkarni et al., 2012), suggesting a possible regulatory mechanism of *PDK4* mRNA by DNA methylation. The discrepancies between our results from diet-induced weight loss and previous results in skeletal muscle from weight loss after RYGB (Barres et al., 2013) may be explained by the use of different technique to measure DNA methylation. We used a technique to enrich for methylated DNA and subsequent PCR, though others, including the study of skeletal muscle biopsies after RYGB, used techniques including bisulfite treatment of the DNA. This implies that we may have measured different regions of the gene promoter. Other factors may also have affected the degree of methylation, such as a greater weight loss after RYGB, surgery itself, or larger metabolic disturbances like in type 2 diabetes.

In our cohort, *PDK4* mRNA is increased after the low calorie diet, consistent with an earlier study (Pilegaard, Saltin, & Neufer, 2003). PDK4 is an enzyme that inhibits the pyruvate

dehydrogenase complex, which results in decreased carbohydrate oxidation and a shift in fuel selection towards lipid metabolism. Similarly, high fat diet (Peters et al., 2001) and exercise induces PDK4 expression in the normal glucose tolerant subjects (Pilegaard & Neufer, 2004), but no effect of exercise is seen in type 2 diabetic subjects (Kulkarni et al., 2012). *PDK4* mRNA levels are higher in type 2 diabetic patients (Kulkarni et al., 2012; P. Wu et al., 1999) and obese subjects (Barres et al., 2013) in comparison to healthy, lean controls. Insulin reduces *PDK4* mRNA expression in normal-glucose tolerant individuals but not in type 2 diabetics (Y. I. Kim, Lee, Choi, Lee, & Youn, 2006). In a state of type 2 diabetes with hallmarks of hyperinsulinemia and hyperglycaemia as well as high fatty acid levels, increased PDK4 expression leads to increased fatty acid oxidation but by inhibiting carbohydrate oxidation the hyperglycaemia is elevated even further, leading to a vicious circle. This process illustrates the metabolic dysfunction associated with type 2 diabetes.

In conclusion, these results demonstrate that changes in DNA gene promoter methylation in response to interventions altering body weight are tissue specific and underscores the importance of studying DNA methylation directly in different tissues (Bell, 2017). Thus, it does not appear possible to use promoter DNA methylation in peripheral blood as a surrogate marker for gene methylation in skeletal muscle. Rather, changes in DNA methylation in peripheral blood can be used for predicting general metabolic changes. The subpopulation of blood cells will influence the epigenetic pattern in blood and thus sorting of blood cells could overcome this issue. Promoter methylation of metabolically important genes may constitute a molecular regulatory mechanism contributing to the metabolic improvements after RYGB surgery.

Peripheral blood is readily accessible and does not require invasive techniques. Nutritional markers in blood are warranted as this could enable a strategy of risk evaluation and personalised medicine. However, DNA methylation pattern differ between tissues and thus a blood sample may not fully substitute for the study of the specific tissues. Investigation to increase knowledge about gene regulation in specific tissues contributing to metabolic dysfunction is of major importance for enabling better treatments. However, peripheral blood could be used as general metabolic markers and as guidance for personalised health advice and risk assessment where invasive sampling techniques are unsuitable.

In **paper 1**, we studied the promoter DNA methylation of metabolically important genes after negative energy homeostasis induced by weight loss. In **paper 2**, in contrast, we were interested in the acute effects of insulin and glucose ingestion on DNA methylation in skeletal muscle.

4.1.2 DNA methylation is altered acutely by insulin and glucose exposure

In **paper 2**, we hypothesized that insulin treatment could acutely alter the genomic DNA methylation pattern in human skeletal muscle. Intact human skeletal muscle strips derived from open muscle biopsies from healthy, lean donors (**Table 3**) were incubated *in vitro* with or without (basal) insulin for one hour. The muscle strips were then frozen immediately for subsequent DNA extraction and bisulfite conversion. The differentially methylated genes were studied using a genome-wide Illumina 450k array.

Insulin treatment induced broad DNA methylation changes throughout the genome. A KEGG analysis of all the differentially methylated genes revealed that pathways of insulin signalling, type 2 diabetes and metabolic pathways were significantly changed. This suggests that acute insulin treatment does affect the methylation signature of metabolic genes and that this could be a regulatory mechanism for changed gene expression important for metabolic homeostasis.

One specific gene target, *DAPK3* (Death-associated protein kinase 3), was selected for further investigation due to consistent changes in DNA methylation between basal and insulin treated muscle strips (18% change between beta-values, p=0.006). DAPK3 is known to play a role in autophagy (Komatsu & Ikebe, 2004) and apoptosis (Kawai, Matsumoto, Takeda, Sanjo, & Akira, 1998) and has been identified as a tumor suppressor gene, frequently mutated or methylated in several cancer types (Brognard, Zhang, Puto, & Hunter, 2011). However, DAPK3 is previously unknown to be involved in skeletal muscle metabolism. Pyrosequencing was performed to validate the changes in the array and confirmed that the methylation of one specific CpG site (cg08498987) in the gene body of *DAPK3* was increased in the insulin treated samples (**Figure 17, left**). However, mRNA of *DAPK3* remained unchanged in the muscle strips (**Figure 17, right**).

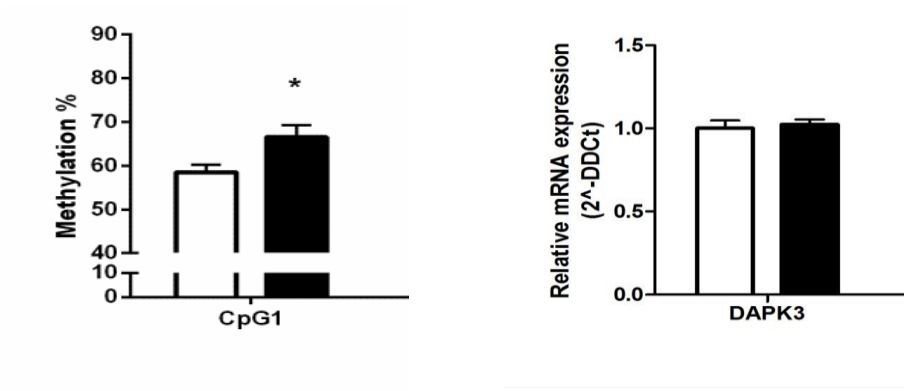

Figure 17. *DNA methylation changes of DAPK3 (left) mRNA of DAPK3 (right), n=10.*

DNA methylation of *DAPK3* was then studied in skeletal muscle biopsies, from a separate cohort including normal glucose tolerant and type 2 diabetic men, before and 2-h after an oral glucose challenge (**Table 3**). The methylation level of DAPK3 at the same CpG site was lower at baseline in the type 2 diabetic than in the normal glucose tolerant subjects (**Figure 18, left**). After the glucose challenge, the methylation level was reduced in both groups. The blood glucose concentration at the 2-h time point after the glucose challenge was inversely correlated with the methylation level of DAPK3 (**Figure 18, right**).

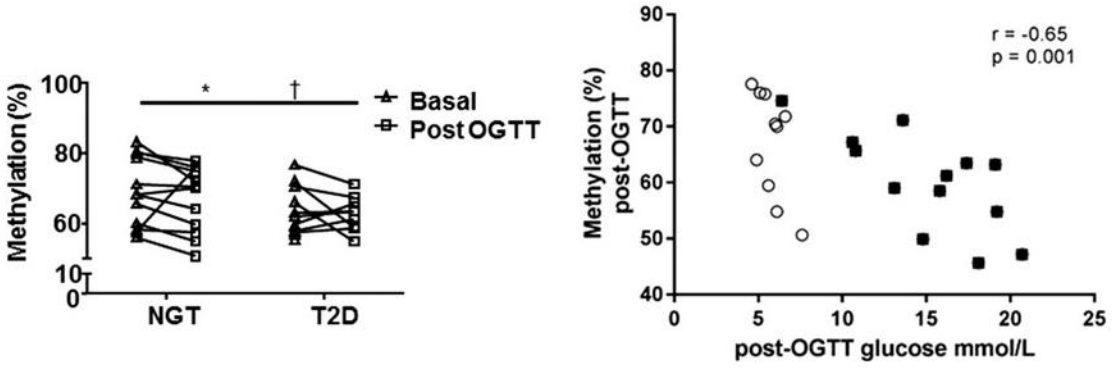

Figure 18. *DAPK3 DNA methylation in skeletal muscle from subjects with normal glucose tolerance (NGT) and type 2 diabetes (T2D) before and 2-h after an oral glucose tolerance test (OGTT) (left) DAPK3 methylation changes after OGTT correlated with post OGTT glucose values (right). Open circles, NGT and closed squares, T2D. n=12. * Main effect of OGTT. † indicates the main effect of T2D.*

Collectively this identifies that DNA methylation of a previously unknown gene target is regulated by changes in glucose and insulin levels and altered in type 2 diabetes. Moreover, this study demonstrates the rapid and transient changes in DNA methylation, induced by insulin and glucose, illustrating the dynamic regulative mechanism of DNA methylation.

To investigate the metabolic relevance of DAPK3 in skeletal muscle, we silenced *DAPK3* using siRNA in primary human myotubes and performed metabolic assays. Glucose incorporation into glycogen was unchanged in primary myotubes transfected with siRNA against DAPK3. Conversely, both basal and AICAR-challenged lipid oxidative capacity was increased when *DAPK3* was silenced, suggesting that DAPK3 could be involved in suppressing lipid oxidation in skeletal muscle.

The mRNA of *DAPK3* was not changed in any of the samples, highlighting the complex relationship between DNA methylation and subsequent effects on gene regulation. Perhaps the level of DAPK3 DNA methylation changes noted here is not sufficient to regulate transcription. The muscle strips were frozen after 1h insulin incubation and it is possible that the mRNA expression changes in the muscle occurred at a later time point. However, DNA methylation does not always correlate with changes in mRNA (Barres et al., 2013; Multhaup et al., 2015). Additional involvement of other epigenetic modifications, such as histone modifications, may be required to fully regulate *DAPK3* mRNA expression.

This study underscores the dynamic features of DNA methylation. It demonstrates that both short insulin treatment *in vitro* of intact human muscle strips, and glucose ingestion *in vivo* can induce acute DNA methylation changes in skeletal muscle. Further studies are needed to reveal the relevance of these transient and dynamic methylation changes for skeletal muscle metabolism and health.

In summary, both **paper 1** and **paper 2** highlight DNA methylation as a potential regulatory mechanism for metabolically important genes. We, and others, demonstrate that environmental factors such as weight loss, surgical stress, exercise and nutrition can induce tissue-specific rapid, transient, but also more long-term, epigenetic marks that can influence metabolic health. This enables the identification of potential targets for disease prevention and treatment. However, epigenetic regulation is complex and multileveled and there are still questions about the precise mechanism of regulation and its causative role in metabolic disease.

4.2 AICAR-induced AMPK activation and cytokine mRNA in skeletal muscle

Energy homeostasis and inflammation are tightly regulated and perturbations in one system affect the other. Activation of AMPK, a central regulator of cell energy homeostasis, has anti-inflammatory effects, but the underlying mechanisms are not fully elucidated. In **paper 3**, we aimed to study the regulatory mechanisms of AICAR-induced AMPK activation on cytokine mRNA expression in healthy skeletal muscle. Study of skeletal muscle from people with normal glucose tolerance can aid in the identification of the mechanisms by which energy and inflammatory systems are interconnected, and dissect dysfunction in metabolic disease.

Thirteen healthy, non-obese men were recruited for an open muscle biopsy (**Table 3**). The muscle biopsy was subsequently dissected into intact muscle strips and incubated *in vitro* with or without AICAR (2 mM) for one hour and subsequently frozen in -80 °C for further mRNA studies. AICAR is often used in experimental settings as an exercise-mimicking agent as it, in similarity to exercise, strongly activates AMPK (Corton, Gillespie, Hawley, & Hardie, 1995). Exercise induces *PGC1α, PDK4* and *TFAM* mRNA expression (Egan et al., 2010; Short et al.,

2003), and thus we were expecting to see an increased mRNA response also with AICAR. Surprisingly, the mRNA of *PGC1α*, *PDK4* and *TFAM* remained unchanged by the AICAR treatment (**Figure 19**).

AICAR is an AMP-analogue, mimicking a signal of low cellular energy, which is only part of the exercise stimuli. Indeed, our group has previously shown that *PDK4* mRNA is induced in skeletal muscle cells by calcium release induced by caffeine incubation, but not by AICAR incubation (Kulkarni et al., 2012). Thus, another factor other than increased AMP/ATP ratio is the regulative stimuli for *PDK4* expression. Maybe calcium release, which is also part of the exercise stimuli, is responsible for the exercise-induced change in *PDK4* expression. In contrast, AMPK is a specific inducer of *PGC1α* mRNA (Jager, Handschin, St-Pierre, & Spiegelman, 2007) and this AMPK-mediated induction of *PGC1α* is suggested to play a major role in the improved mitochondrial function in response to exercise.

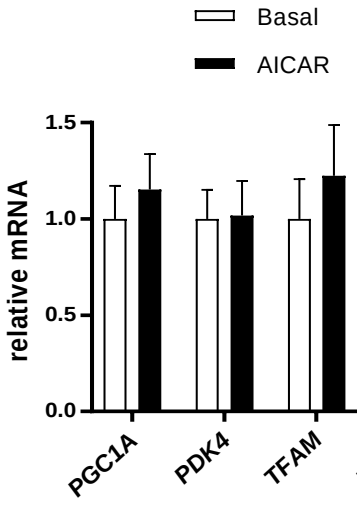

Figure 19. *mRNA of metabolic targets in human skeletal muscle biopsies treated with and without AICAR (2 mM) for one hour, n=13.*

Since the muscle strips were only incubated with AICAR for one hour and the experiment was terminated immediately after the incubation, longer incubation time with AICAR or a later termination time point may have revealed changes in *PGC1α* and *TFAM* mRNA. This is in line with previous results from our group demonstrating that *TFAM* and *PGC1α* mRNA do not increase until two hours and three hours post *ex vivo* contraction in mice, and at three hours post exercise (*PGC1α*) in human subjects (Barres et al., 2012) respectively. In conclusion, it is likely that we studied mRNA of the above metabolic targets at a time point that was too early to see any significant changes.

In contrast to the metabolic targets, mRNA of all the cytokines we tested were reduced (**Figure 20**). Cytokine mRNAs are constitutively expressed in skeletal muscle at low levels as demonstrated in the figure below based upon publically available expression arrays (**Figure 21**). AICAR exposure has previously been shown to reduce baseline cytokine mRNA and protein in mice skeletal muscle and human skeletal muscle cells (Glund et al., 2009). We confirmed these findings through *in vitro* incubations of intact human muscle strips, as well as human skeletal muscle cells and rat L6 cells.

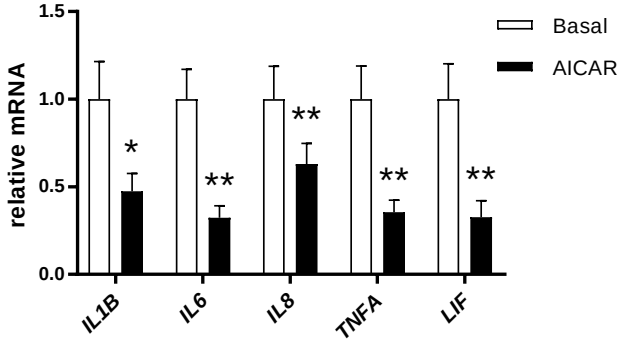

Figure 20. *mRNA of cytokine targets in human skeletal muscle biopsies treated with and without AICAR (2 mM) for one hour, n=13.*

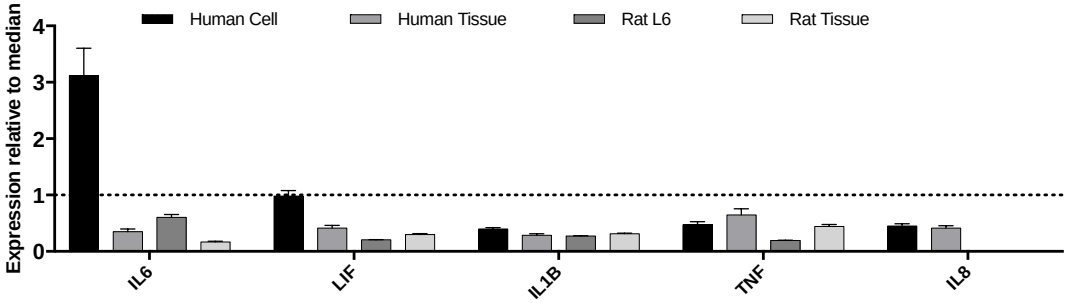

Figure 21. *Cytokine gene expression in Human and Rat tissue and cells based on publically available array databases generated by a free web-based tool (Pillon, 2017).*

This acute effect on cytokine expression could constitute a mechanism by which AMPK reduces tissue inflammation. The inflammatory process requires energy and since the main role of AMPK is to restore ATP in the cell, reducing production of cytokines may be an efficient mechanism to save energy.

We aimed to elucidate the mechanism by which AICAR reduces cytokine mRNA. We decided to focus on IL-6 and LIF as these cytokines were expressed in both human and rat cells, and are typical "immunometabolic" genes in comparison to the more pro-inflammatory genes IL-1β and TNFα. IL-8 is not expressed in rat and was not studied further. IL-6 and LIF belong to the same structural class of cytokines and have overlapping activities as they have specific binding receptors, but share a common signal-transducing receptor gp130 (Taga & Kishimoto, 1997). LIF has mainly been appointed a function in muscle development and regeneration (Brandt et al., 2015; Broholm et al., 2011; Spangenburg & Booth, 2006) but recently LIF has also been suggested a role in skeletal muscle metabolism (Brandt et al., 2015).

To determine if AICAR is likely to decrease cytokine mRNA by degradation or by inhibiting transcription, we designed an experiment where we exposed rat L6 muscle cells to either the transcription-inhibiting agent Actinomycin D alone or together with AICAR. Actionmycin D reduced IL-6 mRNA as expected. Adding AICAR to Actinomycin D, did not decrease the mRNA further than with Actinomycin D alone (**Figure 22**). Thus, we concluded that AICAR is likely reducing cytokine mRNA through inhibiting transcription.

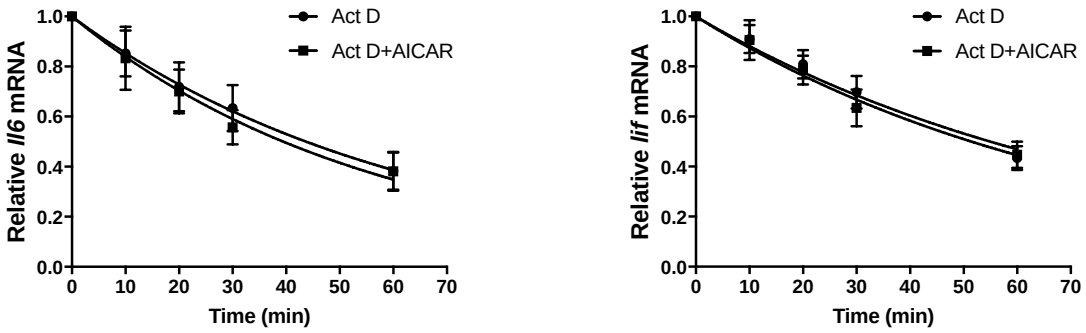

Figure 22. *L6 cells were treated with Actinomycin D alone or with AICAR and mRNA expression of Il6 (left) and lif (right) was assessed. Results are mean±SEM (n=4).*

Transcription of eukaryotic genomic DNA to mRNA requires the enzyme RNA polymerase II and several transcription factors and cofactors. Transcription factors are proteins that bind to a promoter sequence and initiate a series of interactions with other cofactors that together form the transcriptional complex, to which RNA polymerase II can bind and transcribe the specific sequence. Sequence specific transcription factors are considered the most important and diverse mechanism of gene regulation. Other regulatory mechanisms that can affect transcription are epigenetic marks as discussed earlier in this thesis.

A classical inflammatory signalling pathway includes translocation of the p65/*RELA* subunit of the NFκB transcription factor into the cell nucleus and subsequent binding to NFκB response elements, which will initiate transcription of inflammatory genes such as *IL6*. However, exercise-induced IL-6 production can be regulated independently of the NFκB-pathway by the JNK/AP-1-pathway in mouse skeletal muscle (Whitham et al., 2012) and by the p38 MAPK/CREB pathway in cardiac fibroblasts (Du et al., 2005). The *IL6* promoter contains known binding sites for several transcription factors NFκB, AP-1, CREB and C/EBP and RBPJ (Luo & Zheng, 2016; Vanden Berghe, De Bosscher, Boone, Plaisance, & Haegeman, 1999; Welc & Clanton, 2013). Thus several transcription factors may play a role in the AICAR-induced transcriptional effect upon cytokine mRNA.

Complementary to the transcription factors known from the literature to regulate *IL6* and *LIF* mRNA expression, we were interested in finding previously unknown factors that could be involved in the transcription of these targets. Thus, we used an unbiased bioinformatics approach. Taking advantage of the fact that AICAR reduces *IL6* and *LIF* in two different species, in human and rat, we identified conserved regions in the promoters of *IL6* and *LIF* between human and rat. We listed the transcription factors that bind to these promoter regions of *IL6* and *LIF* respectively and then overlapped the list, reducing the candidates to 15 common transcription factors. The previously known transcription factors from the literature were added to the list. Using a database with publically available expression arrays we could then exclude the candidates that were not expressed in skeletal muscle, which finally resulted in a list of 8 transcription factor candidates. This work process identified three, in the context of metabolism, new transcription factors, Nfyc, Sp1 and Zbtb14 (**Figure 23**).

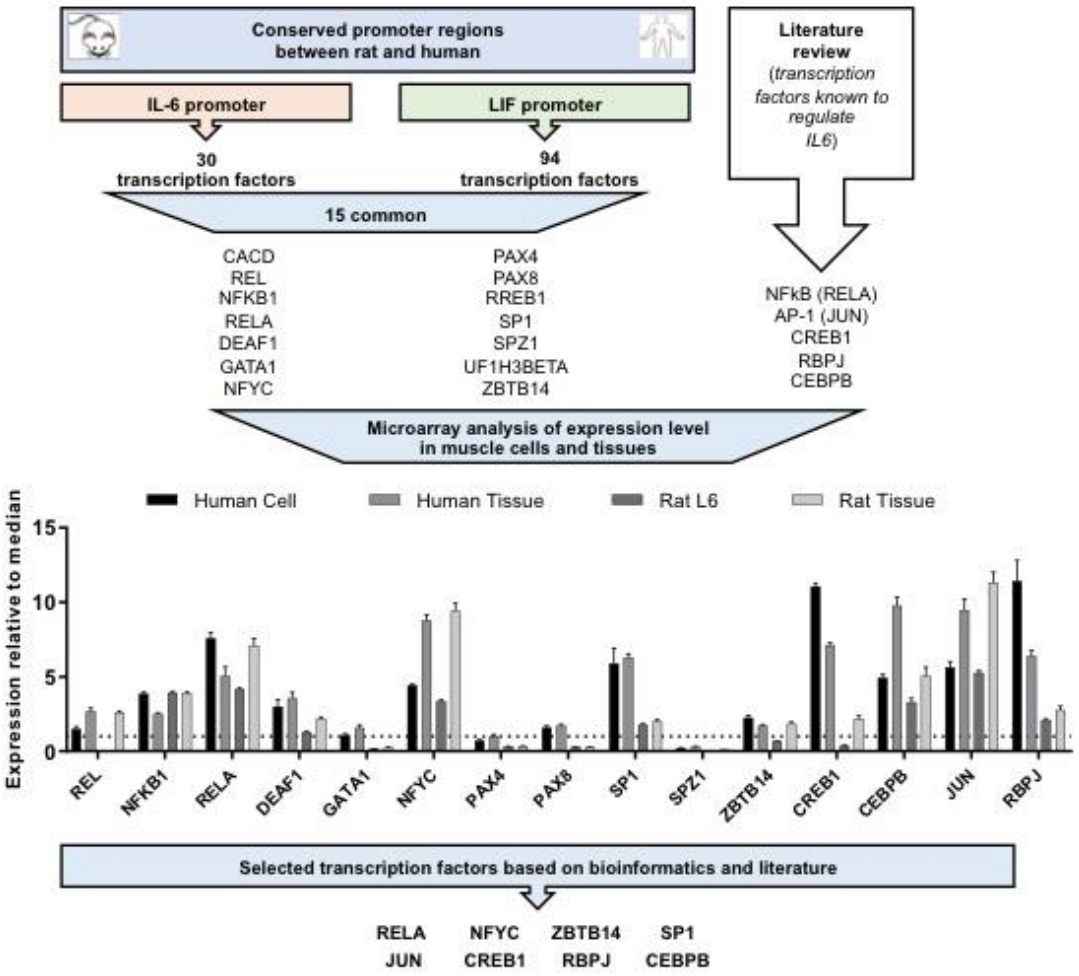

Figure 23. *Schematic illustration of the process of identifying new potential transcription factors.*

Using specific siRNA sequences, we silenced each one of the eight transcription factors candidates in L6 cells and subsequently treated them with AICAR to determine the *IL6* or *LIF* mRNA. Silencing *RELA*, *JUN*, *NFYC*, *CREB1*, *ZBTB14*, *RBPJ* and *CEBPB*, was without effect in the AICAR-mediated decrease of *IL6* and *LIF* mRNA. However, when silencing *SP1*, the AICAR-reducing effect on *IL6* mRNA was blunted, indicating that this transcription factor may be involved (**Figure 24**).

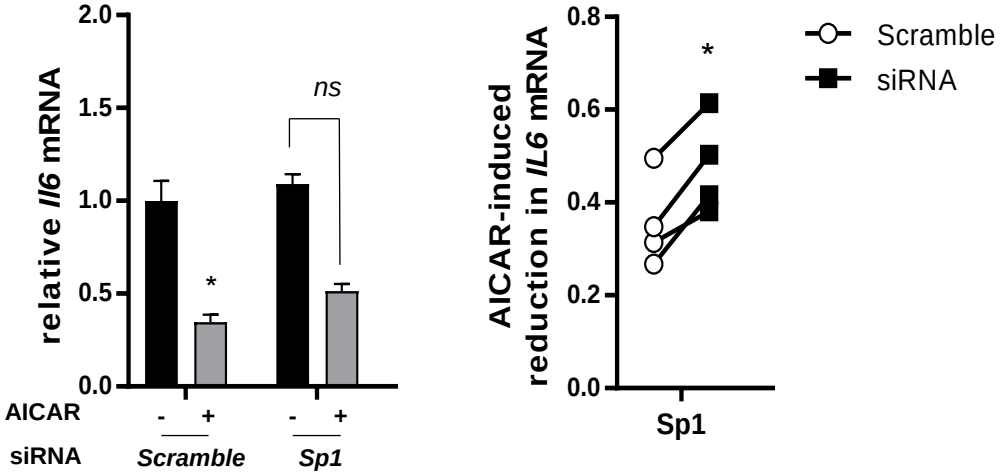

Figure 24. *Il6 mRNA in rat L6 skeletal muscle cells treated with siRNA for Sp1 and then incubated with or without AICAR (2 mM) for 1 hour (left). Individual change in IL6 mRNA reduction with AICAR-treatment (right), n=4.*

Sp1 was identified in the 1980's and was the first ever transcription factor to be purified (Kadonaga & Tjian, 1986). Sp1 is a housekeeping-type of transcription factor, meaning that it is involved in constitutively transcribed genes. It interacts with NFκB/Rela factors and can provide a regulative factor for constitutive expression of NFκB genes (Hirano et al., 1998). The silencing effect of Sp1 achieved in the L6 cells was about 50%, however, despite this modest reduction on Sp1, there was a significant blunting of the AICAR effect on *IL6* mRNA. More pronounced reduction of Sp1, may have resulted in a more complete inhibition of the AICAR effect. However, silencing more than 50% of housekeeping genes are often difficult to achieve, and not compatible with cell survival (personal communication with Nicolas J Pillon).

Interestingly, when silencing the relatively unknown transcription factor Zbtb14, the baseline *LIF* mRNA was increased three-fold (**Figure 25**), suggesting that this factor is involved in the regulation of LIF expression. LIF has, besides its involvement in muscle and nerve regeneration, also gained attention in the cancer field as a bad prognostic factor in several cancers including colorectal and breast cancer (Nicola & Babon, 2015; Yue, Wu, & Hu, 2015). Our observation, of the involvement of ZbtB14 in the regulation of LIF may be of importance in the cancer field in understanding the oncogenic properties.

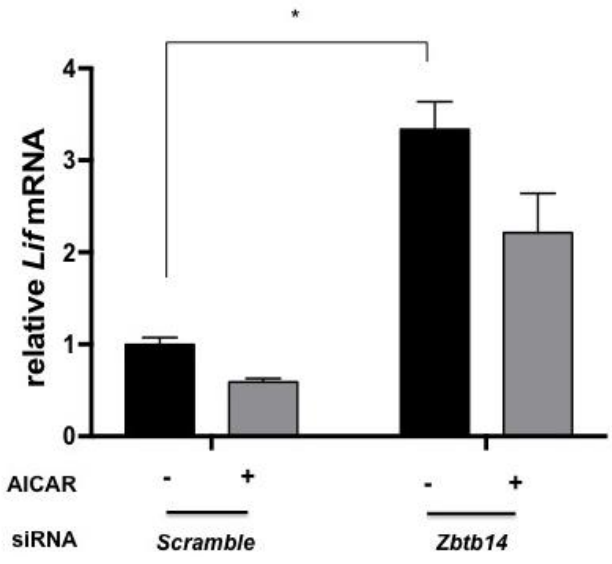

Figure 25. *Lif mRNA in rat L6 skeletal muscle cells after treatment with siRNA against the transcription factor Zbtb14 and subsequent incubated for 1 hour with or without AICAR (2 mM), n=4.*

In conclusion, we demonstrate that AICAR, mimicking a low energy state in skeletal muscle, robustly reduces baseline mRNA of several cytokines. Through a bioinformatics approach and siRNA silencing experiments we identify Sp1 transcription factor as involved in the regulatory mechanism of AICAR-induced reduction of IL-6. In addition, we identify a new transcription factor Zbtb14 as involved in the transcriptional regulation of LIF.

4.3 Obesity, diet and effect on mRNA and lipid profile in skeletal muscle

Skeletal muscle metabolism is impaired in the obese state, resulting in insulin resistance. There is evidence for the involvement of intra-myocellular lipid accumulation and tissue inflammation in this process. In **paper 1**, we demonstrated that obese, insulin resistant subjects improve their fasting insulin levels and HOMA-IR after substantial weight loss. In **paper 4**, we aimed to investigate if weight loss in obese insulin resistant subjects could result in altered skeletal muscle lipid and gene expression profiles. Eight obese, non-diabetic but insulin-resistant subjects were recruited from the waiting list of patients scheduled for RYGB surgery at Danderyd Hospital to enrol in the study. The subjects underwent a three-week low calorie diet intervention (1,000 kcal/day). Muscle biopsies were obtained after a 12 h fast and clinical chemistry was determined before and after the diet intervention (**Table 3**).

The subjects lost significant weight (average 8±3.5 kg) and improved fasting insulin, HbA1c, triglycerides, cholesterol and LDL levels after the diet intervention (**Table 3**). Thus, the three week low calorie diet was efficient in improving whole-body metabolic health similar to results of other diet intervention studies in insulin resistant and type 2 diabetic subjects (Jazet et al., 2008; Lara-Castro et al., 2008; Lim et al., 2011). A three-week diet intervention was also as expected, more efficient in reducing insulin levels than a two-week diet intervention but not as powerful as the weight loss after RYGB as described in **paper 1** (**Table 3**).

We performed a gene expression microarray analysis to identify altered gene expression after the diet intervention. KEGG analysis showed enriched pathways of oxidative phosphorylation and surprisingly also inflammation. Most of the genes in the expression array were upregulated (35 genes that were significantly up and one down) (**Figure 26**).

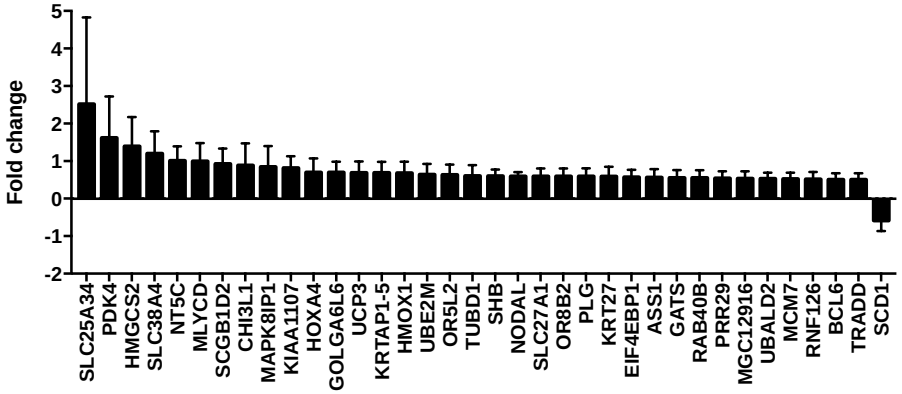

Figure 26. *Diet-induced changes in mRNA expression in human skeletal muscle. Mean±SD, n=8.*

The diet-induced weight loss and concomitant whole-body metabolic changes were associated with a substantial effect on gene transcription. More specifically, genes involved in fatty acid transport or metabolism were among the most changed in the expression array. SLC27A1, also named FATP1, is a long fatty acid transporter. Skeletal muscle from obese adults is associated with increased uptake of long fatty acids (Bonen et al., 2004). Overexpression of FATP1 in rat skeletal muscle increased fatty acid uptake concomitant with increased fatty acid

oxidation (Holloway et al., 2011). In contrast, overexpression of another fatty acid transporter, FAT/CD36, similarly increases the fatty acid uptake, but instead increases the esterification and storage of fatty acids (Garcia-Martinez et al., 2005). Thus, this indicates that an increased FATP1 expression in skeletal muscle after the diet intervention could reflect an increased fatty acid uptake of lipids that are destined for fatty acid oxidation rather than storage.

Mitochondrial malonyl-CoA decarboxylase (MLYCD), that catalyses the conversion of malonyl-CoA to acetyl-CoA, is upregulated in skeletal muscle after the diet intervention. Deletion of MLYCD shifts oxidation of metabolic substrates from lipids to glucose (Bouzakri et al., 2008). PDK4 suppresses conversion of pyruvate to acetyl-coA, the rate-limiting step in glucose oxidation, diverting fuel selection towards fatty acid oxidation. Thus, increased expression of both MLYCD and PDK4 promotes fatty acid oxidation.

The uncoupling protein 3 (UCP3) is upregulated in skeletal muscle in response to the weight loss in our study, consistent which the literature (Hildebrandt & Neufer, 2000). UCP3 is skeletal muscle specific and suggested to have a role to protect the muscle against oxidative damage induced by mitochondrial production of reactive oxygen species (Echtay et al., 2002). More specifically, UCP3 may act as a transporter of fatty acids out of the mitochondria, to ensure that the delivery of fatty acids into the mitochondria matrix does not exceed the capacity to oxidize (Dulloo & Samec, 2000). As fatty acid oxidation in the mitochondria is likely to be increased in the skeletal muscle after the diet intervention, the increased expression of UCP3 could enable a protective mechanism against reactive oxygen species that are produced by the mitochondria as a by-product.

Stearoyl-CoA desaturase-1 (SCD1), an enzyme involved in the biosynthesis of fatty acids, is downregulated in the muscle biopsies after the diet intervention, which has been described previously (Dube et al., 2011). Obesity is associated with increased SCD1 expression, accumulation of triglycerides and reduced β-oxidation in skeletal muscle (Hulver et al., 2005). However, also highly trained athletes exhibit an increased SCD1 expression (Goodpaster, He, Watkins, & Kelley, 2001) and exercise training increases SDC1 expression, associated with improved insulin sensitivity (Schenk & Horowitz, 2007). The decreased SCD1 levels in our study may reflect the shift toward increased oxidation and thus decreased storage of fats. Collectively our gene expression data indicates that genes promoting lipid oxidation are increased in the skeletal muscle after the diet intervention (**Figure 27**). However, we do not know if the altered gene expression is a cause or a consequence of the decreased insulin levels.

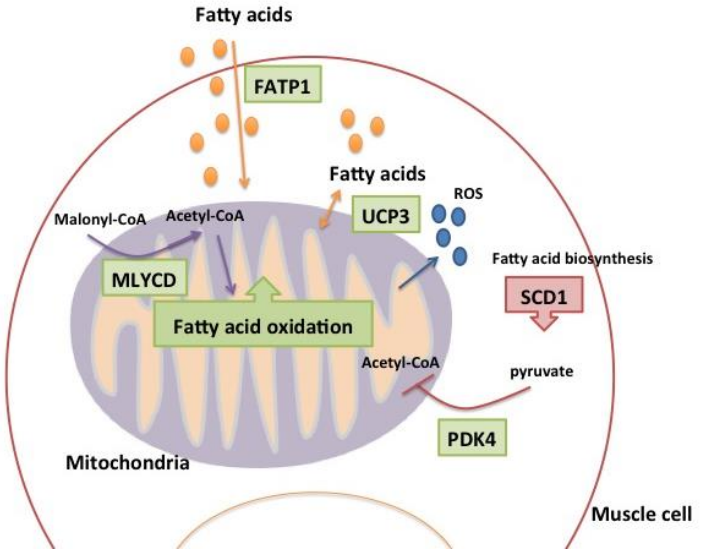

Figure 27. *Expression of genes promoting fatty acid oxidation is up-regulated after the diet.*

To study the change in skeletal muscle lipid profile after the diet intervention, we performed a lipidomic analysis. We found that total lipids within each lipid class were unaltered after the dietary intervention. However, several specific lipid species were significantly reduced after the diet intervention. Most of the changed lipid species were from the phosphatidylcholine (PC) and triglyceride (TG) class (**Figure 28**).

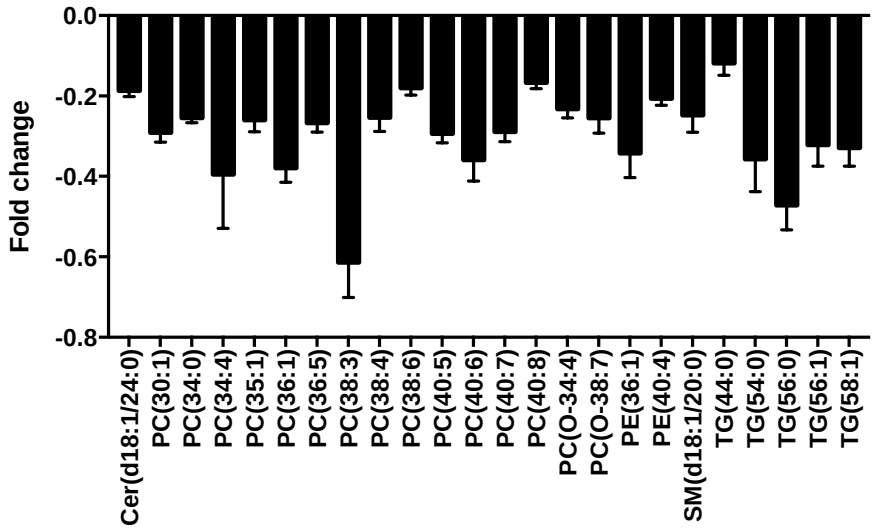

Figure 28. *Alterations in specific lipid species in human skeletal muscle after dietary intervention. Mean±SD, n=8.*

PC and phosphatidylethanolamine (PE) are phospholipids important for membrane composition and integrity and stands for 50% and 20-30% respectively of the phospholipid pool of the membrane (Takagi, 1971). An altered relationship of the phospholipids can change the membrane fluidity and thereby the signalling and transportation of molecules and nutrients across the cell membranes. Alterations of the PC and PE species are associated with insulin resistance (Newsom et al., 2016).

Ceramide and sphingomyelin (SM), both belonging to the sphingolipid class, are important for cell membrane structure, but have also been recognized as signalling molecules (Hannun & Obeid, 2008). Mainly ceramide species, but also sphingomyelins, have been appointed relevance in the development of insulin resistance (Chavez et al., 2014; Li et al., 2011). Diacylglycerol (DAG) species have also been implicated in the development of insulin resistance (Itani, Ruderman, Schmieder, & Boden, 2002). However, in our cohort DAG species were unaltered.

To estimate the relevance of the identified 24 lipid species in our cohort and the relationship to clinical parameters, we compared each one of the lipid species to LDL, HDL, triglycerides, insulin, BMI and HOMA-IR (**Figure 29**).

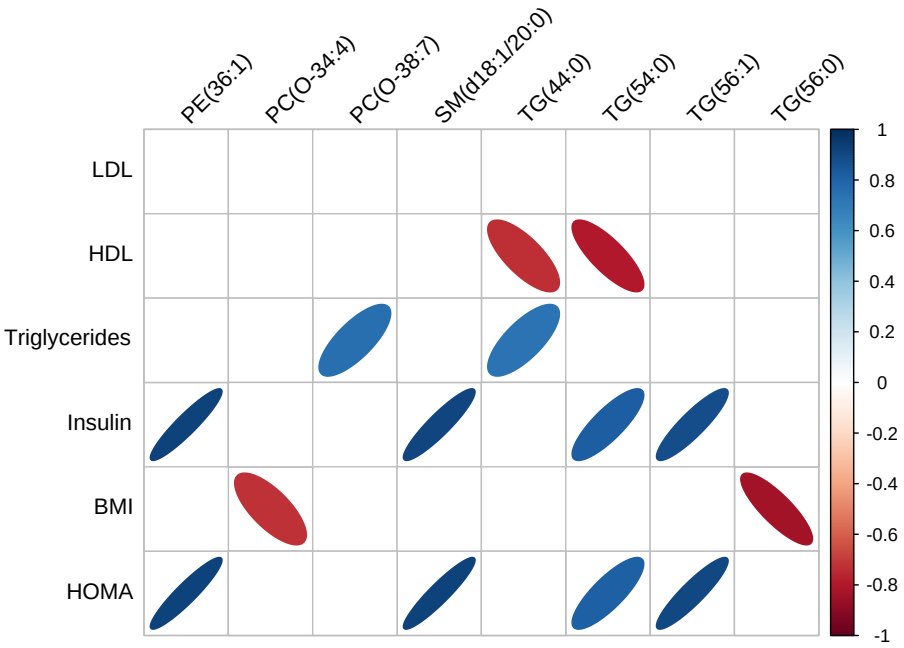

Figure 29. *Altered lipid species after dietary intervention and their correlation to clinical parameters. Mean±SD, n=7-8.*

Four species, PE(36:1), SM(d18:1/20:0), TG(54:0) and TG(56:0) were correlated with insulin levels and HOMA-IR, indicating that these specific lipid species may play a role in the ameliorations of insulin resistance after the diet intervention. These lipid species are not previously described in the literature, and further investigation is needed to fully understand their relevance for skeletal muscle metabolism and development of insulin resistance.

Recently several papers on the muscle lipid profile have been published. Skeletal muscle and plasma lipid profiles were compared between lean and obese subjects with and without insulin resistance (Tonks et al., 2016). The authors found that Cer(d18:1/18:0) was the only species associated with insulin resistance in skeletal muscle. However, they identified PC(0-34:3) in skeletal muscle as associated with obesity *per se* (Tonks et al., 2016). Interestingly, in our cohort, PC(0-34:4), a closely related lipid species, correlated inversely with BMI, but not insulin. Another study of obese subjects undergoing RYGB surgery with or without a prior exercise regime, identified a total decrease of skeletal muscle ceramide content after the RYGB intervention that was even greater with prior exercise (Coen et al., 2015). This decrease was associated with improved insulin sensitivity in the skeletal muscle. In addition they identified Cer 18:0 as reduced after RYGB and exercise but not after RYGB only (Coen et al., 2015). Interestingly, lipidomic analysis showed that in skeletal muscle from mice fed with high fat diet for 3 weeks to induce insulin resistance, Cer 18:0 was the only lipid species that significantly increased (Turner et al., 2013). Collectively, this draws attention to this specific ceramide species that is also altered in the skeletal muscle biopsies of our cohort. Further investigation is needed to identify the relevance and relation of specific lipid species in the development of insulin resistance.

Our results show that a relatively short diet intervention in obese insulin resistant subjects has a substantial impact on weight loss and insulin levels. This improvement could be a result of increased fatty acid uptake in the skeletal muscle and thus clearance of fatty acids from the circulation, as well as increased mitochondrial fatty acid oxidation, as indicated by the altered gene expression. In addition, the diet intervention induced changes in the lipid profile of skeletal muscle, which could have an impact, with four specific lipid species decreasing in an inverse correlation with insulin sensitivity.

This study pinpoints the importance of studying the specific lipid species to understand the influence of lipids upon skeletal muscle metabolism, as there was no change in total lipids within each lipid class. Although the main consensus today is that total intra myocellular lipid content is not of major importance (Coen & Goodpaster, 2012), it would have been of interest to measure the change in our cohort. This was not possible though, taken the limited amount of skeletal muscle tissue from the biopsy.

We only had access to a small cohort (n=8) and the diet intervention only endured for three weeks. With a larger cohort and longer intervention we might have seen more dramatic changes in lipid profiles. Small alterations in lipid species level could have been masked by the individual differences. We rely on insulin levels and HOMA-IR to estimate the change in insulin sensitivity. However, the most accurate way to evaluate skeletal muscle insulin sensitivity is by euglycaemic clamp, which we did not have the opportunity to perform. However, whole-body insulin sensitivity is associated with skeletal muscle insulin action (Tripathy, Almgren, Tuomi, & Groop, 2004), suggesting that HOMA-IR can be used as a reflection also of peripheral insulin sensitivity.

Interestingly, the KEGG analysis showed that inflammatory gene pathways were upregulated in skeletal muscle after the diet intervention. This is in contrast out hypothesis since obesity and insulin resistance are associated with inflammation, and one would have thought that by reducing weight and reducing insulin levels, inflammation would decrease. We did not measure inflammatory parameters in the blood, but mRNA of *IL6* was increased (**Figure 16**) which supports the KEGG analysis data. Other studies of diet- and RYGB interventions that efficiently induced weight loss and improved insulin sensitivity, have described decreased C-reactive protein (CRP) levels but unaltered levels of IL-6 and TNF in adipose tissue and in blood respectively (Barres et al., 2013; Fritzen et al., 2015). CRP is produced in the liver, which may suggest that improved inflammation of the liver could be of higher importance than skeletal muscle and also adipose tissue, for the overall improved inflammation seen after weight loss.

Improvements in insulin sensitivity after a diet intervention was associated with increased AMPK activity in adipose tissue (Fritzen et al., 2015). In another study, AMPK activity was lower in visceral adipose tissue from obese, insulin resistant individuals than BMI-matched obese insulin sensitive individuals (Gauthier et al., 2011). In addition, insulin resistance and decreased AMPK activity were associated with increased inflammatory markers and infiltration of immune cells in the insulin resistant individuals (Gauthier et al., 2011). Calorie restriction induces AMPK skeletal muscle activity in mice (P. Wang et al., 2012). A deletion of the AMPKα2 subunit in high fat fed mice, prevented improvements in insulin resistance after calorie restriction (P. Wang et al., 2012). Thus, AMPK may be an important target in the development of insulin resistance. In the light of this, it would be of interest to investigate the AMPK activity also in the skeletal muscle biopsies from our cohort and to link this to mRNA and plasma levels of inflammatory markers.

5 SUMMARY AND CONCLUSION

The work of this thesis focuses on the effects of external factors such as weight loss, insulin or glucose exposure on DNA methylation and gene regulation in clinical samples obtained from insulin resistant and type 2 diabetic subjects. We further assessed the effect of pharmacological activation to mimic low energy signals on cytokine expression in healthy skeletal muscle and we studied changes in lipidomic and gene expression profile in skeletal muscle in response to diet-induced weight loss. In summary, this thesis provides insight to how changes in energy homeostasis affects gene regulation and lipid profile in skeletal muscle.

Results from this thesis may have future implications for developing new strategies for treatment of obesity. Delineating molecular changes in relevant clinical tissues could pinpoint novel regulatory sites and targets for pharmacological interventions. Improvement of insulin sensitivity *per se* may also avoid complication of obesity, such as type 2 diabetes, even in the absence of weight reduction.

The specific conclusions are;

1. Promoter DNA methylation of metabolic and inflammatory targets, is altered by low calorie diet (*PGC1α*) and RYGB surgery (*IL6, IL1β, TNF and PDK4*) in peripheral blood. The changes in promoter DNA methylation could play a role in whole-body metabolic health. However, DNA methylation of these targets in peripheral blood appears to be tissue specific and may not be a suitable proxy of DNA methylation mark in skeletal muscle.

2. Short insulin and glucose exposure alters the DNA methylation profile in skeletal muscle, especially of gene pathways involved in metabolism. DAPK3 DNA methylation is different between type 2 diabetic and normal glucose tolerant individuals, and is altered by insulin and glucose.

3. The AMPK activator AICAR reduced *IL6* and *LIF* mRNA in skeletal muscle by inhibiting transcription, in particilar for *IL6* mRNA, likely through interaction with the transcription factor Sp1. We identified the transcription factor Zbtb14 as a novel regulator of basal *LIF* mRNA in skeletal muscle.

4. A three-week diet intervention induced weight loss and improved insulin sensitivity. The diet-intervention increased expression of genes involved in promoting fatty acid oxidation in skeletal muscle. The diet also induced an altered lipid profile with the specific lipids PE(36:1), SM(d18:1/20:0), TG(54:0) and TG(56:1) being inversely correlated to insulin sensitivity measured by HOMA-IR.

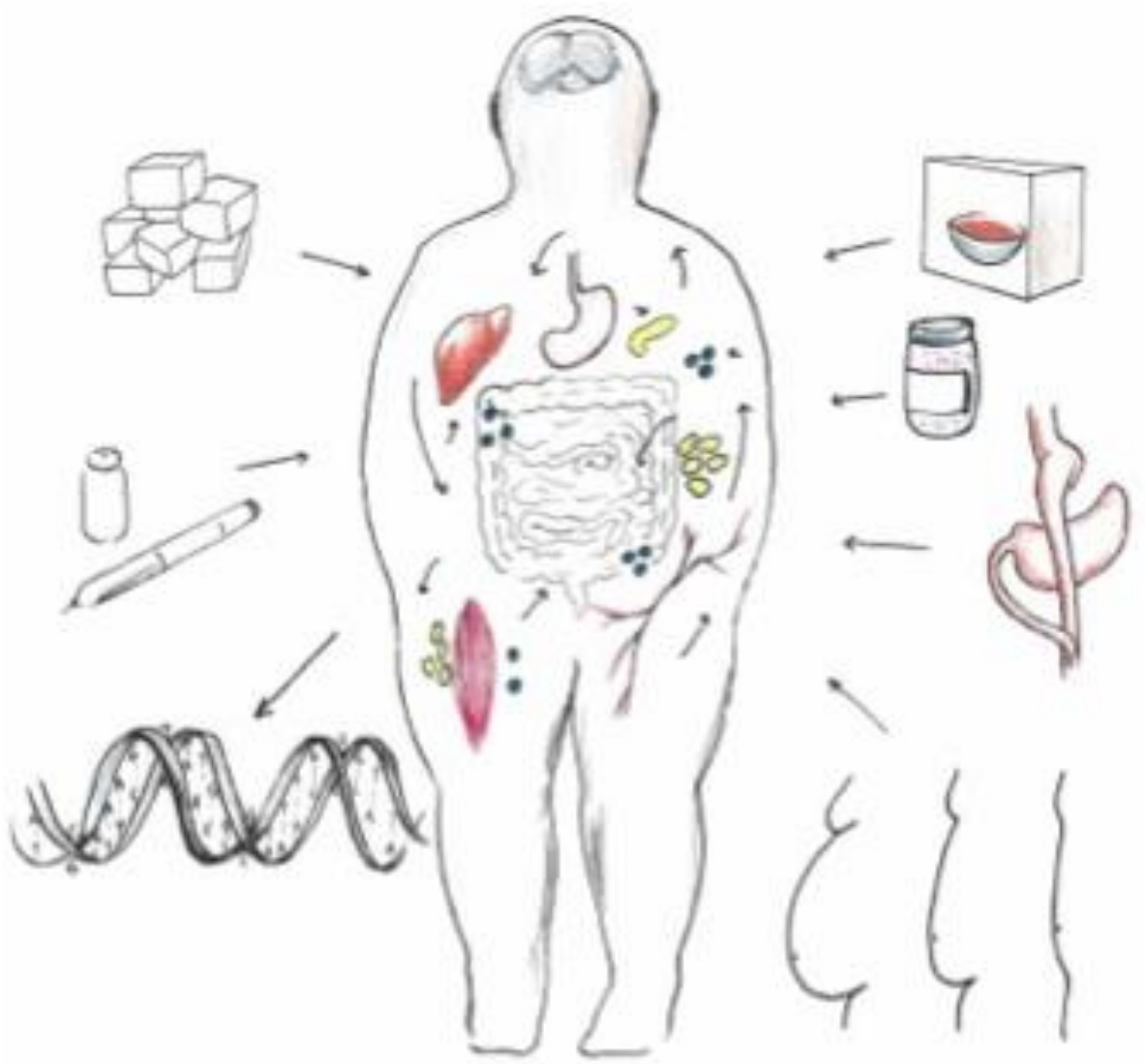

Figure 30. *Summary of the external factors, affecting skeletal muscle metabolism studied in this thesis.*

6 FUTURE PERSPECTIVES

This thesis has focused on several aspects of skeletal muscle metabolism, using different techniques such as gene and methylation arrays, lipidomic analysis and *in vitro* silencing of genes using siRNA. All these techniques are constantly improving, providing deeper and more refined information, that contributes to new insights and applications in the metabolic field.

6.1 Epigenetic regulation

The field of epigenetics is still in its cradle and the understanding of how epigenetic marks ultimately regulate our phenotype is still unclear. There is evidence that DNA methylation is affected by histone modifications (Baubec et al., 2015) and that small non-coding RNA and transcription factors can guide and regulate the enzymes involved in de novo methylation (Chan et al., 2004). Simultaneous studies of gene expression, DNA methylation, non-coding RNA and histone modifications, particularly with singe cell techniques, will most likely add new information to this field. In **paper I**, we concluded that in our cohort, DNA methylation in peripheral blood was not a useful proxy for skeletal muscle DNA methylation. DNA methylation is tissue specific but also heterogeneous within a tissue (reviewed in (van Dijk et al., 2015) and (Bell, 2017)). Single cell sequencing analysis will be helpful also in understanding how genetic and epigenetic regulation lead to tissue phenotype.

6.2 Gene silencing

In addition to the siRNA technique used in **paper 2 and 3**, there are other, new tools to silence genes, TALENs and CRISPR-Cas9 systems. These systems enable complete silencing of a gene, in contrast to the less efficient silencing techniques of siRNA, and are thus useful tools to test gene function in cells and organisms. CRISPR-Cas9 has potential also in future gene therapy for disease, however there are still not completely resolved issues concerning off target effects.

6.3 Lipidomic analysis

With the insight that specific lipid species, their localization and turnover matter more to metabolic function that the total amounts of lipids, the implication of lipidomic analysis in medicine is clear. Today, total plasma triglycerides, LDL and HDL as well as total cholesterol are routinely measured in the clinical setting, as an estimate of metabolic health. Recently, a Finnish group performed plasma lipidomics in a large prospective study and identified specific lipid species in the plasma that could predict which patients who would develop type 2 diabetes (Suvitaival et al., 2017). Lipidomic analysis may provide new diagnostic and prognostic markers, and thus have useful and important implication for obese patients, to help guide and personalise dietary and exercise regimes to prevent insulin resistance and type 2 diabetes.

6.4 Personalised medicine

Obesity and insulin resistance are consequences of numerous different factors affecting metabolism. There are currently tools to map the genetic, epigenetic, metabolomic and gut microbiotic profiles of an individual. Thus, with the possibility of deep and personalised information, future challenges lie not only in deconvoluting the data, to predict future risk and personalized intervention, but also in determining the cost/benefit of the different possible analysis.

7 SVENSK POPULÄRVETENSKAPLIG SAMMANFATTNING

Fler människor på jorden är i dag överviktiga än underviktiga. Typ 2-diabetes, hjärt- och kärlsjukdomar, och flera typer av cancer är starkt förknippade med fetma. Lågkaloridiet kombinerad med beteendeterapi kan visserligen leda till viktnedgång men den enda riktigt effektiva och långsiktiga behandlingen för fetma är i dag kirurgi. Fetmaepidemin kan till viss del förklaras av samspelet mellan gener och miljö, där våra gener har selekterats för att öka vår överlevnad under perioder av svält. Forskning har visat att miljöfaktorer även kan påverka våra gener genom att olika epigenetiska markörer.

Skelettmuskeln är det organ i kroppen som omsätter störst mängd näringsämnen. Vid övervikt och fetma har kroppen ett överskott på kolhydrater och fetter som ska lagras och omsättas framför allt i skelettmuskeln. Om skelettmusklerna får för mycket näringsämnen som de inte kan omsätta, leder detta till stress i de system i skelettmuskelcellerna som hanterar ämnesomsättning och lagring. Denna stress, samt förekomsten av överskott på näringsämnen i sig, leder till inflammation. Inflammationen försämrar i sin tur cellernas förmåga att hantera näringsämnen, vilket leder till en ond spiral.

Epigenetik betyder ”ovanpå” (från grekiskan *epi*) generna och är ett sätt att reglera hur våra gener uttrycks. Epigenetik omfattar bland annat DNA-metylering, den mekanism som studerats i denna avhandling. Genom metylering sätts ”små flaggor” på DNA-sekvensen vilket förändrar dess förmåga till uttryck, det vill säga om genen så småningom blir översatt till protein. Olika faktorer, från miljögifter, till vad vi äter och hur vi tränar, påverkar vilka gener som uttrycks och blir till protein i kroppen. Detta påverkar sedan risken att drabbas av olika sjukdomar.

Det övergripande målet med denna avhandling är att studera hur externa faktorer så som rubbningar i energibalansen, insulin eller glukos, påverkar genuttryck. Syftet är att förstå de mekanismer som leder till försämrad förmåga att hantera näringsämnen i skelettmuskeln. Insulin är ett centralt hormon som styr ämnesomsättningen. En oförmåga hos skelettmuskeln att ta hand om, och omsätta en ökad mängd kolhydrater och fetter är starkt kopplat till en minskad känslighet för insulin i skelettmuskeln, vilket är förstadiet till typ 2 diabetes. Fetma är ett tillstånd starkt förknippat med minskad insulinkänslighet i skelettmuskeln, och utgör därför en viktig modell för våra studier i denna avhandling.

I **studie 1** undersökte vi blod från feta försökspersoner före och efter en lågkaloridiet och sedan även 12 månader efter fetmakirurgi. Försökspersonerna gick i genomsnitt ner 5 kg i vikt efter 14 dagars diet och sedan ytterligare 38 kg efter fetmakirurgin. Försökspersonernas blodsocker, insulin- och blodfettsnivåer förbättrades något efter dieten men mer väsentliga förbättringar sågs efter kirurgin. Vi studerade hur DNA metylering i blodet av utvalda gener förändrades av dieten och kirurgin. Resultaten visar att DNA metyleringen i blodet av gener, som vi tror påverkar vår ämnesomsättning och graden av inflammation, var mer förändrade efter kirurgin än efter dieten.

I **studie 2** undersökte vi hur en timmes insulinexponering påverkade DNA metyleringen av våra gener i skelettmuskelbiopsier. Trots kort insulinexponering, fann vi ändrad DNA metylering av flera genfamiljer involverade i ämnesomsättning och inflammation. Vi identifierade en gen, DAPK3, vars DNA metylering hade förändrats, som inte tidigare identifierats som betydelsefull inom ämnesomsättningen. Graden av DNA metylering av

DAPK3 skilde sig inte bara efter insulinexponering, utan även i skelettmuskeln mellan typ 2 diabetiker och friska, och förändrades ytterligare av en glukosbelastning. Sammanfattningsvis kan en kort exponering för insulin eller glukos påverka DNA metyleringen av gener.

I studie 3 stimulerade vi skelettmuskelbiopsier istället med en AMP-analog, som signalerar till cellen att energi-nivån är låg. Uttryck av gener involverade i inflammation reducerades efter exponeringen. En tolkning av resultaten är att skelettmuskeln vill minska graden av inflammation för att spara energi, eftersom inflammationsprocesser förbrukar energi. Vi fann två, inom området ämnesomsättning, ej tidigare beskrivna transkriptionsfaktorer, Sp1 och Zbtb14, som var involverade i regleringen av inflammationsgenerna *IL6* och *LIF*.

I studie 4 undersökte vi skelettmuskelbiopsier från feta försökspersoner före och efter en tre veckor lågkaloridiet. Försökspersonerna gick i genomsnitt ner 8 kg i vikt och förbättrade sina blodvärden. Vi såg att genfamiljer involverade i fettförbränning och inflammation ändrade sitt uttryck efter dieten. Vi bekräftade att specifika gener viktiga för fettomsättningen ökade i uttryck. Vi undersökte sedan hur fettsammansättningen förändrades i muskeln, och såg att specifika fettyper minskade väsentligt efter dieten. Dessa specifika fettyper var korrelerade med förändring i muskelns insulinkänslighet. Sammanfattningsvis kan dessa fynd ha en betydelse för den förbättrade ämnesomsättningen i skelettmuskeln efter viktnedgång.

Sammanfattningsvis har vi studerat blod och skelettmuskel före och efter viktnedgång, hos feta och normalviktiga, med eller utan nedsatt insulinkänslighet eller typ 2 diabetes. Vi har undersökt vilka mekanismer som kan ligga till grund för den förbättrade insulinkänsligheten efter viktnedgång. Gener, viktiga för ämnesomsättningen i bland annat skelettmuskeln, är förändrat metylerade i blodet efter fetmakirurgi. Vi ser även att gener som ger en ökad fettförbränning i skelettmuskeln ökar, specifika fettyper minskar, samtidigt som insulinvärdet i blodet sjunker vid viktnedgång efter lågkaloridiet. Resultaten i denna avhandling ger ökad insikt i hur förändringar i energibalansen kan påverka genuttryck och lipidsammansättning i skelettmuskeln, vilket i sin ur är förknippat med förändringar i insulinkänslighet.

Det krävs vidare studier för att utreda vilka faktorer som är avgörande för att förbättra insulin känsligheten i skelettmuskeln. Ökad kunskap är avgörande för att kunna erbjuda bättre behandlingsrekommendationer och behandling och därmed bromsa den pågående epidemin av fetmainducerad insulinresistens.

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