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Genetic and epigenetic influence on oxidative phosphorylation, islet function and type 2 diabetes in humans

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Genetic and epigenetic influence on oxidative phosphorylation, islet function and type 2 diabetes in humans

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LUND UNIVERSITY
Faculty of Medicine

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Abstract

The prevalence of type 2 diabetes (T2D) is increasing worldwide. T2D is a heterogeneous disease caused by a complex interplay between multiple genetic, epigenetic and non-genetic factors. The disease is characterised by impaired insulin secretion from pancreatic β-cells and insulin resistance in peripheral tissues. Mitochondrial ATP production by oxidative phosphorylation (OXPHOS) is known to play a critical regulatory role in glucose stimulated insulin secretion (GSIS). The overall aim of this thesis was to explore the influence of genetic and epigenetic variation on OXPHOS, islet function and T2D in humans.

In study I, we identified a single nucleotide polymorphism (SNP) in *TFB1M*, a nuclear-encoded factor involved in the translational control in mitochondria, that is associated with decreased insulin secretion in response to glucose, increased future risk of T2D, and reduced expression of *TFB1M* in human pancreatic islets. In mice with a heterozygous deficiency of *Tfb1m* and in clonal β-cells where *Tfb1m* had been silenced, we found that reduced levels of TFB1M caused impaired OXPHOS and, consequently, reduced insulin secretion. This study concludes that deficiency in TFB1M contributes to the pathogenesis of T2D by causing impaired insulin secretion.

In study II, we showed that SNPs located adjacent to OXPHOS genes are nominally associated with decreased GSIS. Therefore, we cannot rule out the possibility that SNPs in or near genes involved in OXPHOS may influence β-cell function.

In study III, we demonstrated that a set of OXPHOS genes is down-regulated in pancreatic islets from patients with T2D compared to donors not diagnosed with diabetes. Islet expression of multiple OXPHOS genes correlated positively with GSIS. This result suggests that decreased expression of OXPHOS genes in pancreatic islets may contribute to T2D by impaired GSIS.

In study IV, we performed a genome-wide methylation quantitative trait locus (mQTL) analysis to assess the effects of SNPs on DNA methylation in human pancreatic islets. Our results demonstrate that DNA methylation in pancreatic islets is under the control of genetic variability, suggesting the importance of integrating genetic and epigenetic mechanisms when studying the underlying biological effects on complex human diseases, such as T2D.

Taken together, genetic and epigenetic influence on pancreatic islet function and mitochondrial OXPHOS may be involved in the pathogenesis of T2D by affecting insulin secretion.

Key words
Genetics, SNP, Gene expression, Epigenetics, DNA methylation, GWAS, mQTL, Human pancreatic islets, Oxidative phosphorylation, Insulin secretion, Type 2 diabetes

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"Man can learn nothing except by going from the known to the unknown."
Claude Bernard

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Scientific papers contributing to this thesis

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II. **Olsson AH**, Rönn T, Ladenvall C, Parikh H, Isomaa B, Groop L, Ling C (2011). *Two common genetic variants near nuclear-encoded OXPHOS genes are associated with insulin secretion in vivo.* Eur J Endocrinol 164, 765-771.

III. **Olsson AH**, Yang BT, Hall E, Taneera J, Salehi A, Dekker Nitert M, Ling C (2011). *Decreased expression of genes involved in oxidative phosphorylation in human pancreatic islets from patients with type 2 diabetes.* Eur J Endocrinol 165, 589-595.

IV. **Olsson AH**, Volkov P, Dayeh T, Hall E, Sterner M, Ladenvall C, Rönn T, Ling C. *A genome-wide methylation quantitative trait locus analysis identifies SNPs associated with DNA methylation in human pancreatic islets.* Manuscript.

Publications not included in this thesis

Wen J, Rönn T, **Olsson AH**, Yang Z, Lu B, Du Y, Groop L, Ling C, Hu R (2010). *Investigation of type 2 diabetes risk alleles support CDKN2A/B, CDKAL1, and TCF7L2 as susceptibility genes in a Han Chinese cohort.* PLoS One 5 (2), e9153.

Nilsson L, **Olsson AH**, Isomaa B, Groop L, Billig H, Ling C (2011). *A common variant near the PRL gene is associated with increased adiposity in males.* Mol Genet Metab 102, 78-81.

Olsson AH, Rönn T, Elgzyri T, Hansson O, Eriksson K-F, Groop L, Vaag A, Poulsen P, Ling C (2011). *The expression of myosin heavy chain (MHC) genes in human skeletal muscle is related to metabolic characteristics involved in the pathogenesis of type 2 diabetes.* Mol Genet Metab 103, 275-281.

Stamenkovic JA, **Olsson AH**, Nagorny CL, Malmgren S, Dekker Nitert M, Ling C, Mulder H. *Regulation of core clock genes in human islets.* Metabolism. Accepted 29 November 2011.

ABBREVIATIONS

ATP	adenosine triphosphate
BMI	body mass index
CI	confidence intervals
CpG	cytosine guanine dinucleotide
DGI	Diabetes Genetics Initiative
DNA	deoxyribonucleic acid
ETS	electron transport system
$FADH_2$	flavine adenine dinucleotide
FPG	fasting plasma glucose
GSIS	glucose stimulated insulin secretion
GWAS	genome-wide association study
HbA1c	glycated haemoglobin
HOMA-IR	homeostasis model assessment of insulin resistance
kb	kilobase
MAF	minor allele frequency
Mb	megabase
mQTL	methylation quantitative trait locus
mRNA	messenger ribonucleic acid
mtDNA	mitochondrial DNA
NADH	nicotinamide adenine dinucleotide
OGTT	oral glucose tolerance test
OR	odds ratio
OXPHOS	oxidative phosphorylation
PCR	polymerase chain reaction
RNA	ribonucleic acid
ROS	reactive oxygen species
SD	standard error of the mean
SEM	standard deviation
siRNA	small interfering ribonucleic acid
SNP	single nucleotide polymorphism
T2D	type 2 diabetes
WHO	World Health Organization

INTRODUCTION

Type 2 diabetes (T2D) is an endocrine disease that is reaching pandemic proportions. T2D is a polygenic and multifactorial disease, resulting in a complex network of interactions that underlies the phenotypic outcome of the disease. Although substantial efforts have been made to understand the pathophysiology of T2D and identify the disease-affecting genes, the aetiology of T2D is still incompletely understood. This thesis addresses the impact of common genetic variation and DNA methylation on oxidative phosphorylation (OXPHOS), islet function and insulin secretion in the pathogenesis of T2D.

Diabetes mellitus

Diabetes mellitus is a group of disorders that share the common phenotype of chronic hyperglycaemia [1]. Several subtypes of diabetes mellitus have been classified based on the aetiology of the disease: type 1 diabetes (T1D), type 2 diabetes (T2D), maturity onset diabetes of the young (MODY), latent autoimmune diabetes in adults (LADA), mitochondrial diabetes, and gestational diabetes mellitus (GDM). The definition and diagnostic criteria for diabetes mellitus according to the World Health Organization (WHO) is fasting plasma glucose (FPG) $\geq$ 7.0 mmol/l and/or plasma glucose at 2 hours (2hrPG) $\geq$ 11.1 mmol/l after ingestion of 75 g of glucose during an oral glucose tolerance test (OGTT). Impaired fasting glucose (IFG) (FPG 6.1 to 6.9 mmol/l, and if measured, 2hrPG < 7.8 mmol/l) and impaired glucose tolerance (IGT) (FPG < 7.0 mmol/l, 2hrPG $\geq$ 7.8 and < 11.1 mmol/l) are considered prediabetic states [1]. Individuals with IFG and IGT have higher risk of developing T2D [2]. Another measurement recently recommended for the diagnosis of diabetes is haemoglobin A1c (HbA1c) $\geq$ 6.5 % [3]. However, a value of less than 6.5 % does not exclude diabetes diagnosed using glucose tests. HbA1c is a glycosylated form of haemoglobin and the measurement represents the average blood glucose levels over the last eight to twelve weeks.

Insulin is the key hormone for regulation of glucose homeostasis. In the normal physiological state, insulin suppresses glucose output from the liver and enhances glucose uptake into skeletal muscle and adipose tissue. Diabetes mellitus is either caused by defects in insulin secretion from the pancreatic β-cells or insulin action in target tissues, or both, resulting in chronic hyperglycaemia (Figure 1).

Diabetes mellitus is a common disease and its prevalence is rapidly increasing. In 2011, diabetes mellitus afflicted 366 million people worldwide and, if no crucial

action is taken the number is estimated to rise to 552 million by 2030 [4]. The alarming increase of people developing diabetes, especially in developing countries, is due to social trends of changes in diet, physical activity and urbanisation [5, 6]. Diabetes mellitus is a global problem with a devastating socio-economic burden, largely due to the macrovascular and microvascular complications of the disease [7].

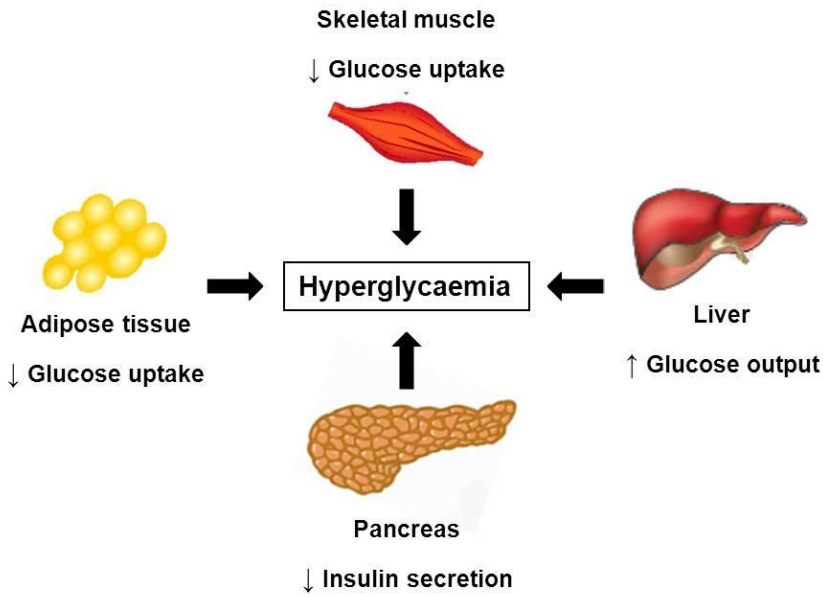

Figure 1. Pathophysiology of hyperglycaemia in diabetes mellitus

Diabetes is either caused by defects in insulin secretion from the pancreas or impaired action in target tissues of insulin, or both, leading to decreased glucose uptake by the skeletal muscle and the adipose tissue and increased glucose output from the liver, resulting in hyperglycaemia.

Type 2 diabetes

Type 2 diabetes (T2D) is the most common form of diabetes mellitus, constituting over 90 % of all diabetes cases globally [7]. T2D typically manifests later in life and is strongly associated with risk factors such as a family history of diabetes, obesity, insulin resistance and reduced insulin secretion [8]. In addition, the incidence of T2D is rising among younger individuals; this is thought to be the result of sedentary lifestyle and obesity [9]. T2D is characterised by pancreatic β-cell failure and insulin resistance in target tissues, which leads to hyperglycaemia (Figure 1). The interplay between insulin action in target tissues and insulin secretion from the pancreatic β-

cells is coordinated in a hyperbolic relationship [10, 11] (Figure 2). In the insulin-resistant state, the β-cell compensates by increasing insulin secretion to maintain normoglycaemia. Secretory defects in the β-cells are present before overt T2D arises [12]. Transition to overt T2D will occur when the pancreatic β-cells no longer can produce sufficient amounts of insulin to compensate for the increasing demands. The order of events leading to T2D is still not fully understood, and it is not possible to exclude any of the mechanisms of insulin secretion and insulin action in the pathogenesis of T2D. However, there is now strong support that failure of insulin secretion is the main culprit in T2D [13, 14].

The aim of T2D treatment is to normalise the glucose levels throughout the day. Hyperglycaemia can cause long-term complications and may lead to cardiovascular disease, retinopathy, nephropathy and neuropathy [15, 16]. Different aspects of treatment are used based on the severity of the disease symptoms. The choice of treatment can range from lifestyle intervention, in the form of exercise and diet control, to oral medications. Anti-diabetic oral medications act to reduce glucose levels either by stimulating endogenous insulin secretion, improving insulin sensitivity in target tissues or decreasing the absorption of carbohydrates from the gastro-intestinal tract. When these treatments fail, injection of exogenous insulin is used to maintain metabolic control.

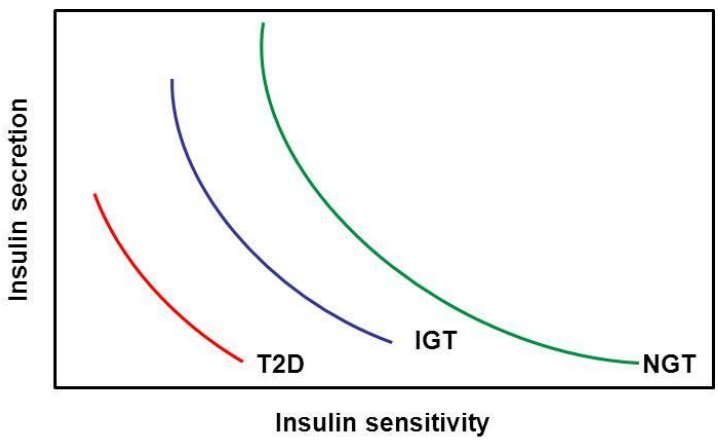

Figure 2. Hyperbolic relationship between insulin secretion and insulin sensitivity

As long as the β-cells in the pancreas can compensate for the decreased insulin sensitivity normoglycaemia is maintained, but with defects in insulin secretion hyperglycaemia and eventually T2D will develop. NGT, normal glucose tolerance; IGT, impaired glucose tolerance; T2D, type 2 diabetes.

T2D is a complex disorder involving interactions between genetic, epigenetic and non-genetic factors. Although non-genetic factors, such as obesity, physical inactivity and ageing, play a key role in the development of T2D, not all people exposed to these risk factors develop the disease. Genetic susceptibility to T2D has been demonstrated in twin studies, where the concordance rate is higher in monozygotic twins compared to dizygotic twins [17, 18]. Moreover, the lifetime risk for an offspring to develop the disease has been estimated at approximately 40 % if one parent had T2D, and even higher if both parents had T2D [19, 20]. The risk to develop T2D is higher if the mother, rather than the father, is affected by the disease [20]. These results highlight a clear genetic component in the pathogenesis of T2D.

Genetics of type 2 diabetes

Genetic susceptibility to T2D is well known, and T2D is considered to be a polygenic and multifactorial disease. Over the past decades extensive efforts have been made to identify and understand the genetic basis of T2D. Genetic mapping aims to identify variants in the genome that increase the risk of a disease or have an impact on a disease-related phenotypic trait. The field of genetics has experienced rapid development in recent years.

The human genome contains approximately three billion base pairs organised in 22 pairs of autosomal chromosomes and two sex-specific chromosomes. The DNA sequence, composed of four different types of nucleotides, stores the genetic information. The human genome contains approximately 20000 - 25000 protein coding genes [21]. The inter-human difference in the nucleotide sequence is approximately 0.1 % [22]. Variations in the DNA sequence can be created by base exchanges, insertions or deletions. Single nucleotide polymorphisms (SNPs), which refer to a substitution of a single nucleotide in the DNA sequence, are the most common form of genetic variation in the human genome [23]. On average, one SNP occurs for every 300 base pairs [22, 24]. Depending on the location of the SNP in the genome, it may or may not have functional consequences. SNPs located adjacent to each other are often inherited together more frequently than would be expected by chance in a population. This phenomenon of non-random association of alleles is called linkage disequilibrium (LD). Therefore, SNPs can capture the genetic information at a nearby locus and serve as a proxy for other genetic variants, so called tagSNPs. The statistical measurement r^2 denotes the allele frequency correlation between the SNPs, and is used to describe LD. The r^2 value can range between zero and one, where a value of one demonstrates complete dependency, i.e., the SNPs are in perfect LD.

Until recently, the main approaches to studying genetic predisposition to T2D included genome-wide linkage studies in affected families and association studies of candidate genes selected based on their direct or indirect involvement in glucose homeostasis. In 2006, the study of the genetics of T2D had a breakthrough with the identification of the genetic variation in *TCF7L2* [25]. The development of large-scale genotyping technologies in recent years has further progressed the field. Genome-wide association studies (GWAS), which investigate, without any prior hypotheses, SNPs distributed across the entire genome in large population samples, have yielded major results. Association studies compare the allele frequencies between non-diabetic and T2D affected participants or analyse the effect of a SNP on a disease-related phenotypic trait. The large number of tests performed in a GWAS leads to many false positive results caused by chance. Therefore, adjustment for multiple testing is used to define a genome-wide significant result.

Since 2007, several GWAS of T2D have been performed in different ethnic populations [26-36] along with large-scale meta-analyses of T2D [37-40]. Today, more than 40 SNPs have been reported to consistently associate with T2D or glycaemic traits. Most of these genetic variants seem to affect insulin secretion through abnormalities of β-cell function and development rather than insulin action. The identified SNPs associated with T2D are common variants that show modest odds ratios (OR) and can explain approximately 10 % of the genetic variance that contributes to the disease [39, 41]. Although several genetic and environmental factors involved in the pathogenesis of T2D have been identified, a better understanding of the underlying biological mechanisms involved in the disease is still needed.

Epigenetics in type 2 diabetes with a focus on DNA methylation

Epigenetics has been described as the study of changes in gene function that cannot be explained by changes in the DNA sequence, which can be mitotically and/or meiotically inherited [42]. Epigenetic factors, including DNA methylation, histone modifications and microRNAs, can influence genome structure during developmental stages and disease progression. Although the cells in the human body share the same genomic sequence, the epigenome varies between different cell types and over time. Therefore, epigenetics can help to explain the diversity of cell types with different phenotypes that share the same genomic sequence. Interestingly, epigenetic traits have been shown to be inherited between generations in animals [43, 44].

DNA methylation is a major epigenetic mark where methyl groups are added to the DNA sequence. In differentiated mammalian cells, this modification occurs primarily on the 5′ position of cytosine followed by a guanine dinucleotide, a so called CpG site. DNA methyltransferases (DNMTs), which act together with regulatory factors, are responsible for the DNA methylation processes (Figure 3). DNMT1 is thought to be responsible for the maintenance of methylation during DNA replication, and DNMT3a and DNMT3b are considered essential for *de novo* methylation [45-47] (see for review [48]).

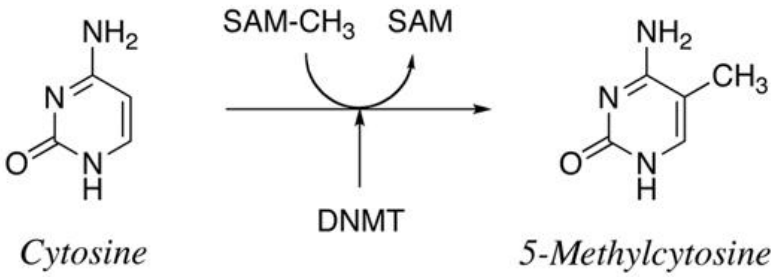

Figure 3. DNA methylation

In differentiated mammalian cells, DNA methylation can take place on the 5′ position of cytosines followed by guanines in so called CpG dinucleotides. DNA methyltransferase (DNMT) is responsible for the DNA methylation process and S-adenosylmethionine (SAM-CH_3) is the main methyl donor for the methyltransferase reaction.

DNA methylation has been widely related with embryonic development, X-chromosome inactivation, imprinting and cell-specific control of gene expression. DNA methylation of CpGs in gene promoters is generally associated with transcriptional silencing, either by repressing binding of transcription factors or by recruiting methyl-CG-binding proteins, which additionally attract histone deacetylases (HDACs) and co-repressors (Figure 4).

A close relationship has been described to exist between epigenetic modifications and the influence of environmental factors as well as ageing [49]. Because DNA methylation is considered to be established during embryonic development, the intrauterine environment may affect epigenetic patterns that may impact disease progression later in life [50, 51]. However, twin studies have shown that genetic

factors also have to be considered in establishing intra-individual differences in the epigenome [52, 53].

The effect of epigenetic modifications has been linked to the pathogenesis of T2D [54, 55]. There are also several examples of epigenetic involvement in the regulation of gene expression and the effect on insulin secretion in human pancreatic islets [56-58]. Moreover, a decrease of S-adenosylmethionine has been reported in diabetic patients. S-adenosylmethionine is the main methyl donor for methyltransferase reactions, and a decrease of this compound was also associated with the progression of disease complications [59].

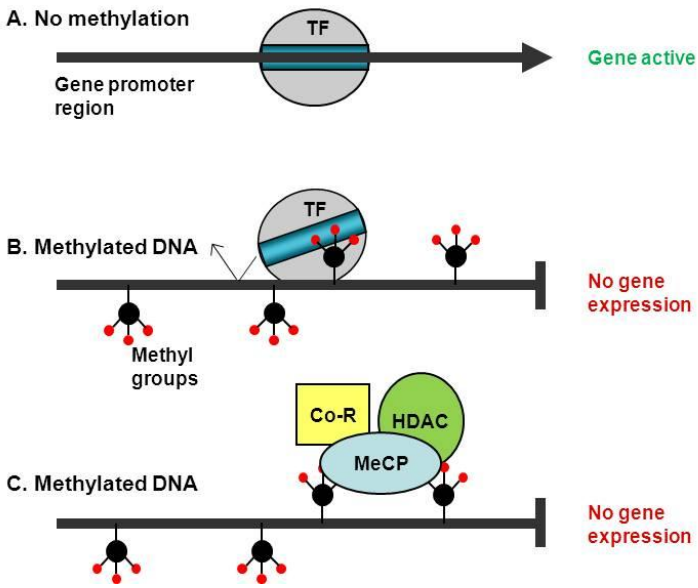

Figure 4. DNA methylation of gene promoter regions and regulation of gene transcription

A. No methyl group is attached to the gene promoter region and a transcription factor (TF) can access to the DNA and activate gene transcription. **B.** DNA methylation of CpGs in the gene promoter region repress binding of TFs and no activation of gene transcription occurs. **C.** Methylated CpGs recruits methyl-CG-binding proteins (MeCP) which act to repress gene transcription by attracting histone deacetylases (HDACs) and co-repressors (Co-R).

Changes in DNA methylation patterns may have a regulatory role in biological processes and contribute to the development of human diseases. Given this, it is of great interest to study how alterations in DNA methylation levels are controlled in different tissues. Because epigenetic modifications are dynamic they have potential as therapeutic targets [60].

Insulin secretion

Insulin, the key hormone for regulation of blood glucose, is produced by the β-cells in the pancreatic islets of Langerhans. Insulin secretion from the pancreatic β-cells is regulated in a biphasic manner by fuel metabolism of various secretagogues, where glucose is the most prominent source. Other factors, such as hormones, autonomic nerves and several neurotransmitters, are also involved in regulating insulin secretion (see for review [61]).

Pancreatic β-cells have a high efficacy of aerobic metabolism and approximately 80 % of glucose carbon is fully oxidised to CO_2 and H_2O [62]. The high rate of aerobic metabolism in the β-cell is most likely due to low expression levels of lactate dehydrogenase [62, 63] and plasma membrane lactate/monocarboxylate transport activity [64, 65] together with elevated levels of mitochondrial glycerolphosphate dehydrogenase [63, 66]. Therefore, the oxidation of pyruvate in the tricarboxylic acid cycle (TCA cycle) by mitochondria is favoured over its conversion to lactate.

Pancreatic β-cells express glucose transporters (GLUTs; predominantly GLUT1 in humans and GLUT2 in rodents) at the cell membrane, which transport glucose into the cytosol with high capacity and low-affinity. When plasma glucose levels rise, glucose is transported into the β-cell, phosphorylated into glucose-6-phosphate by glucokinase and further metabolised into pyruvate. This enzymatic breakdown of glucose into pyruvate is called glycolysis, which gives rise to the reduced electron carrier NADH, and ATP. Pyruvate enters the mitochondria where it is oxidised in the TCA cycle, yielding the more reduced electron carriers NADH and $FADH_2$. These reduced electron carriers are reoxidised in the electron transport system (ETS) resulting in the production of ATP by OXPHOS. The generated ATP is transported from the mitochondria to the cytosol, which causes a rise in the cellular ATP/ADP ratio, resulting in closure of ATP-sensitive K^+ (K_{ATP}) channels and depolarisation of the cell membrane. Consequently, voltage-gated calcium channels open and Ca^{2+} influx into the cell. Increased intracellular Ca^{2+} concentrations trigger exocytosis of insulin by the fusion of insulin granules with the cell membrane. This K_{ATP}-dependent pathway of insulin secretion, also called the triggering pathway, constitutes the first phase of insulin release after glucose stimulation [67] (Figure 5).

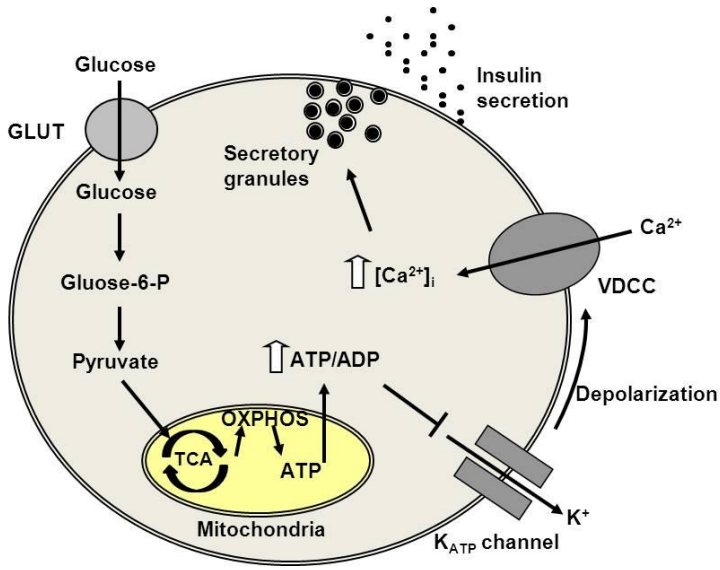

Figure 5. Glucose stimulated insulin secretion (GSIS) from the pancreatic β-cells

Glucose enters the cell by the glucose transporter (GLUT) and is metabolized to pyruvate through glycolysis. Pyruvate enters the tricarboxylic acid cycle (TCA) in the mitohcondria, which promotes ATP production by oxidative phosphorylation (OXPHOS). ATP is transported from the mitochondria to the cytosol, increases the ATP/ADP ratio, and closes the K_{ATP} channel. This leads to membrane depolarization, which opens voltage gated calcium channels (VDCC) and calcium (Ca^{2+}) flux into the cell and triggers the release of insulin granules.

The K_{ATP}-independent [68-70], or amplifying, pathway seems to be important for the second and sustained phase of insulin secretion, which is provoked by the triggering pathway [67]. Mitochondria-derived signals, other than changes in ATP/ADP ratio, have been implicated to play a crucial role for this amplifying signal. The source of coupling signals could potentially involve anaplerotic reactions where generation of intermediates, reducing equivalents or GTP, stimulates the amplifying pathway (see for review [71]).

Insulin secretion in the first and second phases of insulin secretion has been suggested to originate from different pools of insulin granules [72]: the readily releasable pool and the reserve pool, respectively. The mobilisation and priming of insulin granules in the reserve pool is an ATP-dependent process [73], indicating the importance of ATP for insulin secretion in the amplifying pathway as well.

Free fatty acids (FFAs) are also essential fuel for pancreatic β-cells, and oxidisation of FFAs leads to mitochondrial ATP production. FFAs can act by potentiating glucose stimulated insulin secretion (GSIS) [74, 75]. However, chronic exposure to elevated levels of glucose and FFAs are known to induce pancreatic β-cell dysfunction. Studies suggest that "glucolipotoxicity", rather than chronic exposure to each of these nutrients alone, leads to the impairment of β-cell function (see for reviews [76, 77]).

It is clear that failure of GSIS by pancreatic β-cell dysfunction is critical for the progression of T2D [14, 78]. Mitochondrial metabolism is fundamental for the regulation of insulin secretion, and mitochondrial dysfunction, therefore, may be of importance in the pathogenesis of T2D.

Mitochondria

Mitochondria are cytoplasmic organelles harbouring the OXPHOS system. In eukaryotic cells, most of the cell's energy, in the form of ATP, is generated in these organelles. Mitochondrial ATP is used to drive a variety of metabolic reactions, e.g., insulin secretion by pancreatic β-cells [79].

Evidence suggests that mitochondria originate from prokaryotic endosymbionts [80]. Mitochondria have their own genome (mtDNA), a circular genome of approximately 16.6 kb in length, which is predominantly maternally inherited. Each mitochondrion has several copies of mtDNA and the number of mtDNA copies varies between different cell types. The mtDNA encodes 13 genes of the OXPHOS system, 22 transfer RNAs (tRNAs) and 2 ribosomal RNAs (rRNAs). However, the majority of the proteins necessary for mitochondrial biogenesis and function are encoded by the nuclear genome and, therefore, need to be transported to mitochondria.

Transcription of mtDNA requires a specialised machinery where the regulation of mtDNA expression is controlled by nuclear-encoded factors [81]. These factors include mitochondrial RNA polymerase (POLMRT), which forms a complex with the mitochondrial transcription factor A (TFAM) and the mitochondrial transcription factor B2 (TFB2M) [82-84]. The mitochondrial transcription factor B1 (TFB1M), which is a paralogue of TFB2M, is most likely not a transcription factor of mtDNA [83, 84]. TFB1M, alternatively known as mitochondrial dimethyltransferase 1 (mDMAT1), may instead have a role in the translation of mtDNA-encoded proteins. In this role TFB1M dimethylates two highly conserved adenines of the rRNA of the small mitochondrial ribosomal subunit [84]. This modification is essential for the assembly of the mitochondrial ribosome and mitochondrial translation is abolished in its absence [84]. TFB1M has been reported to bind to S-

adenosylmethionine, the main physiological methyl donor for methyltransferase reactions [85].

Because each mitochondrion contains several copies of the mtDNA molecule, a mutation in mtDNA gives rise to heteroplasmy. Mutations in the mtDNA have been implicated in the development of diabetes, which often relates to β-cell dysfunction and defects in insulin secretion (see for reviews [86, 87]). The most frequent mutation associates with a base pair substitution in the mtDNA-encoded $tRNA^{Leu}$ and has been described as the cause of maternally inherited diabetes and deafness (MIDD) and mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke (MELAS) [88-90].

mtDNA may be vulnerable to reactive oxygen species (ROS) generated by the mitochondria. An age-related decline in mtDNA copy numbers has been reported in skeletal muscles [91] and pancreatic islets [92] of humans. The decreased number in skeletal muscle was reported to be associated with reduced mitochondrial ATP production [91]. Ageing, together with hyperglycaemia and hyperlipidaemia, may have a deleterious effect on mitochondrial function due to cumulative or accelerated ROS production. Experimental studies have demonstrated that expression of mtDNA is crucial for insulin secretion [93].

Oxidative phosphorylation

Oxidation of most cellular fuels takes place in the mitochondria. Therefore, proper cellular uptake of these nutrients is important. Glucose is transported across cell membranes via different glucose transporters (GLUTs). GLUT1 facilitates passive transport of glucose into the insulin producing pancreatic β-cells in humans. On the other hand, uptake of glucose into skeletal muscles and adipose tissues in humans is mediated by GLUT4, which is regulated in response to insulin.

Mitochondrial oxidation of cellular fuels generates the reducing equivalents NADH and $FADH_2$, molecules containing electrons with high transfer potential. The energy released when these reduced electron carriers are reoxidised in the ETS is used to produce cellular energy in the form of ATP, a process called OXPHOS. This process takes place in the inner membrane of the mitochondria and is the main source of energy production in human cells. The production of ATP is crucial for insulin secretion by pancreatic β-cells to occur, as well as to maintain metabolism in tissues of the whole body, such as skeletal muscle and fat.

The OXPHOS system, including the ETS and ATP synthase, consists of multiprotein complexes of approximately 90 known protein subunits [94]. The OXPHOS system

requires a unique coordinated expression of the nuclear genome and mtDNA for its biogenesis and function [94].

The ETS is grouped into four complexes (I – IV), where NADH enters in complex I and $FADH_2$ enters in complex II. The flow of electrons finally reduces O_2 to H_20 at complex IV. The energy of the electrons that flows through the ETS is coupled with proton pumping out of the mitochondrial matrix by complex I, III and IV. This process results in a proton gradient across the inner mitochondrial membrane. Subsequently, the protons flow back down this gradient through ATP synthase, and the energy released is used to generate ATP from ADP (Figure 6).

In addition, the ETS can be uncoupled from ATP production. In this situation, protons flow back into the mitochondrial matrix, independent of ATP synthase, generating heat instead of ATP. Obesity and ROS production have been suggested to stimulate the activity of uncoupling protein-2 (UCP-2) in pancreatic β-cells, resulting in decreased ATP production and impaired insulin secretion [95-97].

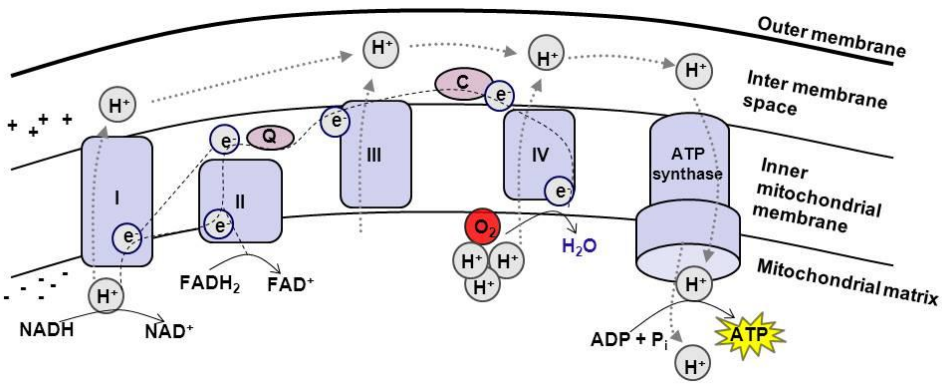

Figure 6. Oxidative phosphorylation (OXPHOS) system is the main source of energy production in human cells

The OXPHOS system, including the electron transport system (ETS) and ATP synthase, is located in the inner mitochondrial membrane and consists of multiprotein complexes. The ETS is grouped into four complexes (I – IV) and two electron carriers: complex I, NADH dehydrogenase; complex II, succinate dehydrogenase; complex III, ubiquinol-cytochrome c oxidoreductase; complex IV, cytochrome c oxidase; Q, coenzyme Q ubiquinone; C, cytochrome c. The energy of the electrons that flow through ETS is coupled to proton (H+) pumping out of the mitochondrial matrix, which results in a proton gradient across the inner mitochondrial membrane. The energy released when the protons flow back down this gradient through ATP synthase is used to generate ATP from ADP.

Impaired ATP production by reduced OXPHOS is considered a common denominator for the defects in glucose homeostasis, both in insulin secretion and action, observed in patients with T2D. Mitochondrial dysfunction in skeletal muscle has been observed in elderly, as well as in insulin-resistant and T2D patients [98-102]. Moreover, the expression of nuclear-encoded genes involved in OXPHOS is down-regulated in skeletal muscle [103, 104] and adipose tissue [105] of patients with T2D. Gene expression of *PPARGC1A*, a master regulator of mitochondrial genes, is reduced in pancreatic islets from T2D patients and this reduction correlates with reduced GSIS [56]. The decline in *PPARGC1A* expression has been explained by both genetic and epigenetic factors [56]. In addition, reduced expression of OXPHOS genes may be the result of integration between genetic and epigenetic factors in an age dependent manner [100].

AIMS OF THIS THESIS

The overall aim of this thesis was to explore the role of SNPs as well as alterations in gene expression and DNA methylation in the pathogenesis of T2D with focus on OXPHOS, islet function and insulin secretion. The project takes advantage of genome-wide approaches in this field.

The specific aims were as follows:

Studies I and II	To identify SNPs in nuclear-encoded genes involved in the OXPHOS system that are associated with insulin secretion, insulin resistance and/or T2D.
Study III	To examine whether OXPHOS genes show differential gene expression and DNA methylation levels in pancreatic islets from patients with T2D compared with donors not diagnosed with diabetes mellitus.
Study IV	To assess the effects of SNPs on DNA methylation levels in human pancreatic islets by performing a genome-wide methylation quantitative trait locus (mQTL) analysis.

STUDY PARTICIPANTS

All studies were approved by the regional ethical committees and conducted according to the *Declaration of Helsinki*.

Diabetes Genetics Initiative

The Diabetes Genetics Initiative (DGI) is a collaboration of the Broad Institute of MIT and Harvard, Lund University and Novartis Institute for BioMedical Research. The goal of the project was to identify genetic risk factors for T2D and related metabolic traits [26] by performing a whole genome association study using the Affymetrix Human Mapping 500K GeneChip®. The DGI consisted of patients with T2D, geographically matched controls and discordant sib-ships that were selected from Finland and Sweden. The study included 1464 patients with T2D and 1467 controls defined as normal glucose tolerant (Table 1), where 1008 control subjects had available insulin data from oral glucose tolerance tests (OGTTs). Population based controls had no first degree relatives with T2D [26].

Prevalence, Prediction and Prevention of Diabetes – Botnia study

The PPP-Botnia study (Prevalence, Prediction and Prevention of diabetes) is a population-based study from the Botnia region of Western Finland. The study includes approximately 10 % of the population aged 18 - 75 years randomly selected from the Finnish Population Registry [106]. The aim of the study is to study the prevalence and risk factors for diabetes, impaired glucose tolerance and metabolic syndrome in the general population. Information on lifestyle and family history of diabetes is obtained by questionnaire. All participants underwent an OGTT with plasma glucose and insulin measurements. Up to 4553 non-diabetic subjects (FPG < 7.0 mmol/l and 2hrPG < 11.1 mmol/l) were included in this thesis (Table 1).

Helsinki Birth Cohort Study

The Helsinki Birth Cohort Study (HBCS) includes 2003 subjects born as singletons at Helsinki University Central Hospital in 1934 - 1944 who participated in clinical examinations in 2001 - 2004, including a standard OGTT with measurements of

plasma glucose and insulin [107]. 1618 non-diabetic subjects were included in this thesis (Table 1).

METabolic Syndrome In Men

The METabolic Syndrome In Men (METSIM) study is a population-based cross-sectional study from the town of Kuopio in Eastern Finland including men aged 45 - 70 years that were randomly selected from the population registry [108]. All participants underwent clinical examinations including plasma glucose and insulin measurements from an OGTT. In this thesis, 6602 non-diabetic subjects with clinical data were included (Table 1).

Malmö Preventive Project

The Malmö Preventive Project (MPP) is a large population-based prospective study from the city of Malmö in Sweden, including Swedish subjects participating in a health screening from 1974 - 1992 (men were included from 1974 - 1990 and women were included from 1980 - 1992) [109]. This thesis comprises 16061 subjects who did not have diabetes at baseline and participated in a re-screening visit in 2002 - 2006 (Table 1). Among these, 2063 subjects (12.8 %) developed T2D during a median follow-up period of 24.8 years [110]. Diagnosis of diabetes was confirmed from patient records or based on fasting plasma glucose concentrations (FPG $\geq$ 7.0 mmol/l). Information about lifestyle factors, measurements from physical examinations and plasma glucose levels from an OGTT are available for the subjects included in MPP.

Human pancreatic islets

Human pancreatic islets and clinical information about the deceased donors were obtained by the Nordic Network for Islets Transplantation by the courtesy of Professor Olle Korsgren. The islets were processed at the Human Tissue Laboratory within EXODIAB / Lund University Diabetes Centre. Here, GSIS was measured and DNA and RNA were extracted from the human pancreatic islets. Moreover, gene expression was quantified using the Affymetrix microarray. Up to 94 donors not diagnosed with diabetes mellitus as well as nine donors with T2D were included in the different studies of this thesis (Table 2).

Table 1. Clinical characteristics of participants in the Diabetes Genetics Initiative (DGI); the Prevalence, Prediction and Prevention of Diabetes (PPP-Botnia); the Helsinki Birth Cohort Study (HBCS); the METabolic Syndrome In Men (METSIM) and the Malmö Preventive Project (MPP)

	DGI (controls)	DGI (T2D)	PPP-Botnia	HBCS	METSIM	MPP
N (male/female)	1467 (707/760)	1464 (741/723)	4553 (2134/2419)	1618 (713/905)	6602 (6602/-)	16061 (10416/5645)
Age (years)	58.8 ± 10.1	64.4 ± 10.2	48.4 ± 15.6	61.6 ± 3.0	57.1 ± 6.9	45.5 ± 6.9
BMI (kg/m^2)	26.6 ± 3.7	28.5 ± 4.5	26.3 ± 4.3	27.1 ± 4.3	26.8 ± 3.8	24.3 ± 3.3
FPG (mmol/l)	5.3 ± 0.5	9.5 ± 3.1	5.3 ± 0.6	5.5 ± 0.6	5.7 ± 0.5	5.5 ± 0.6
2hrPG (mmol/l)	5.6 ± 1.3	14.7 ± 5.1	5.3 ± 1.6	6.9 ± 1.7	6.0 ± 1.7	6.4 ± 1.7

Data are expressed as mean ± SD. FPG, fasting plasma glucose; 2hrPG, plasma glucose at 2 hours after ingestion of glucose during an oral glucose tolerance test.

Table 2. Characteristics of donors of human pancreatic islets

	Donors not diagnosed with diabetes	Donors with T2D
n (male/female)	89 (55/34)	9 (5/4)
Age (years)	57.0 ± 10.5	57.0 ± 13.1
BMI (kg/m^2)	25.8 ± 3.4	28.5 ± 4.7
HbA_1c (%)	5.7 ± 0.7	7.3 ± 1.2

Data are expressed as mean ± SD.

METHODOLOGY

Phenotype characterisation

Participants' body weight and height were measured and body mass index (BMI) was calculated as weight in kilograms (kg) divided by height in meters squared (m^2). Glucose tolerance was determined by a 75 g OGTT and classified in accordance with the WHO criteria [1]. An OGTT is a method used to determine how quickly glucose is cleared from the blood and is widely used for the diagnosis of diabetes [111]. In an OGTT, a subject is given a solution of 75 g of glucose to drink after an overnight fast. Blood samples are then collected at certain time points over the following two hours for analysis of glucose and insulin levels.

There are several different formulas used to calculate insulin secretion and action, both from fasting values of glucose and insulin and from values obtained during an OGTT. In the studies presented in this thesis, β-cell function and GSIS were assessed by the insulinogenic index during an OGTT ((insulin at 30 min – insulin at 0 min) / (glucose at 30 min – glucose at 0 min)). The basal insulin resistance state was estimated by homeostasis model assessment (HOMA-IR) ((fasting glucose x fasting insulin) / 22.5). β-cell function was also assessed as disposition index, where insulin secretion is corrected for insulin resistance (insulinogenic index / HOMA-IR).

Genotyping

Different methods of SNP genotyping were used in this thesis: TaqMan genotyping to obtain data on individual SNPs and IlluminaBeadChip technology using the Infinium assay for genome-wide genotyping. For studies I and II, genotype data from an existing GWAS were used [26].

TaqMan allelic discrimination

TaqMan allelic discrimination assay is a sequence-specific method for SNP genotyping that contains two different fluorescently labelled probes, one for each allele of the SNP. Each probe consists of an oligonucleotide with a fluorescent dye at the 5′ end and a quencher at the 3′ end. During PCR amplification, the TaqMan probes hybridise only to perfectly matching DNA, and Taq polymerase with 5′ to 3′ exonuclease activity cleaves the hybridised probes. This cleavage separates the quencher from the reporter, allowing the fluorescence of the reporter dye to be detected. The measured emission represents the genotype of each sample [112]. Emission of only one of the signals corresponds to homozygosity, whereas emission of

both signals corresponds to heterozygosity. Allelic discrimination was performed using the ABI 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA). This genotyping method was used in studies I and II.

HumanOmniExpressBeadChip

The HumanOmniExpressBeadChip (Illumina, Inc. CA, USA) is an array with genome-wide coverage of common SNP variations used for whole-genome genotyping. The BeadChip used in study IV of this thesis covers >730K SNPs in a twelve-sample per array format. By using tagSNP content from the International HapMap project, the chip covers 91 % ($r^2 > 0.8$) of common SNP variation with minor allele frequency (MAF) > 5 % in the CEU (Utah residents with ancestry from northern and western Europe) population.

The whole-genome Inifinium Assay with Illumina's BeadArray technology makes it possible to assay multiple genotypes of an individual at once. The technology is based on small silica beads, where each bead is covered with multiple copies of an oligonucleotide probe that targets a specific locus in the genome. As DNA fragments pass over the BeadChip, each probe will hybridise to a complementary sequence in the sample DNA. Allele specificity of the locus of interest is conferred by an enzymatic single base extension that incorporates labelled nucleotides. Once laser excited, the nucleotide label emits a signal that is detected by the Illumina iScan. Intensity values give information about the allelic ratio of a given locus. The Infinium Assay produces two-colour readouts (one colour for each allele) for each SNP in a genotyping study. Genotype calling is performed with GenomeStudio software (Illumina, Inc. CA, USA) [113].

Gene expression

To identify changes in expression of specific genes, messenger RNA (mRNA) levels were quantified by TaqMan real-time PCR in study I and III. Affymetrix oligonucleotide microarray method (Affymetrix, Santa Clara, CA, USA) was also applied in study III. Microarray is a powerful technique used for gene expression profiling, which allows for quantification of most gene transcripts in the sample. Both of these methods, TaqMan real-time PCR and Affymetrix microarray, require RNA to be converted into complementary DNA (cDNA) by reverse transcription.

TaqMan real-time PCR

TaqMan real-time PCR quantifies differences in cDNA levels between samples by continuous measurements of fluorescence throughout the PCR reaction. The technique is based on the 5′ to 3′ exonuclease activity of Taq polymerase. The probe, which has a reporter fluorescent dye attached to its 5′ end and a quencher attached to

its 3′ end, is designed to anneal to the target strand that is amplified by a set of primers. When the probe is intact, the quencher suppresses the reporter dye from emitting fluorescence. During PCR amplification, the exonuclease activity of Taq polymerase cleaves the probe, resulting in the release of the reporter dye. The reporter dye then emits fluorescence, which is detected and measured in each PCR cycle. The real-time PCR was performed using the ABI7900 HT detection system (Applied Biosystems, Foster City, USA). The cycle number when the fluorescence exceeds a threshold value is recorded as the Ct-value, which can be compared between different samples [114]. The transcript quantity of the studied genes was normalised to the mRNA level of the endogenous control, *Cyclophilin A*. In study I, data were calculated using the standard curve method. For each probe / primer set, a standard curve was generated and was confirmed to increase linearly with increasing amounts of cDNA. In study III, the relative gene expression levels were expressed as the differences in Ct-values (ΔCt).

Affymetrix microarray

The Affymetrix oligonucleotide microarray method (Affymetrix, Santa Clara, CA, USA) is based on slides with a solid surface filled with spots containing thousands of nucleotide probes. On the Affymetrix arrays, the probes are 25 nucleotides long, and the sequence of each probe is specific for a certain transcript. First, the samples to be analysed are labelled with a fluorescent dye and are then hybridised to the array. Once laser excited to the array, the hybridised transcript emits a signal that is detected as the measure of the transcript level. The signal emitted can be used to analyse differences in gene expression between groups of samples. On the GeneChip Human Gene 1.0 ST used in study III, all well annotated genes are represented on the array with approximately 26 probes each [115]. Robust Multi-array Analysis (RMA) by software "Expression Console" (Affymetrix) was employed for probe summarisation and data normalisation [116].

DNA methylation

DNA methylation measurements can be either gene-specific or global. This thesis has focused on both aspects by using two different methods for analysing DNA methylation. Both methods use bisulfite-treated DNA for identifying specific methylation patterns and provide quantitative analysis of CpG methylation.

Bisulfite treatment

Treatment of DNA with sodium bisulfite converts cytosine residues to uracil, but does not affect 5-methylcytosine residues, i.e., DNA methylated CpG sites. The bisulfite-treated DNA is subjected to DNA amplification where methylation dependent sequence changes in the DNA template level are generated. In the

genomic sequence produced after DNA amplification, a cytosine residue denotes a methylated site, whereas a thymine represents an unmethylated site (Figure 7).

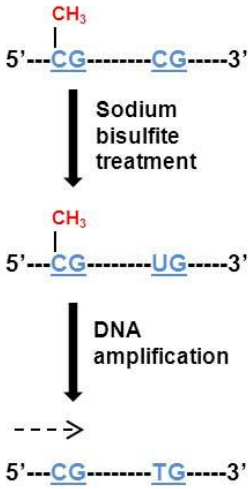

Figure 7. Bisulfite treatment of DNA followed by DNA amplification

EpiTYPER

EpiTYPER using SequenomMassARRAY system (Sequenom, San Diego, CA, USA) was used in study III for DNA methylation analysis of specific genes in human pancreatic islets. The starting point of this method is bisulfite treatment of genomic DNA, followed by PCR amplification of selected regions with incorporation of T7-promoter tags. Next, *in vitro* transcription of the reverse strand is performed where the generated transcript is used for enzymatically base-specific cleavage by RNase A. Due to the sequence changes generated through bisulfite treatment, the cleavage products differs in mass between the methylated and the non-methylated template. The mass of the cleavage products is determined by matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS). Comparison of the signal intensity of the mass signal between the methylated and non-methylated template gives information about the relative amount of DNA methylation [117].

Infinium HumanMethylation450 BeadChip

The Infinium HumanMethylation450 BeadChip (Illumina, Inc. CA, USA) was used for global DNA methylation profiling of human pancreatic islets in study IV. The BeadChip probes > 480K CpG sites with 99 % coverage of RefSeq genes in a twelve sample per array format [118]. DNA methylation is measured in bisulfite-treated genomic DNA. The Infinium methylation array uses beads with target-specific probes for examination of individual CpG sites. The HumanMethylation450 BeadChip employs two assays: Infinium I and Infinium II.

The Infinium I methylation-specific assay exploits two different probes per CpG site, corresponding to either the methylated or unmethylated state of the CpG site. Thus, the 3′ terminus of the probes is designed to match either the methylated (C) or unmethylated (T) residue resulting from bisulfite conversion of the sample DNA. Both probes will incorporate the same type of labelled nucleotide after the targeted CpG locus and therefore will be detected in the same colour (Figure 8A).

The Infinium II methylation-specific assay requires only one probe per CpG site, for allowing detection of both the methylated and unmethylated states of the CpG site. The 3′ terminus of the probe has a degenerate R (purine) base corresponding to the underlying "C" in the CpG site. The methylation state is detected by a single base extension, which results in the incorporation of a labelled nucleotide (A) at an unmethylated site (T) and another labelled nucleotide (G) at a methylated site (C). Each CpG locus will be detected in two colours (Figure 8B).

The BeadChips are imaged in the IlluminiScan, and the methylation level of each CpG site can be calculated from the intensity values of the methylated and unmethylated alleles. The methylation score of each CpG site is represented as Beta-values calculated using GenomeStudio (Beta-value = intensity of the methylated allele (M) / intensity of the unmethylated allele (U) + intensity of the methylated allele (M) + 100) [118].

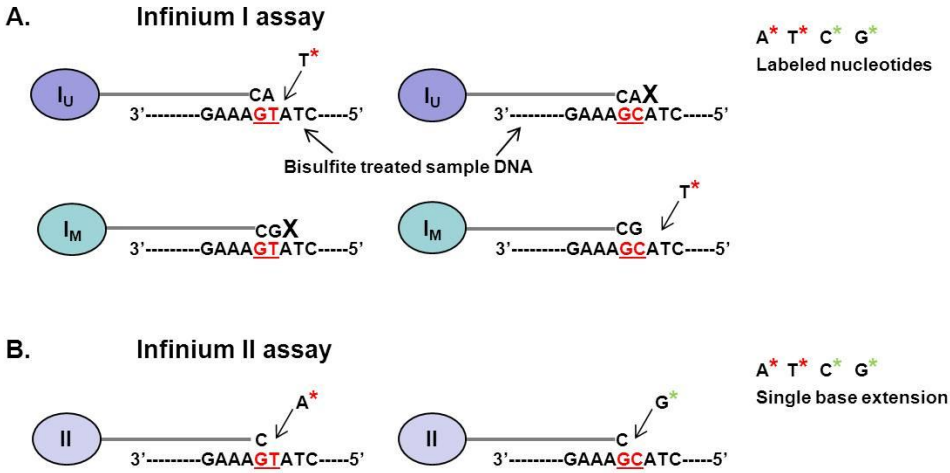

Figure 8. Overview of the Infinium methylation assays

A. Infinium I assay employs two bead types per CpG locus; one bead type to methylated C (Im), and another to unmethylated T (Iu) state of the CpG site resulting from bisulfite conversion of the sample DNA. Both probes will incorporate the same type of labelled nucleotide after the targeted CpG locus. **B.** Inifnium II assay uses one bead type to each CpG locus. Methylation state is detected by single base extension.
GT represents an unmethylated locus of the sample DNA; GC, represents a methylated locus of the sample DNA. Adapted from Bibikova *et al.* 2011 [118].

Western blotting

Western blotting was used to detect and quantify certain proteins in study I. In this method, the proteins in a sample are separated according to size by gel electrophoresis. The proteins in the gel are then transferred onto a membrane, followed by immunoblotting of the protein of interest.

Animal model

Wild type (*Tfb1m*$^{+/+}$) and heterozygous (*Tfb1m*$^{+/-}$) male mice that were 2-3 months of age [84] were examined in study I.
All experiments were approved by the local Animal Ethics Committee.

Intraperitoneal glucose tolerance test

Intraperitoneal glucose tolerance tests were carried out in fasted mice. First, the mice were anaesthetised; then, blood was collected at 0, 10, 30, 60 and 120 min after intraperitoneal injection of glucose (2 g/kg body weight). The plasma glucose concentration was measured by glucose oxidase reagent (Thermo Electron Corporation, Victoria, Australia). Insulin concentration was assayed by insulin enzyme-linked immunosorbent assay (ELISA) (Mercodia, Uppsala, Sweden).

Cell model

Rat INS-1 832/13 clonal β-cells were used for studies of β-cell function in study I. These cells are derived from the INS-1 β-cell line [119] and are transfected with a plasmid containing the human insulin gene and neomycin resistance cassette [120]. The 832/13 cells are robust secretors of insulin in response to glucose concentrations in the physiological range, and both the triggering pathway (K_{ATP}-dependent) and the amplifying pathway (K_{ATP}-independent) of GSIS are present in these cells [120]. Therefore, the 832/13 cells can serve as a model for a normal β-cell [120].

RNA interference

RNA interference (RNAi) can occur naturally and is a process in which double-stranded RNA (dsRNA) inhibits the expression of genes with a complementary nucleotide sequence [121]. Long dsRNAs are degraded by the endoribonuclease Dicer into small effector molecules called small interfering RNAs (siRNAs). siRNAs are double-stranded RNA molecules, 21-23 nucleotides in length with 3′ two-nucleotide overhangs on each strand. The siRNAs enters the RNA-induced silencing complex (RISC), where one strand is discarded and the other strand remains to guide the silencing complex to the target mRNA to amplify gene silencing. Synthetic siRNAs, which mimics the natural Dicer products, can be exogenously introduced into cell lines by transfection. In study I, siRNAs directed to *Tfb1m* (Thermo Scientific) were transfected into the glucose-responsive 832/13 clonal β-cell line using a lipid-based transfection method (Dharmacon, Lafayette, CO, USA). Cells were cultured in medium for 96 hours at 37°C in the presence of 50 nM siRNA. Subsequently, the consequences of reducing the expression of *Tfb1m* were examined.

Stimulated insulin secretion

Isolated human pancreatic islets were prepared by collagenase digestion and density gradient purification. Islets were hand-picked under a stereomicroscope and GSIS from the human pancreatic islets was measured *in vitro* in static incubations. Each incubation vial contained 12 size-matched islets and the islets were incubated for 1 hr at 37°C in a medium containing either 1.0 (basal secretion) or 16.7 (stimulated secretion, GSIS) mM glucose. Immediately after incubation an aliquot of the medium was removed for analysis of insulin using a radioimmunoassay (RIA) kit (Euro-Diagnostica, Malmö).

Islets from *Tfb1m*$^{+/+}$ and *Tfb1m*$^{+/-}$ mice were isolated by collagenase digestion and hand-picked under a stereomicroscope. Freshly isolated mouse islets in groups of three islets or the INS-1 832/13 clonal β-cells transfected with siRNA were incubated at 37°C for 1 hr in buffer containing 2.8 or 16.7 mM glucose. The buffer was removed and secreted insulin was determined using insulin ELISA (Mercodia) for mouse islets and Coat-A-Count kit (DPC, Los Angeles, CA) for 832/13 β-cells, which recognises human insulin and cross-reacts approximately 20 % with rat insulin [120].

ATP measurements

ATP measurements were performed with a firefly luciferase-based luminescence system (BioThema, Handen, Sweden). Chemiluminescence is the emission of light as the result of a chemical reaction. The luciferase reaction is dependent on ATP and molecular oxygen. The sample light emission makes it possible to continuous measure ATP levels in a sample. Glucose-induced cellular ATP level was determined in 832/13 β-cells upon a 1 hr stimulation by 2.8 mM or 16.7 mM glucose. The rate of mitochondrial ATP-production was measured in digitonin-permeabilised mouse islets and clonal β-cells in response to a metabolic substrate mixture of 50 mM pyruvate, 50 mM glutamate and 25 mM malate.

Statistical analyses

Phenotype characteristics are presented as the mean ± standard deviation (SD), the mean ± standard error of the mean (SEM) or as the median with interquartile range (IQR).
Multiple logistic regression analysis was used to assess the association between genotype and risk of developing T2D, with adjustment for potential confounding effects of other covariates, such as age, sex, BMI and geographic origin. Multiple

linear regression analysis was used to determine associations between genotype and quantitative traits after adjusting for different covariates. Meta-analyses were performed with fixed effect models using an inverse weighting method. All analyses between genotype and phenotype have been performed under additive genetic models, unless otherwise stated. Non-normally distributed data were logarithmically transformed before analysis.
Comparisons between two groups were assessed by Student's t-test or a Mann-Whitney U-test. Correlations between two variables were calculated using the Spearman correlation coefficient.
A χ^2-test was used for analysis if a set of OXPHOS genes show significantly reduced expression in pancreatic islets from T2D patients. Moreover, a gene set enrichment analysis performed in DAVID [122, 123] was used to identify key biological pathways of down-regulated genes in T2D islets. KEGG database was used as a reference for identification of genes in the pathways (http://www.genome.jp/kegg). Methylation Beta-values, assessed by Infinium HumanMethylation450 BeadChip, were converted to M-values before further statistical analysis ($M = \log_2 (Beta / (1 - Beta))$[124]. However, Beta-values were included in the final report for its biological interpretation ($Beta = 2^m / (2^m + 1)$) [124]. The methylation data were normalised using quantile normalisation and batch correction within the methylation data was performed using COMBAT [125]. In the global mQTL analysis, associations between SNPs and DNA methylation of CpG sites were tested using a linear regression model with biological covariates. The mQTL analysis was performed by using the Matrix eQTL R package [126]. *P*-values from the mQTL analysis were adjusted for multiple testing using Bonferroni correction for the total number of tests.

Statistical analyses were performed using PASW Statistics for Windows (SPSS, Chicago, IL, USA), STATA (StataCorp LP, College Station, Texas) and R software.

RESULTS

Study I

A common variant in TFB1M is associated with reduced insulin secretion and increased future risk of type 2 diabetes

In view of the important role of OXPHOS and ATP production in metabolic homeostasis, the aim of this study was to investigate whether SNPs in or near genes of the OXPHOS system and the mtDNA transcription and translation machinery are associated with β-cell dysfunction, insulin resistance and/or T2D.

To identify genetic loci associated with these clinical factors or the disease, we examined data from a GWAS, the DGI [26]. In this study, a common SNP (rs950994 G/A) located in intron 2 of the *TFB1M* gene was found to be associated with a decreased insulinogenic index (β = -0.17 ± 0.050, $P = 0.0007$), an elevated 2 hr glucose during an OGTT (β = 0.12 ± 0.042, $P = 0.007$), and a nominally increased risk of T2D (OR = 1.13, 95 % CI = 1.02 - 1.25, $P = 0.017$) in DGI.

Next we examined whether the associations of rs950994 with decreased insulinogenic index and increased 2 hr glucose could be replicated in additional cohorts (PPP-Botnia, HBCS and METSIM for insulinogenic index and PPP-Botnia, HBCS, METSIM and MPP for 2 hr glucose). In accord with the effect of the SNP on insulin secretion in the DGI, A-allele carriers showed a decreased insulinogenic index in the PPP-Botnia study (β = -0.045 ± 0.012, $P = 0.01$). However, the SNP was not significantly associated with the insulinogenic index in the METSIM study or in the HBCS ($P > 0.05$). Moreover, no significant association was found between the SNP and 2 hr glucose levels in any of the separate replication studies ($P > 0.05$). Because the METSIM study only includes males, we tested if gender may affect the associations by performing a gender stratified meta-analysis of all studied cohorts. In support of a gender effect, the pooled estimate effect for the A-allele of the SNP was both significantly associated with a decreased insulinogenic index and an increased 2 hr glucose in the meta-analysis only including females ($P = 0.006$ and $P = 0.008$, respectively). However, in the meta-analysis that only included males, the pooled estimate effect for the A-allele was not associated with any of these traits ($P > 0.05$).

We also asked whether the *TFB1M* SNP could predict future T2D in a prospective study, the MPP study. The frequency of the A-allele of the SNP was higher in individuals who developed T2D compared to those who did not, showing a modestly increased future risk of T2D (OR = 1.12, 95 % CI = 1.04-1.20, $P = 0.002$).

To identify a mechanism whereby the risk SNP of *TFB1M* contributes to the development of T2D, we examined the effect of the SNP on gene expression in human pancreatic islets. mRNA levels of *TFB1M*, assessed by TaqMan real-time PCR, were 24 % lower in pancreatic islets from risk A-allele carriers compared with GG carriers ($P < 0.01$; Figure 9A). Gene expression of *TFB1M* correlated positively with GSIS in the human pancreatic islets *in vitro* ($r = 0.40$, $P < 0.05$). Moreover, protein levels of TFB1M ($P < 0.05$; Figure 9B) and mtDNA-encoded OXPHOS subunits were also decreased in islets of A-allele carriers ($P < 0.05$).

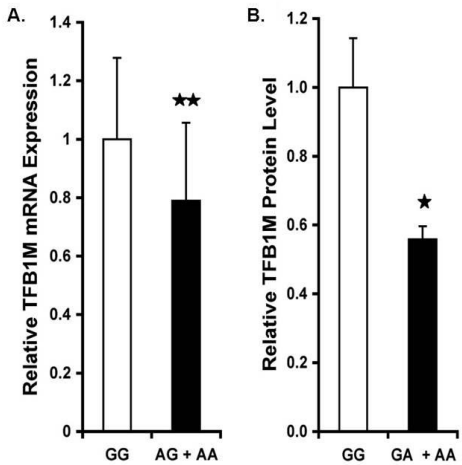

Figure 9. Effects of rs950994 on human pancreatic islets

A. *TFB1M* mRNA levels were lower in pancreatic islets from risk A-allele carriers (n=24) compared with G/G carries (n = 26) of *TFB1M* SNP rs950994. Bars represent mean ± standard deviation (SD). **B.** Protein levels for TFB1M were decreased in risk A-allele carriers (n = 4) compared with G/G carriers (n=4). Bars represent mean ± standard error of the mean (SEM).
P-values were based on a dominant model. * $P < 0.05$, ** $P < 0.01$.

To mimic the situation of reduced TFB1M expression observed in humans, we examined mice heterozygous for a germ line deletion of *Tfb1m* (*Tfb1m*$^{+/-}$), as total deficiency is known to be embryonically lethal [84]. Pancreatic islets from *Tfb1m*$^{+/-}$ mice contained less TFB1M protein levels compared to wild-type littermates ($P < 0.05$). Therefore, the *Tfb1m*$^{+/-}$ mouse is suggested to be a suitable model to study the role of TFB1M deficiency in islet function. In *Tfb1m*$^{+/-}$ *mice*, plasma glucose levels were higher while insulin secretion tended to be lower during an intraperitoneal glucose tolerance test *in vivo* (Figure 10A and B). The ratio of total insulin secretion during the challenge (insulin-area under the curve (AUC)) related to glucose (glucoseAUC) was decreased in the *Tfb1m*$^{+/-}$ mice compared to the control mice ($P < 0.05$; Figure 10C), showing that *Tfb1m*$^{+/-}$ mice have reduced insulin secretion in response to glucose *in vivo*. Furthermore, insulin secretion in response to glucose was significantly reduced in isolated pancreatic islets from the *Tfb1m*$^{+/-}$ mice (1.36 ± 0.19

versus 0.87 ± 0.14 ng insulin/islet/hr, P = 0.04 in control versus *Tfb1m*$^{+/-}$ mice, respectively). Finally, the rate of ATP production in response to a mixture of mitochondrial metabolic substrates (pyruvate, glutamate and malate) was also reduced in isolated islets from the *Tfb1m*$^{+/-}$ mice (34.1 ± 2.5 versus 25.4 ± 0.9 pmol ATP/min/islet, P = 0.04 in control versus *Tfb1m*$^{+/-}$ mice, respectively).

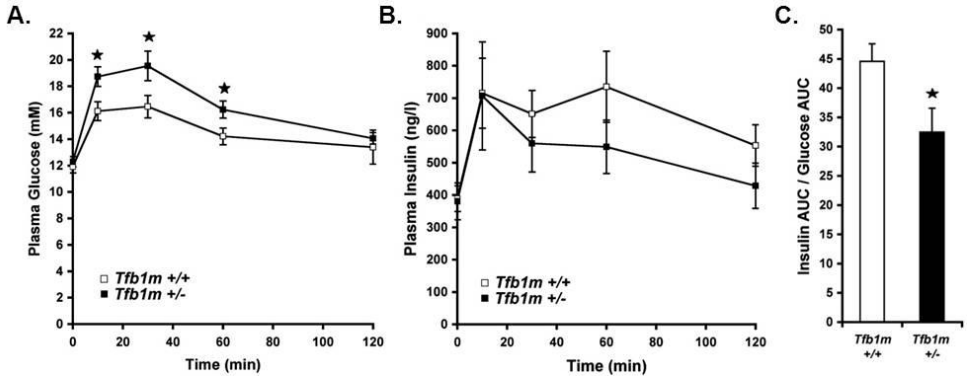

Figure 10. Reduced TFB1M levels in heterozygous mice (*Tfb1m*$^{+/-}$) result in impaired insulin secretion during a intraperitoneal glucose tolerance test (IPGTT) *in vivo*

A. Plasma glucose and **B.** plasma insulin levels in the *Tfb1m*$^{+/+}$ (n = 10) and *Tfb1m*$^{+/-}$ (n = 11) during an IPGTT. **C.** The ratio of insulin area under the curve (InsulinAUC) to glucose area under the curve (GlucoseAUC) for *Tfb1m*$^{+/+}$ and *Tfb1m*$^{+/-}$ mice during the IPGTT reflects the capacity of the mice to release insulin in response to glucose.
Bars represent mean ± SEM. * P < 0.05.

To further model the situation found in human and mouse islets and to explore the mechanisms of TFB1M deficiency on mitochondrial β-cell function, we silenced *Tfb1m* in the glucose-responsive 832/13 clonal β-cell line with siRNAs. By using TaqMan real-time PCR and Western blotting, we found a reduction in the *Tfb1m* mRNA level to 24.2 % ± 0.6 % (P = 0.0001) and in the protein level to 44.2 % ± 1.8 % of control (P = 0.0006) after transfection with siRNA directed to *Tfb1m*. TFB1M deficiency in clonal β-cells leads to reduction in GSIS (P < 0.01; Figure 11A). Because TFB1M is thought to primarily control translation of mitochondrially encoded proteins [84], we further examined OXPHOS protein levels in mitochondria from clonal β-cells. A panel of antibodies directed to both mitochondrially encoded (ND5 in complex I, CytB in complex III and COXI in complex IV) and nuclear-encoded (NDUFB8 in complex I, SDHB in complex II, QCR2 in complex III and ATPA in ATP synthase) OXPHOS proteins were selected and analysed by Western blotting. All mitochondrially encoded subunits, together with nuclear-encoded NDUFB8 in complex I, showed significantly reduced protein levels in cells with TFB1M deficiency compared to control cells (P < 0.05; Figure 11B). However, the other nuclear-encoded subunits were not significantly reduced in β-cells where TFB1M had

been silenced. Finally, the negative impact of TFB1M deficiency was further confirmed by a 27.6 % ± 4.8 % decrease in the glucose-induced cellular ATP level in the TFB1M-deficient cells compared to control cells ($P = 0.01$; Figure 11C).

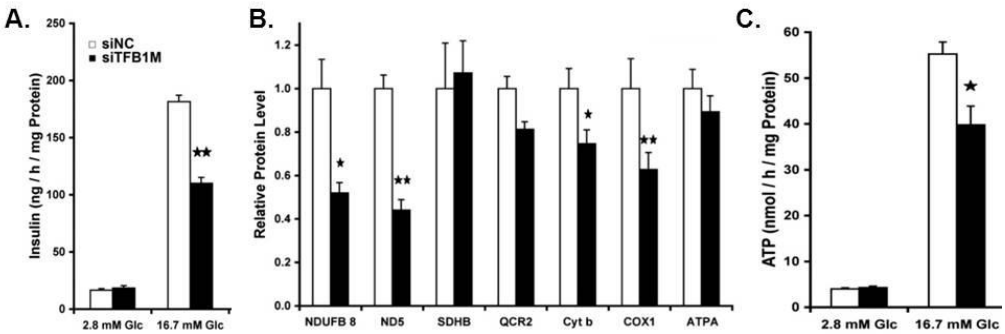

Figure 11. Reduced TFB1M levels in glucose-sensitive clonal β-cells (INS 832/13) result in impaired insulin secretion, mitochondrial OXPHOS and ATP generation

A. Insulin secretion in response to 2.8 and 16.7 mM glucose (Glc) (n = 10) was determined in a 1 hr static incubation of the 832/13 β-cells. **B.** Analysis of OXPHOS protein levels (NDUFB8, complex I, nuclear-encoded; ND5, complex I, mitochondrially encoded; SDHB, complex II, nuclear-encoded; QCR2, complex III, nuclear-encoded; Cyt b, complex III, mitochondrially encoded; COX1, complex IV, mitochondrially encoded; ATPA, ATP synthase, nuclear-encoded) in mitochondria from clonal β-cells (n = 4). **C.** Accumulation of cellular ATP was determined in 832/13 β-cells upon a 1 hr stimulation by 2.8 and 16.7 mM glucose.
Bars represent mean ± SEM. White bars represent control 832/13 β-cells (siNC) and black bars represent 832/13 β-cells after ninety-six hours transfection with siRNA to *Tfb1m* (siTFB1M). $*P < 0.05$, $**P < 0.01$.

In summary, our data from studies in humans, mice and clonal β-cells provides genetic and functional evidence that the *TFB1M* gene is associated with an increased risk of T2D by causing impaired insulin secretion. Our data suggest a model where the risk SNP (rs950994) confers lower TFB1M protein expression and, consequently, the synthesis of mitochondrially encoded proteins is reduced. This is followed by restrained OXPHOS, reduced ATP production and impaired stimulated insulin secretion from the β-cell, which leads to an increased risk of T2D (Figure 12).

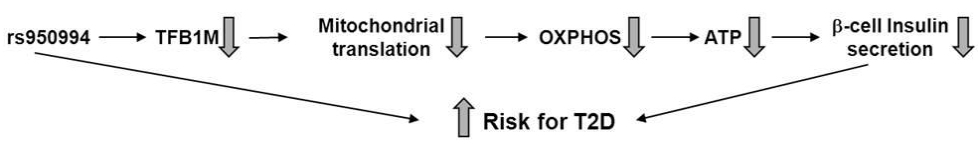

Figure 12. Model for possible role of TFB1M in the development of T2D

Study II

Two common genetic variants near nuclear-encoded OXPHOS genes are associated with insulin secretion in vivo

Our premise is that some genetic factors may modulate mitochondrial function and, consequently, the capacity of the pancreatic β-cells to secrete insulin, thereby increasing the risk of T2D. Therefore, the aim of this study, similar to study I, was to identify genetic loci in or near nuclear-encoded genes of the OXPHOS pathway that are associated with insulin secretion *in vivo*. In addition, we wanted to follow-up our findings in an independent cohort.

To find SNPs associated with GSIS assessed as the insulinogenic index, we examined data from the DGI GWAS of non-diabetic individuals [26]. We included a region of 25 kb regions both up- and downstream of each of the 76 nuclear-encoded genes contributing to the OXPHOS system, which included 413 studied SNPs in DGI GWAS with a MAF $\geq$ 0.05. The identified SNPs were ranked based on the association between genotype and phenotype. Out of these, nine SNPs representing six different genes showed nominal association with the insulinogenic index ($P \leq$ 0.01; Table 3). *P*-values are based on linear regression analyses, assuming an additive genetic model, with gender, age, BMI and the recruiting region included as covariates. Based on the lowest *P*-values, two SNPs were selected for follow-up: the first SNP (rs606164) was located 12 kb upstream of *NDUFC2,* and the second one (rs1323070) was located 24 kb downstream of *COX7A2* (Table 3). The major C-allele of rs606164 and the minor G-allele of rs1323070 are nominally associated with decreased GSIS during an OGTT (insulinogenic index) in non-diabetic subjects of DGI (P = 0.0009 and P = 0.003, respectively; Table 3). However, none of the variants were associated with T2D in the case-control based study of DGI or insulin resistance (HOMA-IR) in non-diabetic subjects of DGI [26].

Next, we used a more extensive cohort to replicate our findings, the PPP-Botnia study. In line with the results from DGI, we found that C-allele carriers of the SNP upstream of *NDUFC2* (rs606164) and G-allele carriers of the SNP downstream of *COX7A2* (rs1323070) showed a decrease in the insulinogenic index in the PPP-Botnia study (rs606164: β = -0.070 ± 0.022, P = 0.002; rs1323070: β = -0.040 ± 0.021 P = 0.05). Moreover, we also calculated the disposition index, which is an additional assessment of β-cell function that takes into account the insulin resistance. Again, C-allele carriers of rs606164 and G-allele carriers of rs1323070 showed a decrease in insulin secretion in PPP-Botnia, adjusted for insulin resistance (rs606164: β = -0.066 ± 0.023, P = 0.007; rs132070: β = -0.040 ± 0.042, P = 0.03). However, neither rs606164 nor rs1323070 was associated with HOMA-IR in PPP-Botnia ($P >$ 0.05).

With this work, we have identified two common polymorphisms that are associated with GSIS *in vivo*. These SNPs are located near nuclear-encoded OXPHOS genes. This leads to the possibility that genetic variation near genes involved in OXPHOS may influence the risk of T2D by impaired insulin secretion.

Table 3. Identified SNPs from DGI GWAS located in a region of ~25 kb up- or downstream of OXHPOS genes with an association to insulinogenic index in non-diabetic individuals of DGI with $P \leq 0.01$ and MAF ≥ 0.05

SNP	Chr	Nearest OXPHOS gene	Alleles[a] (Major/Minor)	MAF	*P*-value
rs606164	11	~12 kb upstream *NDUFC2*	**C**/G	0.16	$9{\cdot}10^{-4}$
rs1323070	6	~24 kb downstream *COX7A2*	A/**G**	0.36	$3{\cdot}10^{-3}$
rs10793285	11	~20 kb upstream *NDUFC2*	**T**/G	0.36	$6{\cdot}10^{-3}$
rs1133322	15	~0.3 kb downstream *COX5*	A/**G**	0.49	$7{\cdot}10^{-3}$
rs2643338	8	Intron *UQCRB*	**A**/G	0.47	$1{\cdot}10^{-2}$
rs7827095	8	~3 kb downstream *UQCRB*	**T**/C	0.47	$1{\cdot}10^{-2}$
rs10734905	12	Intron *ATP6OA2*	**G**/T	0.32	$1{\cdot}10^{-2}$
rs1264913	1	~15 kb upstream *ATP5F1*	A/**G**	0.11	$1{\cdot}10^{-2}$
rs2845556	11	~20 kb downstream *NDUFC2*	**C**/T	0.49	$1{\cdot}10^{-2}$

In Diabetes Genetics Initiative (DGI) *P*-values are based on linear regression to test association between genotype and insulinogenic with the covariates gender, recruiting region, age, BMI and type of insulin measurement.
MAF, minor-allele frequency.
[a]Allele denoted in bold associated with decreased insulinogenic index.

Study III

Decreased expression of genes involved in oxidative phosphorylation in human pancreatic islets from patients with type 2 diabetes

Altered gene expression in target tissues for T2D might influence cellular functions and biological pathways and, thus, promote the disease. In this study, we examined whether genes involved in OXPHOS show differential gene expression and DNA methylation in human pancreatic islets from T2D patients compared with donors not diagnosed with diabetes mellitus.

We analysed microarray data of human pancreatic islets from 55 donors not diagnosed with diabetes and nine T2D donors. We identified 17 nuclear-encoded and four mtDNA-encoded OXPHOS genes showing nominally reduced expression in pancreatic islets from T2D patients ($P < 0.05$; Table 4). Although alterations in gene expression of individual genes were of relatively small magnitude (range in fold change: 6 - 22 %; range in *P*-values: 0.0041 – 0.045), the number of observed over expected OXPHOS genes with decreased expression was enriched ($\chi^2 = 26.37$, $P = 2.81 \cdot 10^{-7}$). Additionally, when performing DAVID pathway analyses [122, 123] with KEGG pathway annotations, the OXPHOS pathway was the only significantly enriched pathway among all down-regulated genes in T2D islets ($P = 0.003$).

Next, the microarray data were technical validated by TaqMan real-time PCR for four selected OXPHOS genes: *NDUFA5*, *NDUFA10*, *COX11* and *ATP6V1H*. All four OXPHOS genes were significantly down-regulated in islets from patients with T2D (n = 9) compared with donors not diagnosed with diabetes (n = 47) using TaqMan real-time PCR ($P < 0.01$).

In a correlation analysis including both donors not diagnosed with diabetes and T2D donors, HbA1c levels correlated negatively with the expression levels of *NDUFA5*, *COX11* and *ATP6V1H* (r = -0.32 and $P = 0.03$, r = -0.38 and $P = 0.01$, r = -0.36 and $P = 0.01$, respectively), but not with *NDUFA10* (r = -0.16 and $P = 0.29$). Moreover, GSIS in the human islets correlated positively with the expression levels of all four OXPHOS genes when including donors not diagnosed with diabetes (*NDUFA5*: r = 0.53 and $P = 0.00065$; *NDUFA10*: r = 0.54 and $P = 0.00044$; *COX11*: r = 0.36 and $P = 0.03$; *ATP6V1H*: r = 0.51 and $P = 0.001$). Gene expression levels used for the correlation analyses with HbA1c and GSIS were assessed by TaqMan real-time PCR.

Table 4. Microarray expression patterns of 21 genes involved in OXPHOS (17 nuclear-encoded genes and 4 mtDNA-encoded genes) from pancreatic islets of T2D donors (n = 9) compared with donors not diagnosed with diabetes (n = 55)

Gene symbol	Fold change (%)	*P*-value
NDUFA10	-7.4	0.011
mtND5	-12.0	0.011
NDUFA5	-13.3	0.016
NDUFS1	-10.6	0.039
mtND1	-8.8	0.043
NDUFS5	-9.5	0.045
UQCRFS1	-7.0	0.037
mtCYB	-6.1	0.026
COX11	-21.7	0.0082
COX7A2L	-12.2	0.014
mtCOX2	-8.1	0.032
COX4I1	-11.0	0.035
ATP6V1H	-14.9	0.0041
ATP6V1E2	-13.3	0.011
ATP6V1G1	-12.1	0.011
ATP6V1E1	-11.6	0.012
ATP6V1C1	-9.7	0.026
ATP5L	-11.0	0.028
ATP5G3	-14.1	0.037
ATP5E	-13.8	0.043
ATP6V1A	-13.6	0.045

Fold change in % based on gene expression mean values with non-diabetics as reference. *P*-values calculated with Mann-Whitney U-test. *P*-values without correction for multiple testing.

Finally, DNA methylation was analysed for six CpG sites for *NDUFA5*, 12 CpG sites for *COX11* and 16 CpG sites for *ATP6V1H* in the human pancreatic islets using EpiTYPER. All CpG sites were located upstream of the respective transcription start. However, none of the analysed CpG sites in any of the three regions showed differences in DNA methylation in islets from donors with T2D compared with donors not diagnosed with diabetes.

In summary, pancreatic islets from patients with T2D show decreased expression of a set of OXPHOS genes, which may contribute to T2D by impaired insulin secretion.

Study IV

A genome-wide methylation quantitative trait locus analysis identifies SNPs associated with DNA methylation in human pancreatic islets

DNA methylation has a regulatory role in biological processes and aberrant patterns of DNA methylation may contribute to the development of human diseases. To gain better knowledge about how alterations in DNA methylation levels are controlled, this study aimed to assess the effects of common genetic variations on DNA methylation patterns in human pancreatic islets. We performed a global methylation quantitative trait loci (mQTL) analysis in pancreatic islets of 89 donors not diagnosed with diabetes mellitus. In this study, we relate genotype data of 591016 SNPs with DNA methylation levels of 483025 individual CpG sites in the pancreatic islets, analysed by HumanOmniExpressBeadChip and Infinium HumanMethylation450 BeadChip, respectively (Illumina, Inc. CA, USA).

A linear regression model was used to test the association of each SNP-CpG pair in a *cis*-acting manner with a distance of ≤ 1 Mb between the SNP and the CpG site. In total, we found 111861642 SNP-CpG pairs located in *cis*. Out of these, 30691 SNP-CpG pairs showed significant associations with $P < 0.05$ after Bonferroni correction, comprising 20579 unique SNPs and 5311 individual CpG sites (Table 5).

The unique SNPs of significant *cis*-mQTLs are widespread throughout the genome, but an enrichment of SNPs are located on chromosome 6 (Figure 13). The median distance between SNPs and CpG sites of significant *cis*-mQTLs was 23.9 kb (minimum = 0 kb; 1st quartile = 7.2 kb; median = 23.9 kb; 3rd quartile = 70.0 kb; maximum = 997 kb).

In conclusion, our results demonstrate that common SNPs may influence DNA methylation levels of nearby CpG sites in human pancreatic islets. These findings suggest that genetic variation should be considered when studying the impact of DNA methylation on complex human diseases, such as T2D.

Table 5. Summary counts of number of SNPs, CpG sites and SNP-CpG pairs analyzed in total as well as showing significant *cis*-mQTLs

	In total	Significant
SNPs	591 016	20 579
CpG sites	483 025	5311
cis-pairs	111 861 642	30 691

Significance threshold < 0.05 after Bonferroni correction. The total number of tests performed = 111 861 642.

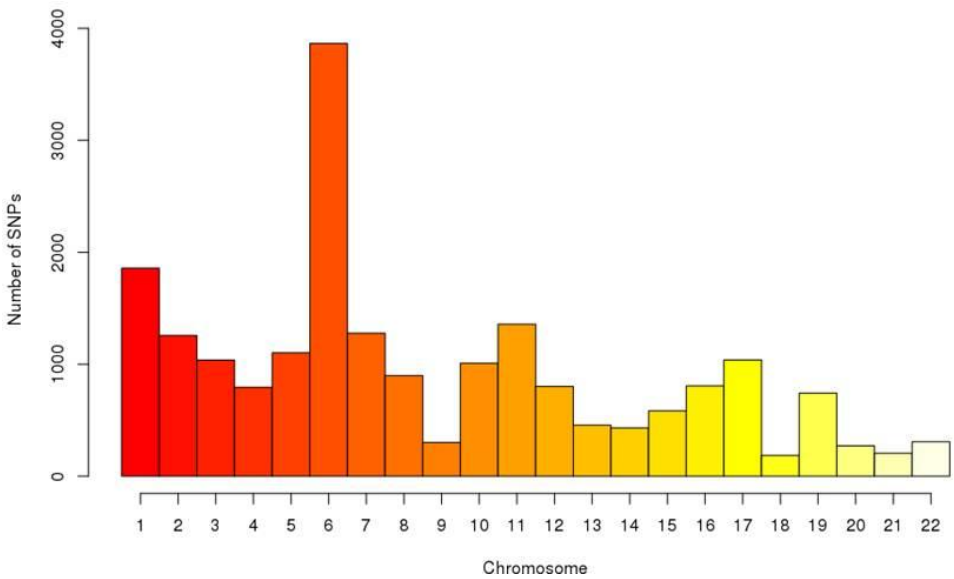

Figure 13. Number of unique SNPs significant associated with DNA methylation in human pancreatic islets across the genome

The unique SNPs (n = 20579) found to be associated with DNA methylation of nearby CpG sites, *cis*-mQTLs, are plotted based on their chromosome number.

DISCUSSION

Type 2 diabetes (T2D), the most common form of diabetes mellitus, is a multifactorial and polygenic disorder. In recent years, the GWAS techniques have generated a vast amount of information about the genetics of T2D. Many of the genes linked with the disease seem to be highly relevant for β-cell function and development. Accumulative evidence suggests that impaired insulin secretion is the main culprit in T2D [13]. However, a better understanding about the functional abnormalities in pancreatic islets that contribute to T2D still needs to be provided. Therefore, in the studies presented here, we investigated the role of genetics and epigenetics on OXPHOS, islets function and insulin secretion in the pathogenesis of T2D.

Mitochondrial ATP production by OXPHOS is a key requirement for GSIS by pancreatic β-cells [79]. While several mitochondrial genes encoded by the nuclear genome have been implicated in the pathogenesis of T2D [103, 104], only a few polymorphisms in these genes have been associated with the disease [56, 100-102]. By mining the DGI GWAS [26] for genes involved in OXPHOS, we identified a SNP (rs950994) in *TFB1M* associated with impaired insulin secretion and T2D. TFB1M is a nuclear-encoded factor involved in the translational control in mitochondria [82, 84]. Follow-up studies showed that the *TFB1M* SNP is associated with an increased future risk of T2D in the prospective MPP study. The effect of rs950994 on future risk of T2D is of a similar magnitude to approximately 20 SNPs that have been previously reported to be associated with T2D [26, 110]. Moreover, carriers of the risk allele showed a reduction in both mRNA and protein levels of TFB1M in human pancreatic islets. To find support for the role of *TFB1M* in the pathogenesis of T2D, we examined the effect of TFB1M deficiency in both a mouse model and a cell model. We found that stimulated insulin secretion and ATP levels were reduced in both *Tfb1m*$^{+/-}$ mice and clonal β-cells where TFB1M had been silenced. A rise in the β-cell ATP/ADP ratio in response to elevated plasma glucose levels is known to be the main trigger for insulin secretion [127]. The impairments of ATP production and insulin secretion in TFB1M deficiency is probably explained by insufficient regulatory actions of TFB1M on the translation of mitochondrially encoded OXPHOS protein subunits in islet β-cell mitochondria. Experimental studies have shown that these subunits are essential for the OXPHOS pathway in the pancreatic β-cells and disruption of mtDNA expression leads to impaired insulin secretion [93]. Indeed, TFB1M deficiency resulted in a decline in mtDNA-encoded protein levels. The reduced levels of both mitochondrially encoded ND5 and nuclear-encoded NDUFB8 in complex I in cells with TFB1M deficiency may be explained by the particularly crucial function of the mitochondrially encoded subunits during the assembly of complex I [128, 129]. This result suggests that the assembly of complex I

is disrupted because of TFB1M deficiency. However, the reductions in GSIS and ATP production are not as profound as the decline in the protein levels of complex I, which may suggest an overcapacity of the OXPHOS system or the use of complex II as the entrance for reduced electron carriers into the ETS. Protein levels of complex II, with all subunits encoded in the nucleus, were unaffected by TFB1M deficiency. By the studies in this thesis, we provide genetic, clinical, and experimental *in vivo* and *in vitro* evidence that deficiency in TFB1M and impaired mitochondrial function contributes to the pathogenesis of T2D by impaired insulin secretion from the pancreatic β-cell.

In another study we provide evidence that some common SNPs located adjacent to OXPHOS genes may increase susceptibility to impaired GSIS in non-diabetic individuals, even when the same OXPHOS SNPs show no association with T2D. The identification of these SNPs was based on one trait, and these SNPs were near the genes of one biological pathway. In total, we identified nine SNPs near OXPHOS genes showing nominal association ($P < 0.01$) with the insulinogenic index in non-diabetic participants of the DGI GWAS [26]. In this exploratory follow-up study, two top SNPs were selected for replication in an independent cohort, although they were not genome-wide significant in DGI. These results are supported by a recent study in a Danish cohort of non-diabetic individuals, where they observed a nominal influence of SNPs in or near OXPHOS genes on indices of GSIS [130]. Additionally, they were able to replicate a nominal association between SNPs in OXPHOS genes and T2D from a recent genome-wide meta-analysis [37] in a case-control study of Danish individuals [130]. Although these data show no genome-wide associations between common SNPs in OXPHOS genes and T2D or related traits, we cannot rule out the possibility that common SNPs in or near genes involved in OXPHOS may be of importance in insulin secretion and/or the pathogenesis of T2D.

Insulin secretion in response to glucose is impaired in pancreatic islets from patients with T2D compared to controls, which is reflected by lower ATP levels and impaired hyperpolarisation of the mitochondrial membrane in the diabetic islets [131]. These effects are mirrored in mice and clonal β-cells with TFB1M deficiency, as shown in this thesis. Moreover, it is possible that a coordinated down-regulation of multiple OXPHOS genes affects the overall ATP production in pancreatic islets. In this thesis, we demonstrate that a set of OXPHOS genes, both nuclear- and mtDNA-encoded, is down-regulated in human pancreatic islets of patients with T2D. We also show that islet expression of multiple OXPHOS genes correlates positively with GSIS. Based on the data shown in this study, we cannot exclude the possibility that altered cell composition in pancreatic islets from T2D may affect the changes we find in insulin secretion and OXPHOS expression. However, it is debated to what extent β-cell mass is reduced in T2D and how this affects β-cell function. Some investigators have reported a decrease in β-cell mass up to 60 % in T2D [132], meanwhile others have observed considerably less or no changes [57, 133]. Although a loss of β-cell mass may play a role in the pathogenesis of T2D, it is likely to do so in combination with

impaired β-cell function. Our studies in TFB1M silenced clonal β-cells and human pancreatic islets with T2D together support that reduced OXPHOS levels lead to impaired GSIS. Similar results have been found in pancreatic islets from a diabetic mouse model, the MKR mouse, where several proteins in OXPHOS exhibited reduced expression [134]. Studies in the same mouse model demonstrate that many of the observed impairments of islet mitochondrial function and reduced GSIS occur just before overt hyperglycaemia [135]. These data suggest that improving mitochondrial β-cell function may be an important therapeutic approach for T2D.

Epigenetic changes have been suggested to influence the pathogenesis of T2D [54]. However, the role of differential DNA methylation in the development of the disease remains unclear. Recent studies propose that DNA methylation can affect mRNA expression of OXPHOS genes in human skeletal muscle and, subsequently, human metabolism [100, 101]. In this thesis, we were unable to detect any differences in DNA methylation for the analysed CpG sites upstream of the selected OXPHOS genes in human pancreatic islets from T2D patients compared with donors not diagnosed with diabetes. One limitation is the small number of genes and CpG sites analysed in our study. The assays only cover approximately 400 – 500 bp of the respective gene. Therefore, we cannot exclude the possibility that some of the OXPHOS genes and CpG sites not analysed might show differential DNA methylation in pancreatic islets from T2D donors. Interestingly, another study in human pancreatic islets showed increased levels of DNA methylation upstream of *PPARGC1A* in T2D donors compared to non-diabetic donors [56]. The increase in DNA methylation was associated with decreased *PPARGC1A* mRNA expression in pancreatic islets from patients with T2D, which correlated with impaired GSIS [56]. *PPARGC1A* is a transcriptional co-activator that regulates expression of genes involved in mitochondrial oxidative metabolism. Other studies have also suggested that DNA methylation may influence gene expression and, subsequently, insulin secretion in human pancreatic islets [57, 58, 136]. Together, these results underscore the important involvement of epigenetic dysregulation in diabetic islets.

A previous study in human skeletal muscle showed that genetic, epigenetic and non-genetic factors integrate to influence the expression of a gene involved in OXPHOS, which in turn was associated with insulin sensitivity [100]. This study demonstrates the complexity of how some biological processes are controlled. The development of quantitative genome-wide technologies has made it possible to study these types of integrations from a global perspective. Genome-wide methylation quantitative trait locus (mQTL) studies in brain samples [137, 138] and HapMap cell lines [139] suggest that SNPs influence the inter-individual variation in DNA methylation across the genome. However, because the epigenome varies between different cell types, it is important to perform these studies in individual cell systems or tissues. In this thesis, we have performed a global mQTL analysis in human pancreatic islets. We have only analysed *cis*-mQTLs and the effect of SNPs located a maximum distance of 1 Mb from the CpG site; the median distance between the SNP and CpG site of genome-

wide significant *cis*-mQTLs were found to be 23.9 kb. An enrichment of *cis*-acting SNPs on DNA methylation has been shown in previous mQTL studies [137-139]. The study present in this thesis is, to our knowledge, the first to show associations between SNPs and DNA methylation in human pancreatic islets, an important target tissue for T2D. These results demonstrate that genetics need to be considered when studying variation in DNA methylation patterns on islet function. The complexity of genetics and epigenetics in biology have been further supported by a recent study, which suggests that genetic variants not associated with a phenotype could still contribute to variations of that phenotype through epigenetic mechanisms [140].

SUMMARY AND GENERAL CONCLUSION

A vast amount of information about the genetics of T2D has been obtained during my time as a PhD-student. This progress in genetics, together with the implications of epigenetics, has been an important contribution to the field of T2D but has also raised new questions that need to be explored. Unravelling the regulation and functional role of genes involved in T2D will provide a better understanding of the mechanisms behind the disease development and potential therapeutic targets. Studies presented in this thesis have contributed to the research field of T2D by dissecting the role of genetic and epigenetic influence on OXPHOS, pancreatic islet function and insulin secretion, which are all important factors in the pathogenesis of T2D. This thesis demonstrates the following points:

- *TFB1M* can be added to the list of genes implicated in the risk of developing T2D. TFB1M is a nuclear-encoded factor involved in translational control in mitochondria. The role of TFB1M deficiency in the pathogenesis of T2D is given by the critical role of mitochondrial function in control of insulin secretion from the pancreatic β-cell.

- Common SNPs located in or near genes involved in OXPHOS may influence GSIS *in vivo*.

- Pancreatic islets from patients with T2D show decreased expression of a set of OXPHOS genes, which may lead to impaired insulin secretion.

- DNA methylation in human pancreatic islets is under the control of common genetic variability. SNPs should be considered when studying the impact of DNA methylation on phenotypic outcomes and human diseases.

At the same time that the scientific study of T2D is making great progress, the prevalence of the disease is rapidly growing worldwide. Although multiple genes and multiple aetiologies are known to be involved in T2D, there is much work left to do until we have a clear understanding of the underlying disease mechanisms. Incorporating studies of human islets, analysis of mouse and cell models and *in vivo* studies in humans may address some of these questions. Studying the interplay between genetic, epigenetic and non-genetic factors might also provide important insight into the complexity of factors involved in the pathogenesis of T2D.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Diabetes är en grupp av sjukdomar som kännetecknas av förhöjda halter av glukos (socker) i blodet. Förekomsten av diabetes ökar i snabb takt i hela världen. År 2011 var 366 miljoner människor drabbade av diabetes, och denna siffra beräknas till 522 miljoner år 2030 om ingen avgörande åtgärd genomförs. Typ 2 diabetes är den vanligaste formen av diabetes och utgör ungefär 90 % av alla diabetesfall. En ökad förekomst av typ 2 diabetes är starkt förknippad med ökad grad av fetma, en konsekvens av en livsstilstrend med högre energiintag och mindre motion. Kroniskt höga blodglukoshalter som ses vid diabetes kan leda till utveckling av en rad komplikationer, till exempel njurproblem, synfel och kardiovaskulära sjukdomar, varav den sistnämnda är en av de största dödsorsakerna vid typ 2 diabetes.

Människokroppen strävar efter att hålla blodglukoshalten på en jämn nivå. Stigande glukoshalter i blodet, till exempel efter en måltid, regleras med hjälp av hormonet insulin. Insulin utsöndras från beta-celler som finns i de Langerhanska öarna i bukspottskörteln. Insulins målvävnader är skelettmuskler och fettväv, där det stimulerar glukosupptag från blodet, samt levern där det minskar glukosproduktion. Glukos som tas upp av kroppens celler används för att producera energi. Typ 2 diabetes uppstår på grund av defekter i insulinfrisättningen från beta-cellerna och/eller minskad effekt av insulin i målvävnaderna, vilket leder till förhöjda nivåer av glukos i blodet. Allt fler bevis tyder dock på att försämrad insulinfrisättning är den stora boven i sjukdomsprocessen.

Frisättning av insulin är en komplicerad process där beta-cellerna fungerar som glukossensorer. Vid ökade halter av glukos i blodet tas detta upp av beta-cellerna och spjälkas till energi i form av ATP-molekyler. Denna energiproduktion sker i cellernas mitokondrier, vars viktigaste funktion är att producera ATP. Mitokondriell ATP-produktion är i sin tur den huvudsakliga utlösaren av insulinfrisättning från beta-cellerna. Om energiproduktionen i mitokondrien inte fungerar optimalt kan frisättningen av insulin försämras, vilket leder till ökad risk för typ 2 diabetes.

De bakomliggande orsakerna till typ 2 diabetes är ännu inte helt kända, men både ärftliga och miljöbaserade faktorer påverkar risken att utveckla sjukdomen. Trots att icke-genetiska faktorer såsom fetma och fysisk inaktivitet spelar roll i utvecklingen av typ 2 diabetes, utvecklar inte alla som är utsatta för dessa riskfaktorer sjukdomen. En stark familjehistoria av typ 2 diabetes tyder på att det finns en genetisk disposition för sjukdomen. Vårt arvsanlag är uppbyggt av en kedja av DNA-molekyler, där ordningen på beståndsdelarna i DNA-sekvensen utgör den genetiska koden. Koden är konstant över tid och likadan i alla kroppens celler. Kroppens celler innehåller två olika DNA. Cellkärnans DNA utgör den stora merparten av arvsmassan, medan

mitokondriens DNA utgör en liten mängd. Mitokondriens DNA kodar 13 gener vilka är helt nödvändiga för att mitokondrien ska kunna tillverka cellernas ATP. Mitokondrien består även av ett flertal enheter som kodas av cellkärnans DNA. Flera genetiska variationer som kan öka risken för typ 2 diabetes har hittills hittats. Genom att identifiera genetiska riskfaktorer för typ 2 diabetes och utvärdera vilken funktionell roll genen har i kroppen kan man inhämta viktig information om de underliggande mekanismerna bakom sjukdomsutvecklingen.

I den här avhandlingen beskrivs fyndet av en genvariant vilken ökar risken för typ 2 diabetes. Genen kallas *TFB1M* och är en translationsfaktor som styr uttrycket av de tretton generna som det mitokondriella DNAt kodar. Därmed påverkar genen ATP-produktionen och vidare insulinfrisättningen från beta-cellerna i bukspottskörteln. Genom analyser i befolkningsstudier, studier i mänsklig vävnad, i försöksdjur samt i odlade celler kan vi påvisa effekten av genen och dess involvering i risken för utveckling av typ 2 diabetes.

Vi har ytterligare identifierat två genetiska variationer som verkar ha samband med försämrad insulinfrisättning hos människa. Båda dessa varianter finns i varsin region av vår arvsmassa. Dessa regioner innehåller gener som är involverade i mitokondriens ATP-produktion.

Vi har även studerat uttrycket av gener som är involverade i cellernas energiproduktion i de Langerhanska öarna. Både gener som kodas från cellkärnans DNA såväl som från mitokondriens DNA studerades. Genom att analysera till vilken nivå dessa gener är uttryckta i patienter med typ 2 diabetes och jämföra dem mot individer som inte har diagnosen diabetes kan man få en bättre förståelse för vad som reglerar dessa geners uttryck och till vilken del de är involverade i sjukdomsutvecklingen. Vi fann att ett flertal gener, som är involverade i mitokondriens ATP-produktion, har lägre genuttryck i de Langerhanska öarna hos patienter med typ 2 diabetes. Vi upptäckte också att ett minskat uttryck av dessa gener bidrog till försämrad insulinfrisättning. De Langerhanska öarna i bukspottskörteln innehåller beta-celler som utsöndrar insulin, och denna vävnad är därmed viktig att studera vid typ 2 diabetes.

Epigenetik är ett fenomen som kan påverka när och hur olika arvsanlag aktiveras. I kontrast till genetiken kan epigenetiska förändringar variera mellan olika celler och över tid. Epigenetiken ändrar inte den genetiska koden, utan involverar istället små ovanpåliggande kemiska förändringar av DNAt som gör att arvsmassan tolkas och uttrycks på olika sätt. DNA-metylering är ett exempel på en så kallad epigenetisk förändring. Det är en reversibel process, där en metylgrupp ($-CH_3$) kan binda till DNA-sekvensen och tas bort igen. Beroende på när och i vilka celler i kroppen DNA-metyleringen sker kan denna epigenetiska förändring påverka hur arvsanlaget uttrycks.

För att få en djupare förståelse för regleringen av arvsanlaget, valde vi att studera hur genetisk variation påverkar DNA-metylering i de Langerhanska öarna hos människa. Vi undersökte hela arvsmassan, det vill säga helgenomsanalys, för att identifiera genetiska variationer som kan påverka bindningen av metylgrupper till DNA-sekvensen. Vi fann att sådan variation påverkar DNA-metylering av flera gener, vilket kan ha betydelse för genuttryck och insulinfrisättning i de Langerhanska öarna.

Sammantaget har vi studerat olika gener och mekanismer som är involverade i typ 2 diabetes, vilket har lett till ökad förståelse av den komplexa bakgrund som bidrar till utveckling av sjukdomen. Genom denna ökade förståelse vill vi bidra till att nya vägar för att behandla eller förebygga typ 2 diabetes utvecklas.

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