Studies of genetic variants in *AHI1*, *UCP2*, and *LMNA* in relation to type 2 diabetes and obesity

Master thesis in biomedicine

by

**Stine Anthonsen**

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Internal supervisor: Louise Torp Dalgaard, Ph.D.
Associate professor, Dept of Science, Roskilde University
External supervisor: Professor Oluf Borbye Pedersen, MD, DMSc and Professor Torben Hansen, MD, PhD, Steno Diabetes Center
Preface and acknowledgement

This master thesis is submitted as part of the science degree in biomedicine at the Roskilde University. The project is elaborated in the period September 2007 to September 2008 in collaboration with Steno Diabetes Center, Gentofte. The thesis is based on original data and the experimental work and genetic analyses included in the thesis were carried out in the laboratory at department of Professor Oluf Perdersen, Md, DmSc at Steno Diabetes Center and in the laboratory of ph.d Louise Dalgaard, associate professor, Dept of Science, Roskilde University.

The study of AHI1 has been published while I have been working on my master thesis. In strong collaboration with the other authors I have performed the analysis and written the paper. In the study about LMNA the RNA-purification and cDNA synthesis was carried out by Lise Wegner before I started my studies. I did the measurements of mRNA expression of lamin A and lamin C.

I would like to thank my external supervisors Professor Oluf Borbye Pedersen MD, DMSc and Professor Torben Hansen, MD, PhD, for the opportunity to work in the research group and for their supervision. Also I would like to thank my internal supervisor Louise Torp Dalgaard for her support for the investigations at Roskilde University. Particularly I would like to thank my daily supervisor at Steno Diabetes Center Lise Wegner for all her support and willingness to help. Finally, I wish to thank the research group for help and creating a pleasant working atmosphere.

Stine Anthonsen, August, 2008
Abstract

The development of type 2 diabetes (T2D) and obesity is a complex interaction of genetics and environmental factors. By identification of susceptible genes, prevention can be approved and personalised treatment might be possible. The aim of this study was to contribute to the identification of genetic variants involved in the development of T2D and obesity.

In this present study three candidate genes, abelson helper integration site 1 (AHI1), uncoupling protein 2 (UCP2), and lamin gene (LMNA) have been investigated for its influence on the development of T2D and obesity. AHI1 encodes the protein jouberin and its function is not clarified. Two variants, rs1535435 and rs9494266, have previously been associated with T2D. In this study their potential associations with T2D have been investigated in 17,521 individuals. UCP2 encodes UCP2 located in the inner mitochondrial membrane and forms a pore through the membrane. This pore is an alternative route for the protons to flux through the membrane and thereby uncouple oxidative phosphorylation instead of synthesizing ATP. The promoter variant rs659366 has previously been studied according to obesity and T2D; however, the results have been inconsistent. In this thesis it was investigated if the rs659366 was associated with obesity in two case-control studies of 3,153 non-obese and 1,550 obese individuals and in 1,567 non-obese and 2,455 obese individuals. Furthermore, it was investigated if rs659366 was associated with T2D in a case-control study of 4,904 T2D cases and 3,214 glucose tolerant controls.

LMNA encodes lamin A and C, they form a polymer that is a part of the nuclear lamina. The LMNA variant rs4641 has shown an association with pre-diabetic traits and a nominal association with T2D. It was investigated in twins if rs4641 was associated with T2D or any T2D-related traits. The rs4641 is located in a splice site in exon 10 and alternative splicing of exon 10 produces two isoforms, lamin A and lamin C. By functional studies it was further investigated if rs4641 influenced the splicing of LMNA by measuring the mRNA expression of lamin A and lamin C and by exon trapping.

The rs1535435 and rs9494266 of AHI1 were not associated with T2D in 17,521 individuals or in a meta-analysis. The UCP2 rs659366 was no associated with T2D or with obesity; however, we found a nominal association with obesity in the ADDITION study. In a meta-analysis we did not find any association with obesity or with T2D. Nevertheless, we found a nominal association with improved insulin sensitivity. We found no association of the LMNA rs4641 with any pre-diabetic traits. Most of the gene expression measurements of lamin A and C did not pass our quality check. Thus it was not possible to draw any conclusion of this study. Unfortunately, it was not possible to fulfill the exon trapping experiment investigating the rs4641 influence on alternative splicing why there are no results from this study.
In conclusion, the rs1535435 and rs9494266 in AH11 were not associated with T2D or any pre-diabetic traits in our populations. The promoter variant in UCP2 was not associated with obesity or T2D in our populations. The LMNA rs4641 was not associated with any pre-diabetic traits in twins and the gene expression ended up with too few individuals to draw any conclusions as well as the exon trapping was not possible to complete why further studies are needed to clarify the effect of rs4641 on alternative splicing of LMNA.
Resume (Dansk)

Udviklingen af type 2 diabetes (T2D) og fedme er et kompleks samspil mellem genetik og miljøfaktorer. Ved at klargjøre den genetiske baggrund kan forebyggelse forbedres og individualiseret behandling blive mulig. Formålet med dette speciale var at medvirke til at identificere genetiske varianter involveret i udviklingen af T2D og fedme. AHI1 kodet for proteinet, jouberin, hvis funktion ikke er klarlagt. To varianter, rs1535435 og rs9494266, er for nylig blevet associateret med T2D. I dette speciale er deres potentielle association blevet undersøgt i 17.521 danske individer. UCP2 kodet for UCP2, som er placeret i den indre mitokondrielle membran, hvor den danner en pore gennem membranen. Dette er en alternativ rute for protonerne, hvorved oxidativ fosforylering afkobles i stedet, for at der syntetiseres ATP, når de passerer igennem membranen. Varianten, rs650366, i UCP2s promotoren er for nylig blevet undersøgt i forhold til fedme og T2D, men resultaterne har ikke været entydige. I dette studie blev det undersøgt, om rs659366 var associeret med fedme i to case-control-studier bestående af 3.153 kontroller og 1.550 fede individer og i 1.567 kontroller og 2.455 fede. Endvidere blev det undersøgt, om rs659366 var associeret med T2D i et case-control-studie bestående af 4.904 T2D-patienter og 3.214 glukosetolerante individer. LMNA kodet for lamin A and C, som sammen danner en polymer, der er en del af nuclear lamina. LMNA-varianten, rs4641, er associeret med pre-diabeteske træk og en nominel association med T2D. I dette studie blev det undersøgt i tvillinger om rs4641 var associeret med T2D eller andre T2D-relaterede træk. Varianten findes i et splice site i exon 10 og alternativ splicing af exon 10 producerer to isoformer, lamin A og lamin C. Ved funktionelle studier blev det undersøgt, om rs4641 påvirkede splicingen af LMNA ved at måle mRNA ekspressionen af lamin A og lamin C samt ved exon trapping.

De to varianter, rs1535435 og rs9494266, i AHI1 var ikke associeret med T2D eller prædiabeteske træk i 17,521 individer eller i en meta-analyse. UCP2 rs659366 var ikke associeret med T2D eller med fedme, men vi fandt en nominel association for G-allelet med fedme i ADDITION. I en meta-analyse fandt vi heller ingen association med præ-diabeteske træk. Derimod fandt vi en nominel association med forbedret insulinfølsomhed. Vi fandt ingen association med LMNA rs4641 med præ-diabeteske træk. De fleste af målerne for geneekspressionsforsøgene af lamin A og C passerede ikke vores kvalitetscheck. Derfor var det ikke muligt at drage nogen konklusioner for dette studie. Desværre var det heller ikke muligt at gennemføre exon trapping-eksperimenterne, hvorfor der ikke er nogen resultater for dette studie. Det kan konkluderes, at rs1535435 og rs9494266 i AHI1 ikke var associeret med T2D eller med nogen præ-diabeteske træk i vores populationer. Promotervarianten i UCP2 var ikke associeret med fedme eller T2D i vores populationer. LMNA rs4641 var ikke associeret med nogen præ-diabeteske træk i tvillinger og studiet af geneekspression endte op med at have for få individer til at drage nogle konklusioner på det. Ligeledes
bled der ikke opnået nogle resultater for exon trapping-experimenterne, hvorfor flere studier behøves for at klargøre effekten af rs4641 på alternative splicing.
Abbreviations

**ABI**: Applied biosystem

**Add**: Additive model

**AGRP**: agouti-related protein

**AHI1**: Abelson helper integration site

**ANOVA**: Analysis of variance

**AUC**: Area under the curve

**BMI**: Body mass index

**CART**: Cocaine and amphetamine-regulated transcript

**CI**: Confidence interval

**DEXA**: Dual-energy X-ray absorption

**DGI**: Diabetes Genetics Initiative

**Dom**: Dominant model

**DZ**: Dizygotic

**ETC**: Electron transport chain

**GDM**: Gestational diabetes

**GOX**: Glucose oxidation (GOX)

**GWA**: Genome wide association

**HDL**: High density lipoprotein

**HGP**: Hepatic glucose production

**HIS**: Histidine

**HLA**: Human leukocyte antigen

**HOMA-IR**: Homeostasis model assessment of insulin resistance

**HOMA-IS**: Homeostasis model assessment of insulin sensitivity

**IFG**: impaired fasting glycaemia

**IGT**: impaired glucose tolerance

**INC**: Incremental

**IRS**: Insulin receptor substrate

**LD**: linkage disequilibrium

**LDL**: Low density lipoprotein

**LEPR**: Leptin receptor

**MAF**: Minor allele frequencies

**MAPK**: Mitogen activated protein kinase

**MC4R**: melanocortin-4 receptor

**MEK**: Map kinase kinase

**MODY**: Maturity-onset of the young

**MZ**: monozygotic

**NF-κB**: Nuclear factor κB

**NGT**: Normal glucose tolerance

**NPY**: Neuropeptide Y

**OGTT**: Oral glucose tolerance test

**OR**: Odds ratio

**PCR**: Polymerase chain reaction

**PI**: Phosphoinositolide

**PI3K**: Phosphatidylinositol phosphate 3 kinase

**PIP**: Phosphatidylinositol phosphate

**PKB**: Protein kinase B

**POMC**: Pro-opiomelanocortin

**Q-PCR**: Quantitative PCR

**QTL**: Quantitative trait loci

**Rd**: Glucose disposal rate

**Rec**: Recessive model

**ROS**: Reactive oxygen species

**SNP**: Single-nucleotide polymorphisms

**T1D**: Type 1 diabetes

**T2D**: Type 2 diabetes

**TNFα**: Tumor necrosis factor

**UCP**: Uncoupling protein

**VLDL**: Very low density protein

**WHR**: Waist to hip ratio

**WT**: Wild type

**WTCCC**: Welcome Trust Case Control Consortium
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1 Introduction

The incidence of type 2 diabetes (T2D) and obesity has reached epidemic proportions due to a marked increase in energy-dense food and a sedentary lifestyle that are some of the main risk factors for T2D and obesity. Because of the increasing incidence of T2D and obesity it is essential to perform basic and clinical research to improve the quality of treatment strategies and prevention. In 2002, an estimated 6.3% of the U.S. population had diabetes, corresponding to approximately 18.2 million people [reviewed in Engelgau et al., 2004]. Approximately 100,000 to 150,000 in Denmark have diagnosed T2D and it is estimated that about the same number have undiagnosed T2D and furthermore, about 10,000 to 20,000 Danish people develop T2D every year [web].

Lately, the prevalence and incidence of T2D have also been increasing explosively in developing countries such as India and China, partly due to an increase in obesity. The lifestyle contributes enormously to the increasing incidence of obesity. Changes in work patterns from heavy labour to sedentary lifestyle with increasing computerisation and mechanisation and improved transportation are just a few of the changes that have had an impact on human health. A busy life often gives rise to an increased intake of more unhealthy, pre-cooked and energy-dense food. Moreover, computer-use and television watching increases the sedentary lifestyle and are often combined with the intake of energy-dense snacks.

The term diabetes mellitus, which in the rest of this report will be referred to as diabetes, covers a group of diseases that have very diverse causes, but are all characterised by chronic hyperglycaemia with disturbances in the metabolism of carbohydrates, fat, and proteins due to defective insulin secretion, insulin action or both. Diabetes was first described in ancient times with the symptoms polyuria, polydipsia, and polyphagia [reviewed in Engelgau et al., 2004]. These symptoms are still valid with polyuria due to high blood glucose levels, polydipsia attempting to compensate for the increased urination and polyphagia because of cell starving to the reason that there is a decreased uptake of glucose and decreased glycogen synthesis.

1.1 Overall aim

The overall aim of this thesis is to contribute to the knowledge of how genetics influence the development of T2D and obesity. By epidemiological and genetic studies variants in the genes \( \text{AHII1}, \text{UCP2}, \) and \( \text{LMNA} \) are investigated to clarify if these variants are associated with T2D and obesity. Furthermore, it is investigated if the \( LMNA \) rs4641 influences the alternative splicing of \( LMNA \) by functional studies.
1.2 Glucose homeostasis

Insulin plays an essential regulatory role in glucose homeostasis by keeping glucose concentrations within narrow limits. Glucose is an important energy source needed to maintain proper function of the body. While low blood glucose concentrations might lead to cellular death, prolonged elevated blood glucose concentrations may result in organ damage. Thus tight regulation of the glucose homeostasis is essential for survival. Two hormones work in opposite direction in the body to regulate blood glucose; insulin and glucagon. In diabetes glucose homeostasis is not properly regulated and the disease is biologically defined as a state where carbohydrate and lipid metabolism are improperly regulated by insulin. This results in elevated fasting and serum glucose concentration that often leads to acute or chronic complications. Knowledge of glucose homeostasis and the action of insulin is essential to understand the pathogenesis of T2D, which this thesis deals with.

1.2.1 Function of insulin

Insulin stimulates the skeletal muscle, fat, and liver cells to take up glucose and store it as glycogen and fat whereas glucagon stimulates the muscle and liver to release glycogen as glucose to the blood. By this regulation of insulin and glucagon fasting plasma glucose concentration remains relatively constant in healthy people at around 5 mmol l\(^{-1}\). Insulin and glucagon are synthesised in the pancreatic islets of langerhans; insulin is synthesised in the β cells and glucagon is synthesised in the α cells. Insulin also activates glycolysis and fatty acid synthesis.

Insulin regulates the plasma glucose concentrations by binding to its receptor, a receptor tyrosine kinase receptor, expressed in almost every tissue, although skeletal muscle, liver and white adipose tissue are regarded as the major organs involved in regulation of glucose homeostasis. The binding activates the intrinsic tyrosine kinase in the cytoplasmic domains resulting in autophosphorylation and phosphorylation of insulin receptor substrates (IRSs) on multiple tyrosine residues. Activation of their molecules triggers the recruitment of multiple downstream signalling proteins, which mediates the unique insulin response depending on the cells or tissues involved [reviewed in Rhodes & White, 2002]. IRS proteins are linked to two main signalling pathways: the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB) pathway, which is responsible for the most of the metabolic actions of insulin, and the mitogen-activated protein kinase (MAPK) pathway, which regulates expression of genes involved in cell cycle and cooperates with phosphatidylinositol-3-kinase (PI3K) pathway to control cell growth and differentiation [Avruch, 1998].

The catalytic subunit of PI3K is activated when it associates with IRS bound to the insulin receptor. Active PI3K phosphorylates the membrane phospholipid phosphatidylinositol bisphosphate 2 (PIP2) to phosphatidylinositol trisphosphate (PIP3). Further activation of downstream effector molecules promotes the intracellular translocation of GLUT-4 to the plasma membrane [Wilcox, 2005]. GLUT-4 is the main...
insulin-responsive glucose transporter in skeletal muscle and adipose tissue, and is therefore crucial for glucose uptake. In the absence of insulin GLUT4 is stored in vesicles inside a lipid bilayer in muscle and fat cells, but when the insulin receptor is activated GLUT4 is translocated to the cell membrane. Insulin increases the rate of GLUT4 vesicle exocytosis and slightly decrease the rate of internalisation, thereby enhancing glucose uptake, and storage of glucose as glycogen or fat [reviewed in Rhodes & White, 2002] (Figure 1.1).

**Figure 1.1.** Insulin signaling through its receptor, mediated by insulin receptor substrate (IRS) leading to conversion of phosphorylated phosphatidylinositol PI (3,4) biphosphate (PIP2) to PIP3, which acts as a co-factor to stimulate pyruvate dehydrogenase kinase and activation of the protein kinase B (PKB). This leads to an activation of transcription in nucleus. The activity of PI3K is opposed by conversion of PIP3 back to PIP2. PIP3 activates protein kinase C (PKC) and by a yet unknown pathway the GLUT4 is translocated to the cell membrane and transports glucose through the cell membrane. IRS also activates the map kinase kinase (MEK) -mitogen activated kinase (MAPK) pathway also leading to an activation of transcription.

### 1.2.2 Glycogen synthesis

When plasma insulin increases, glycogen synthesis becomes predominant; however, a defect in glucose storage is a characteristic finding in all insulin resistant states, including obesity, IGT and in patients with overt diabetes. The synthesis of glycogen in liver and skeletal muscle is a second mechanism of importance in insulin regulated glucose homeostasis. Glycogen synthase kinase-3 is phosphorylated by protein kinase B (PKB) and is thereby inactivated. The glycogen synthase is active in its
dephosphorylated form and is thus indirectly inactivated by PKB. A third event in the regulation of glucose homeostasis is the insulin mediated decrease of hepatic gluconeogenesis. PKB inhibits the expression of gluconeogenic enzymes by regulating the activity of a class of transcription factors [reviewed in Taniguchi et al., 2006]

1.2.3 Synthesis of insulin

The insulin is produced as pre-proinsulin. It consists of a signal sequence and three different parts A, B, and C. The signal sequence is cleaved off and proinsulin makes a specific folding, which is stabilised between the A- and B-chains. The C-chain is cleaved off by a peptidase (figure 1.2). Both the native insulin and the C-peptide are stored in storage granules and they are therefore secreted in equimolar amounts to the blood stream. Because of the equimolar secretion into the blood and because the C-peptide is not removed by the liver, C-peptide can be used to estimate the capacity of the insulin secretion and thereby in diagnosing T2D and insulin resistance [DeFronzo, 1997].

![Figure 1.2 Synthesis of insulin. In the top preproinsulin, in the middle preinsulin and in the bottom insulin [Beta cell Biology Consortium].](image)

1.2.4 Secretion of insulin

Insulin is secreted in discrete pulses into the portal vein in a biphasic pattern and the level of insulin is highly responsive to the prevailing glucose value [Meier et al., 2005]. Insulin release has an early burst within the first 10 minutes followed by a gradually increasing phase of insulin secretion that persist as long as the hyperglycaemic stimulus is present. The first phase insulin response is release of insulin from the granules. The second phase insulin secretion is a prolonged release of newly synthesised insulin [reviewed in Wilcox, 2005].
1.2.5 **Action of insulin**

Glucose is transported into β-cells by the glucose transport protein, GLUT-2, which is primarily expressed in β-cells. In the cytoplasm glucose is converted by glucokinase to glucose-6-phosphate and the ATP/ADP-ratio increases which in turn stimulates the secretion of insulin. This conversion is the first and rate-limiting step in the glycolytic pathway. GLUT-2 expression is tightly regulated in β-cells and markedly decreased in the diabetic state when glucose-stimulated insulin secretion is impaired; however, at some level other glucose transporters, GLUT-1 and GLUT-2, compensate for the absence of GLUT-2 [Thorens, 2006]. The ATP which further is synthesised through oxidative phosphorylation triggers the closure of ATP-sensitive K⁺-channels in the membrane. Furthermore, this leads to depolarisation of the cell membrane with subsequent opening of voltage-dependent Ca²⁺-channels and influx of Ca²⁺. The rise in intracellular Ca²⁺ stimulates the release of insulin from granules in the cells [Rhodes & White, 2006] (Figure 1.3).

![Figure 1.3. Insulin secretion caused by glucose stimulation in β-cells [Thorens, 2006].](image)

### 1.3 Diabetes

Type 2 diabetes (T2D) is the most prevalent form of diabetes; however, a number of other diabetes forms exist. The four main groups of diabetes are T2D, T1D, gestational diabetes and other specific forms of diabetes.

Type 1 diabetes (T1D), also called insulin-dependent diabetes, is due to an autoimmune-mediated destruction of the pancreatic β-cells and the disease has a high heritability. Certain genetic loci are believed to be involved in the pathogenesis; most important is the human leukocyte antigen (HLA) locus, which is a post of the major
Introduction

Histocompatibility complex, important for immune mediated recognition [reviewed in Florez, 2003]. The disease is chronic and progressive and leads to metabolic abnormalities. T1D is one of the most prevalent diseases in childhood and account for about 5-10 % of all cases of diabetes in the U.S [American Diabetes Association, 2004]. Insulin as therapy for T1D was initiated in 1922 and has prevented the majority of deaths due to insulin deficiency abnormalities [reviewed in Castano & Eisenbarth, 1990].

Gestational diabetes (GDM) is a condition in which women without previously diagnosed diabetes have increased blood glucose levels during pregnancy [reviewed in Engelgau et al., 2004], probably due to an impairment of insulin sensitivity, which is believed to be affected by hormones produced during pregnancy. The condition is reversible and it affects approximately 14 % of pregnancies in the U.S [Kim et al., 2002]. No specific causes of GDM have been identified and the disease normally has only a few symptoms. It is most commonly diagnosed by screening during pregnancy by measuring blood glucose levels [reviewed in Engelgau et al., 2004]. Women with GDM have a higher future risk of T2D and children of GDM women have increased risk of complications such as growth abnormalities and low blood glucose levels following delivery. They are also prone to develop obesity with risk of T2D later in life [reviewed in Jovanovic, 2005].

The last group of diabetes includes other specific forms of diabetes such as MODY, diabetes cause by genetic defects in β-cell function and insulin action, and diabetes due to drug-induced diabetes. An example of a monogenic subtype in the group of other specific forms of diabetes is maturity-onset of the young (MODY) with age of onset usually before 25 years. MODY is the most common form in this group and it is an autosomal dominantly inherited disease and is characterised by mild to moderate fasting hyperglycaemia and impaired insulin secretion in response to glucose and other stimuli. Contrary to T2D patients the MODY patients normally do not suffer from insulin resistance [Xu et al., 2005]. A number of mutations in six different genes expressed in β-cells are known to participate in the development of MODY [reviewed in Hansen et al., 2002].

1.3.1 Type 2 diabetes

T2D is the most common form of diabetes, accounting for 90 % of all diabetes cases. It is a chronic multi factorial disease and is a rapidly growing health problem causing increased morbidity and premature mortality. In 2003 it was estimated that 5.1 % of the world’s population suffer form diabetes and that this will increase to 6.3 % in 2025 [Diabetes atlas, second edition, 2003].

T2D is characterised by endocrine dysfunction with abnormalities in insulin action and insulin secretion. T2D is not only a disease among the elderly, overweight individuals but is also seen in younger individuals. This is in part due to the lifestyle in including high intake of energy-dense food and physical inactivity. People that are genetically
predisposed to T2D and who are overweight or obese and/or inactive are at high risk of developing the disease. This T2D arises as a result of complex combinations of many factors such as genetic, environmental factors and physiological dysfunction [reviewed in Kahn et al., 2006].

Insulin resistance is the first detectable sign of T2D characterised by impairment in sensitivity to insulin in the skeletal muscle and/or liver tissue [Zimmet et al., 2001]. To compensate for the insulin resistance the β-cells secrete more insulin, thus maintaining normal or near-normal glucose levels. The insulin secretion will rise but at a certain stage no longer fulfil the requirements, which will lead to hyperglycaemia [reviewed in Kahn et al., 2006; reviewed in Kasuga et al., 2006].

The definition of T2D and the pre-diabetic conditions is based on plasma glucose concentrations after an overnight fast and two hours after 75g oral glucose load, known as an oral glucose tolerance test (OGTT). A fasting venous plasma glucose concentration of less than 6.1 mmol/L and a 2-hour plasma glucose < 7.8 mmol/L is considered as normoglycaemia [WHO, 1999]. Pre-diabetes is divided into two subtypes: Impaired glucose tolerance (IGT) (fasting plasma glucose < 7.0 and 2-hours plasma glucose concentration post glucose load during an OGTT ≥ 7.8 mmol/L) is a condition where the oral glucose load is not well tolerated and causes the plasma glucose to raise more than in normal glucose tolerant individuals but below the diabetic threshold. In impaired fasting glycaemia (IFG) (fasting plasma glucose ≥ 6.1 and 2-hours plasma glucose post glucose load during an OGTT < 7.8 mmol/L), it does not reach the diabetic threshold. Individuals with pre-diabetes are at high risk of progressing to T2D [Knowler, 2002]. T2D is defined as fasting plasma glucose ≥ 7.0 or 2 hours plasma glucose post glucose load during an OGTT ≥ 11.1.

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<tr>
<td>Impaired glucose tolerance</td>
<td>&lt; 7.0 and</td>
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<tr>
<td>Impaired fasting glucose</td>
<td>≥ 6.1 and</td>
<td>&lt; 7.8</td>
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Glucose concentrations are in mmol/l in venous plasma.

The risk of T2D increases with age and about 60 - 90 % of T2D cases are related to obesity. Approximately 20 % of T2D cases are lean and they have a more impaired β-cell function than obese diabetic patients [reviewed in Anderson et al., 2003]. In general, there is 40 - 60 % reduction in the β-cell mass in T2D patients and it is possible to detect the reduction in β-cell function already in the IGT stage [reviewed in Kasuga, 2006].

Insulin sensitivity and β-cell function are proportionally related and their product is referred to as the disposition index, defined as the product of insulin sensitivity and insulin secretion. If the β-cells are able to compensate for increasing insulin resistance...
the disposition index will be constant, why people who are insulin-resistant do not necessarily develop T2D. Both impaired β-cell function and insulin resistance are events that lead to the development of T2D; however, only a minority of T2D patients are diabetic solely due to impaired insulin secretion (Figure 1.3). Likewise, no well-documented cases exist for which impaired insulin secretion is absent and insulin resistance is the immediate cause of T2D [reviewed in Stumvoll et al., 2005]. Environmental causes of insulin resistance are a high-fat diet, decreased physical fitness, increase in visceral fat, smoking, pregnancy, and certain medications.

![Figure 1.4. Relationship between β-cell function and insulin sensitivity. In normal glucose tolerant individuals a hyperbolic relation exists between cell function and insulin sensitivity. When insulin sensitivity decreases, β-cell function will increase. The same relationship exists for impaired glucose tolerant individuals and T2D. NGT-individuals who are at a risk for becoming impaired glucose tolerant represent the upper curve [Stumvoll et al, 2005].](image)

The reduced insulin secretion in T2D can be caused by two different mechanisms; insulin deficiency and disturbed secretion combined with impaired glucose stimulus-secretion coupling. Reduction in insulin secretion can be due to decreased β-cell mass, e.g., caused by hyperglycaemia. Chronic blood glucose concentrations of 5.6 mmol/l or above induce β-cell apoptosis. Normally, β-cells will respond to small elevations in glucose concentrations by secreting more insulin while a higher elevation during a prolonged time-span might lead to proapoptotic signals via the β-cell glucose-sensing pathways. Persistently elevated free fatty acids in individuals predisposed to T2D may contribute to β-cell lipotoxicity progressing to β-cell failure and free fatty acids will inhibit glucose-induced insulin secretion and insulin gene expression. Free fatty acids are fatty acids that are not bound or attached to other molecules, such as triglycerides or phospholipids. In addition, the chronic increase in inflammatory mediators observed in T2D might not only affect insulin-sensitive tissues and blood vessel walls but could also affect pancreatic β-cells [reviewed in Wajchenberg, 2007].
Long-term hyperglycaemia induces the generation of reactive oxygen species (ROS) as the electron transport chain is overloaded resulting in chronic oxidative stress. Especially, the β-cells are exposed to damage of ROS as the level of antioxidant enzymes in the β-cells is slow. ROS can also be generated through other pathways such as the polyol pathway and hexosamine pathway both suggested to upregulate protein kinase C (PKC). Through the pathway of activation of PKC, ROS is also produced [reviewed in Rolo & Palmeira, 2006].

Prevention of T2D can be accomplished by physical activity and wholesome nutrition but requires a public health approach with major changes in society [Zimmet et al., 2001]. Physical activity is a very important approach in prevention as it increases the amount of the glucose transporter, GLUT-4 which transports glucose across the membrane and decreases blood glucose levels and adipokines.

T2D is often associated with chronic complications such as microvascular neuropathy, nephropathy and retinopathy and macrovascular accelerated atherosclerosis resulting in myocardial infarction and stroke. Furthermore, diabetic patients have poor wound healing, particularly of the feet, which can lead to gangrene and may require amputation [reviewed in Zgonis et al., 2008].

1.4 Obesity

There is a strong link between T2D and obesity as the release of pathogenic adipocyt factors such as free fatty acids and adipokines including pro-inflammatory cytokines is increased in obesity which decreases the insulin sensitivity of other metabolic tissue [reviewed in Bays et al., 2008; reviewed in DeFronzo, 2004]

The prevalence of obesity in developed as well as developing countries has increased enormously during the last two decades and is now considered one of the most serious health issues [Warren et al., 2004]. This is due to more plentiful and energy-dense food and physical inactivity. Obesity is caused by a positive energy balance due to excessive energy intake compared with energy expenditure.
1.4.1 Definition and measurement of obesity

Body mass index (BMI) is most often used to measure the degree of obesity. This surrogate gives a prediction of the degree of obesity as it has a good correlation with obesity. BMI is defined as weight (kg)/ height (m)$^2$. WHO has classified weight into five categories as shown in Table 1; however, BMI does not account for the body composition or the fat distribution, which means that a body builder rich in muscle mass, which has a higher density, has a high BMI and would be classified as overweight or even obese according to WHO definitions.
Obesity with visceral fat accumulation associates closely with diabetes, hyperlipidemia, hypertension, and atherosclerosis [reviewed in Okamoto et al., 2006]. Thus, knowledge of the fat distribution accumulation is important for optimisation of BMI measurements, therefore it is advantageous to use BMI in conjunction with waist circumference or the ratio of waist-to-hip as these anthropometric measures correlate well with intra-abdominal fat mass [Deurenberg & Yap, 1999]. A more precise method for measurements of body composition is bio-impedance, which measures the impedance or opposition to the flow of an electrical current through the body. Impedance is low in lean tissue, where intracellular fluid and electrolytes are primarily contained, but high in adipose tissue. Thus, impedance is proportional to body water volume. Lean body mass is estimated using an assumed hydration fraction for lean tissue. Fat mass is calculated as the difference between body weight and lean body mass [reviewed in Mattsson & Thomas, 2006].

Dual-energy X-ray absorptiometry (DEXA) is an enhanced form of X-ray technology and it is more accurate in estimating fat mass than bio-impedance. DEXA measures the whole body composition by sending a thin beam of low-dose X-rays with two distinct energy peaks through the body of the person being examined. One peak is absorbed mainly by soft tissue and the other by bone. The soft tissue amount can be subtracted from the total and what remains is a patient’s bone mineral density. [reviewed in Mattsson and Thomas, 2006].

**Table 1.2. Classification of weight categories according to BMI**

<table>
<thead>
<tr>
<th>Classification</th>
<th>BMI (kg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Under-weight</td>
<td>&lt;18.5</td>
</tr>
<tr>
<td>Normal-weight</td>
<td>18.5-24.99</td>
</tr>
<tr>
<td>Over-weight</td>
<td>≥ 25</td>
</tr>
<tr>
<td>Obese</td>
<td>≥ 30</td>
</tr>
<tr>
<td>Morbidly obese</td>
<td>≥ 40</td>
</tr>
</tbody>
</table>
1.4.2 Prevalence of obesity

Almost one in three adults in the United States are currently defined as clinically obese with a BMI above or equal to 30 kg/m². The prevalence in other developed countries is approaching the same level. Furthermore, the developing countries are adopting the lifestyle of the developed countries and thus have a very high increase in the incidence of obesity. This world-wide expansion in obesity may be considered to be a pandemic [reviewed in Bell et al., 2005]. The WHO estimated that in 2005 around 1.6 billion adults (age 15 years or above) were overweight, at least 400 million adults were obese, and at least 20 million children under the age of 5 years were overweight. If this projection continues: 2.3 billion adults will be overweight and at least 700 million adults will be obese in 2015. Already more people are currently overweight than underweight [WHO, 1999]. The economic burden of obesity is enormous and in combination with its human costs it is one of the most urgent issues in medicine today [Bell et al., 2005]. In Denmark are 33 % of the people overweight whereas 11 % are obese [Ekholm et al., 2005].

1.4.3 Adipose tissue as an endocrine organ

The adipose volume in the body is determined by the balance between lipogenesis and lipolysis. Lipolysis is the breakdown of fat stored in fat cells and free fatty acids are released into the blood stream. The central engine of differentiation of adipose tissues is the peroxisome-proliferator-activated-receptor-γ (PPAR-γ). When it is activated by an agonist ligand in fibroblastic cells it activates a full program of differentiation including morphological changes, lipid accumulation and the expression of almost all genes that are characteristic of fat cells [Rosen & Spiegelman, 2006].

A gain in adipose tissue can result from hypertrophy, hyperplasia or a combination. The number of adipocytes reflects the ability to recruit adipocyte precursors to mature into adipocytes [Drolet et al., 2008].

Two types of adipose tissues exist, brown adipose tissue and white adipose tissue. Excess energy is mainly stored as triglycerides in white adipose tissue and it consists of single lipid droplets. Brown adipose tissue only exists in mammals and is the main fat storage in rodents. Its main function is to generate heat why it is present in newborn humans. The generation of heat without concomitant ATP is made by uncoupling proteins (UCPs), mainly UCP-1, which uncouples the proton gradient and leaks protons through the inner mitochondrial membrane -an alternative route than the ATP synthase [reviewed in Rosen & Spiegelman, 2006]. Brown adipose tissue contains numerous lipid droplets and is rich in mitochondria and it contains more capillaries than white adipose tissues because it has a greater need for oxygen and a high number of mitochondria [reviewed in Duncan et al., 2007].

Adipose tissue functions as an endocrine organ and is required for normal insulin sensitivity and glucose homeostasis. It is important that the size of the adipose tissue
does not enlarge too much as it then secretes execs amounts of secretory proteins and thereby disturbs the homeostasis of glucose and energy balance. Approximately 20-30 %, of the genes expressed in subcutaneous and visceral adipose tissue, are bioactive secretory proteins i.e. adipocytokines, such as tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), and the hormones leptin, resistin, and the most abundant one, adiponectin (Figure 1.5). Adipocytes metabolize only four to five percent of circulating glucose; however the adipokines, including pro-inflammatory cytokines, decrease insulin sensitivity in other metabolic tissues. Expansion of adipose tissue and especially hypertrophy of adipocytes leads to an increase release of pathogenic adipocyte factors including free fatty acids [Rosen & Spiegelman, 2006].

![Figure 1.5. Selected substances released from the human adipocyte.](image)

### 1.4.4 Lipid metabolism

Lipogenesis encompasses the processes of fatty acid synthesis and subsequently triglyceride synthesis from glucose. Triglycerides and cholesterol are transported in lipoproteins, there are four different types of lipoproteins: Chylomicrons, very low density proteins (VLDL), low density protein (LDL), and high density proteins. Chylomicrons, VLDL, and LDL transport cholesterol from the liver to the bloodstream whereas (HDL) transport cholesterol and triglyceride to the liver from the bloodstream. LDL is referred to as the bad cholesterol as it is strongly associated with atheromatous disease and HDL is referred to as the good cholesterol as it transports cholesterol to the liver for excretion away from the blood. Triglycerides can be stored in the liver and adipose tissue and when energy is needed glycagon signals the breakdown of triglycerides to free fatty acids. The brain can not use free fatty acids as an energy source; therefore, the fatty acids are converted to glucose by the

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Page 13
glyconeogenesis. Except from the brain, cells can take up the free fatty acids by a fatty acid transporter and utilize the energy.

### 1.4.5 Inflammatory states of obesity

Adiponectin, leptin, and resistin regulate a number of metabolic processes including glucose regulation and fatty acid catabolism. There is overwhelming evidence that obesity and type 2 diabetes are inflammatory states. The production of TNF-α, IL-6, and resistin are contributing to the inflammation [reviewed in Rosen & Spiegelman, 2006 and in Okamoto et al., 2006] and bone marrow derived macrophages are recruited to the fat pad which also indicates an inflammatory state [reviewed in Okamoto et al., 2006]. There are conflicting results of the inflammation in obese people suggesting that TNF-α and IL-6 are contributing to insulin resistance but also that they are contributing to insulin sensitization [Carey et al., 2006; Rotter et al., 2003]. The cytokines can promote the insulin resistance through several mechanisms, among those, serine phosphorylation of insulin substrate, inhibitor κB kinase activation, and production of ROS [Reviewed in Rosen & Spiegelman, 2006].

### 1.4.6 Obesity and insulin resistance

Liver insulin resistance leads to increased gluconeogenesis and thus increased glucose output because gluconeogenesis fails to be inhibited by insulin. Insulin resistance in skeletal muscles results in decreased uptake of glucose as the muscles do not respond to the insulin stimulation. Because the insulin does not exert its antilipolytic effect the insulin resistance leads to increased release of fatty acids from the adipose tissues [Rosen & Spiegelman, 2006], which are taken up by other metabolic tissues such as liver and skeletal muscle. It is suggested that mitochondrial-derived by-products of lipid oxidation may have a key role in the development of insulin resistance in skeletal muscles. Chronic exposure to elevated lipids in skeletal muscle induces an increase in the expression of genes in fatty acid oxidation; however, it is not coordinated with upregulation of tricarboxylic acids and the electron transport chain, which lead to incomplete metabolism of fatty acids in the β-oxidation pathway and accumulation of lipid-derived metabolites in the mitochondria. In liver the elevated exposure to free fatty acids and the impairment of free fatty acids oxidation lead to hepatic steatosis which further leads to glucose intolerance [reviewed in Muoio & Newgard et al., 2008].

### 1.4.7 Regulation of energy intake

Leptin is indicated to be a sensor for fat mass and acts in a negative feedback loop which regulates the amount of fat in relation to a sudden amount of fat storage via the central nervous system. In the model an increase in energy intake will induce a release of leptin which again will inhibit food intake. The model is well established in rodents, but in human there are still obscure parts to understand the precise mechanisms [Oswal & Yeo, 2007].
Leptin acts through different pathways. Leptin regulates appetite via its main pathway, the melanocortin pathway, shown in figure 6. This pathway includes the direct action of leptin on two descent classes of neurones; one class co-expresses the anorexigenic peptides pro-opiomelanocortin and cocaine and amphetamine-regulated transcript (CART) which reduces food intake while the other classes of neurones co-expresses the orexigenic peptides neuropeptide Y (NPY) and agouti-related protein (AgRP) which reduce satiety and increase food intake [Oswal & Yeo, 2007]. By acting through the central neural pathway combined with direct activation of AMP-activation of AMP-activated protein kinase, leptin improves insulin sensitivity in muscles and it also reduces intra-myocellular lipid levels [reviewed in Rosen & Spiegelman, 2006]. Leptin given to obese people as a drug is not inducing weight loss indicating that obese people are resistant to leptin [reviewed in Oswal and Yeo, 2007]. Leptin activates the PI3K pathway which also plays an important role in regulating energy homeostasis. The activated leptin receptor stimulates the tyrosine phosphorylation of IRS proteins by. Afterwards IRS recruits, phosphorylates, and activates phosphoinositide (PI) 3 kinases, leading to downstream signalling [Oswal & Yeo, 2007].

In opposite to leptin ghrelin stimulates appetite by stimulating the NPY/AGRP neuron and inhibit the POMC/CART neurons. Ghrelin is down regulated in obese people, which is probably a consequence of elevated insulin or leptin, because fasting plasma ghrelin levels are negatively correlated with fasting plasma levels of insulin and leptin. It is suggested that the decrease in plasma Ghrelin concentrations observed in obesity represents a physiological adaptation to the positive energy balance with obesity (Figure 1.6) [Tschop et al., 2001].

Insulin circulates at levels proportional to body fat mass and controls meal size via inhibition of POMC/CART neurons [Schwartz et al., 2000]. The hepatic glucose production in the fasting states is promoted by the counter regulatory hormones glucagon, adrenalin, and corticosteroids. The brain coordinates many of these effects through direct and indirect intake, glucose sensing, and neural outputs to peripheral organs [Reviwed in Rosen & Spiegelman, 2006].
Figure 1.6. Regulation of energy balance in the hypothalamus. Peripheral signals from leptin, insulin, and ghrelin affect neuro peptide Y (NPY) / agouti-related peptide (AGRP) and pro-opiomelanocortin (POMC) / cocaine- and amphetamine-regulated transcript (CART) neurons. These signals in the neurons and interaction with other neurons change food intake and energy expenditure.

1.5 The metabolic syndrome

The metabolic syndrome is a cluster of cardiovascular risk factors including visceral obesity, dyslipidaemia, hyperglycaemia, and hypertension. Obesity and particularly visceral obesity is commonly associated with the development of the metabolic syndrome, which is considered to be one of the major public health inconveniences. Many behavioural factors are involved including a lifestyle of inactivity and food abundance. It is difficult to determine the primary contributor to the metabolic syndrome as all the risk components are closely interrelated. The importance of the metabolic syndrome is that it helps to identify individuals at high risk of cardiovascular diseases [Alberti et al., 2005]. There have been many suggestions to the diagnostic criteria since the metabolic syndrome was defined; however, there have been general consensus regarding the core components of the syndrome which features obesity, especially visceral obesity, insulin resistance, dyslipidaemia, and hypertension. In many of the metabolic cases medical treatment is needed; however, no specific treat-
ment exists hence individual medicine directed against the specific abnormalities is used.

1.6 Genetics

Knowledge of the human genetics underlying metabolic diseases is very important for the understanding of biological pathway as a diagnostic approach may also be helpful when optimising the treatment of patients. Much work is put into identifying new genetic variants involved in the development of common diseases such as T2D. Even though genetics play an important role in the development of T2D, it is a set of complex interactions between genetic and environmental factors that determine whether the phenotype of the disease is expressed.

Two human genomes of approximately 3 billion nucleotides are roughly 99.9 % identical leaving only about 0.1 % of the nucleotides attributable to genomic differences among individuals [Kruglyak & Nickerson, 2001]. The most abundant source of known genetic variation in the genome is single-nucleotide polymorphisms (SNPs) defined as one-base variation in the DNA sequence with an allele frequency of > 1% in a given population. Approximately 10 million SNPs exist in the human population, which is about one site per 300 bases [Kruglyak & Nickerson, 2001]. Sequencing of the human genome provided the location of 2.1 million SNPs with less than 1 % of them resulting in variations in proteins [Venter et al., 2001]; nevertheless, it is estimated that 10-11 million SNPs exist with a minor allele frequency of at least 1 % [The International HapMap Consortium, 2007].

Other forms of genetic variation are large-scale and small-scale insertions and deletions, inversions, mini-and micro satellites and copy number variants [Feuk et al., 2006]. SNPs are distributed throughout the whole human genome; however, many of the variants are synonymous and thus do not change any amino acids. Other SNPs are non-synonymous and result in amino acid substitution. This thesis includes studies of two SNPs located in an intronic region of abelson helper integration site (AHI1), a SNP located in the promoter region of (UCP2), and a SNP placed in a splicing site of LMNA.

1.6.1 Approaches to identify genetic variants predisposing to diseases

Only a decade ago linkage analysis and the candidate gene approach were the common methods applied to identify potential susceptibility variants associated with diseases. These approaches led to the identification of a large number of potential candidate genes and quantitative trait loci (QTL). Technologies have led to a new era where up to $10^6$ genetic markers are analysed on a single chip. During this year genome wide association (GWA) studies have led to a deeper understanding of the genetics of many common diseases. Genes for obesity and T2D have been identified; however, not all these associations have yet been validated. It is expected that more
genes associated with T2D or obesity will be identified in the near future [Li & Loos, 2008].

**The candidate gene approach**

The candidate gene approach identifies susceptibility genes based on knowledge of their biological function or genomic localisation. Genes thought to be involved in the disease are identified based on data from animal models, cellular systems, or extreme forms of the trait in question. Genetic variants are then tested for association with a trait of interest at the population level [Li & Loos, 2008].

**Linkage studies**

In genome-wide linkage studies whole genomes are examined often using microsatellites in order to identify genetic markers that co-segregate with a specific trait. The approach is hypothesis-free and aims to identify the approximate location of genomic regions that co-segregate with a certain trait. Thus the study requires related individuals such as siblings, nuclear families or extended pedigrees. When a region is identified, it is subsequently fine-mapped in order to determine more precisely which specific variants co-segregate with the disease. Linkage studies have been successful for identifying Mendelian disorders, such as MODY; however, they are not suited for detecting common low-penetrance variants believed to be relevant for multifactorial diseases as T2D [reviewed in Lindgren & McCarthy, 2008]. Regions showing significant linkage to inflammatory disease, schizophrenia, and T2D have been identified [reviewed in Hirschhorn & Daly, 2005]. A recent meta-analysis combining 27 linkage studies including data of more than 10,000 families and more than 31,000 individuals of European origin could not explicitly identify a single obesity or BMI locus [Saunders et al., 2007]. Linkage studies have successfully been used for identification of rare monogenic forms of diseases and have been used for identifying of the most validated T2D variants in *TCF7L2* [Cauchi & Froquel, 2008].
Genome-wide association studies

- Selection of a large number of cases and controls
- Genotyping of up to one million variants
- Selection of the top-ranked variants and replication of those in larger case-control studies
- Identification of the variants that remain significant
- Validate the variants in other larger studies

The genome-wide association (GWA) study is a relatively novel approach to identify genetic disease susceptibility variants [Li & Loos, 2008]. Like linkage studies GWA studies are hypothesis-free. The method is very effective as up to 1.5 million SNPs are studied on each chip. GWA studies use SNPs with a minor allele frequency of above 5% and the method has already resulted in the identification of a number of novel variants being associated with different diseases (Figure 1.7) [reviewed in Li & Loos, 2008]. In GWA studies thousands of statistical tests are performed as a result of multiple genetic variants, multiple phenotypes and multiple statistical models including different assumptions regarding the mode of inheritance [reviewed in Salanti et al., 2005]. Thus correction for multiple testing is necessary and usually a very stringent genome-wide significance criteria is required, usually at the significance level of below $10^{-7}$ [Frayling et al., 2007c; reviewed in Seng & Seng].

Case-control studies

Case-control studies compare a group of cases, ascertained for a trait of interest which is presumed to have a high prevalence of susceptibility alleles for the phenotype, with a group of control individuals, not ascertained for the trait and who are considered likely to have a lower prevalence of such risk alleles [McCarthy et al., 2008]. In genetic case-control studies the allele frequency (or haplotype, i.e. a combination of alleles at multiple loci that are transmitted together on the same chromosome) is compared between cases and control individuals. If there is a significant difference in the allele frequency it indicates that this genetic marker may increase the risk or likelihood of the trait or be in high LD with another variant increasing the risk. It is important to include many individuals, the more the better [Li & Loos, 2008]. Furthermore, it is important to match the cases with the controls.

Figure 1.7. Overview of genome wide association study
minimizing the effect of confounders which might lead to false association or missed association [reviewed in Risch, 2000].

**Twin studies**

Twin populations represent some of the best resources for evaluating the importance of genetic variation in susceptibility to disease. MZ twins share all genetic material except for genes for T-cell receptors and immunoglobulins. DZ twins share in average half of their genes. When study population of twins contain both MZ and DZ twins the contribution of genes can be investigated by means of intra-class correlations in MZ and DZ twins. Twin studies allow for control of the influence of genetic factors. Since MZ twins are genetically identical correlation performed between with-in-twin pair differences in phenotypes any association found, is of non-genetic origin. If a trait is highly heritable, MZ twins will be more similar than DZ twins as regards the particular trait. Twins are also excellent for studying the significance of environmental factors on the phenotype.

Studies of twins have shown that genetic as well as environmental factors influence glucose metabolism and insulin sensitivity. In comparison of elderly MZ and DZ, MZ twins had significantly higher plasma insulin during an OGTT. These findings are probably due to an adverse intrauterine environment for MZ twins compared to DZ twins [Poulsen et al., 1999]. This agrees with the finding of lower birth weight for type 2 diabetic twins compared to their non-diabetic co-twin [Poulsen et al., 1997].

**Functional studies**

Genetic association studies are important tools for identifying variants predisposing for diseases such as T2D; however, after replication in independent study samples re-sequencing of the locus and additional association studies are performed. By performing association studies e.g. genome wide association studies, variants might be identified to be associated with a certain trait; however, it is often difficult to determine the exact causative variant. Often a few variants in high LD are identified and the last step of proof for a true association may involve functional studies. Functional studies can be performed by different study designs. In the thesis functional studies of LMNA were performed. Different assays might be used for different hypothesised function. It may involve physiological studies of the impact of the variant on the whole body human level or if the effect size is large enough to do studies in animals; e.g. in transgenic or knock-outs.

**HapMap**

Data from the International HapMap consortium and the completion of the reference sequence of the human genome have been crucial for the performance of GWA studies. The International HapMap Project is an organisation that started with the purpose of creating a haplotype map of the human genome, HapMap, describing the
common patterns of human genetic variation [The International HapMap Project, 2003].

It has become a key resource for researchers aiming to find genetic variants affecting health, disease, and responses to drugs and environmental factors. The publishing of the data has so far comprised three phases; the complete data obtained in phase I by genotyping of one common SNP every 5,000 nucleotides including more than one million SNPs were published in 2005 [The International HapMap Project, 2005] with the following data of more than 2.1 million SNPs from phase II published in 2007 [The International HapMap Project, 2007] and recently the Phase III draft from the HapMap has been released (Box 1.1).

To obtain enough SNPs to create the HapMap a lot of re-sequencing was undertaken. Additional SNP data were submitted to the public dbSNP database. In August, 2006, there were more than ten million SNPs in the database with more than 40% being polymorphic [NCBI]. The Hapmap Project has provided important knowledge of common genetic variants and the linkage disequilibrium (LD) (Box 1.2) structure and this has facilitated impressive progress in the gene-disease mapping strategy [Li & Loos, 2008]. Knowledge of the LD structure makes it possible to select tag SNPs that represent the whole region of interest. Instead of genotyping all SNPs in a region, only some selected tag SNPs are genotyped with the advantage of saving time and money (Figure 1.8). When the association study is performed it is therefore not known if it is the tag SNP itself or the SNPs that it represents that is causative [reviewed in Hirschhorn & Daly, 2005].

**Box number 1.1 HapMap genotyping**

The genotyping in HapMap was done in 270 individuals from four populations:

1) Utah residents with Northern and Western European ancestry (n = 90)  
2) Yoruba people from Nigeria (n = 90)  
3) Han Chinese from Beijing (n = 45)  
4) Japanese from Toyko (n = 45)

**Box 1.2. LD**

LD occurs when two or more genetic variants are collectively inherited more often than by change. Two polymorphic sites are said to be in LD when their specific alleles are correlated in a population. The LD between many neighboring SNPs generally persists because meiotic recombination does not occur at random but is concentrated in recombination hot spots [Gabriel et al., 2002]. The degree of LD is estimated by $r^2$ or $D'$, which are both ranging from 0, indicating no LD, to 1, indicating that the two SNPs are in complete LD [reviewed in Manolio et al., 2008].
1.6.2 Advantages and disadvantages of different study strategies

Linkage analysis and association studies each have their own strengths. Linkage analyses are more powerful than association analysis when identifying rare risk alleles with high penetrance. Furthermore, the analysis is not restricted to already identified SNPs or genes as the analysis results in the narrowing of a chromosomal region [Carlson et al., 2004].

Association analyses are more powerful for detection of common disease alleles that confer modest disease risk because the pattern of allele sharing between unrelated individuals is more striking than the pattern of alleles sharing among affected individuals within pedigrees (Figure 1.9) [Carlson et al., 2004].
One obstacle for linkage studies is the late onset of T2D which prevents the collection of multigenerational families. Candidate gene studies will remain a valuable approach because they allow for more detailed analyses of biologically relevant candidates in interaction with other genes and environmental factors. GWA studies require higher density of the genotyped SNPs than of the micro satellite markers in the linkage analysis. A GWA study requires hundreds of thousands of SNPs while linkage studies generally require fewer than 500 markers across the genome [Carlson et al., 2004].

1.6.3 Methodological issues

Performing an association study aiming to identify genetic variants predisposing to a disease, the study has to be well powered as it reduces the risk of false positives. The power of a study is the probability of successfully detecting an effect of a particular size. Power depends primarily on the magnitude of the effect, upon sample size, the strength of LD with a marker, the frequency of the disease susceptibility allele and the required level of statistical significance [Purcell et al., 2003; Palmer & Cardon, 2005]. Furthermore, inadequate phenotype characterisation, genotyping errors, effect size of the susceptibility locus influences the results of the study [Zondervan & Cardon, 2004].

GWA studies that include a large number of individuals have a high power; however, the change of obtaining false positives is still present. On the other side it is also
possible in GWA studies to miss true findings. When replication of an initial study fails to be replicated this might be due to false-positive finding (type 1 error) or false-negative associations (type 2 error) in the replication study [Lohmueller et al., 2003].

Meta-analyses are studies that combine results and estimate an overall risk. This way of raising the total number of individuals is a way of overcoming lack of statistical power. Unfortunately, heterogeneity between study populations is a problem. The degree of heterogeneity between the studies included in a meta-analysis is tested which might result in a high degree of heterogeneity and the meta-analysis is not reliable. Moreover, the risk of publication bias might be present [Lohueller et al., 2003] and a meta-analysis should only be considered an alternative and not a substitution for a well-designed initial association study.

Finally, epistasis, the synergistical effect of two or more loci on the susceptibility for a trait, is very important when examining the genetic mechanism underlying a complex disease. Thus, if epistasis is present the impact of a susceptibility locus on a trait may be masked or altered by the effect of another locus which may lead to a reduction in the statistical power to detect the true susceptibility locus [Cambien & Tiret, 2007].

### 1.6.4 Genetic variants predisposing to type 2 diabetes

Given the high heritability and public health importance of T2D, the identification of the underlying genes has been a high-priority mission of the scientific community for many years as it may provide information of importance for prevention of the disease and improve the treatment.

Genes predisposing to T2D can affect the progression of the disease at many stages. Variants interacting with risk factors for T2D such as smoking, low birthweight or obesity may also have an impact on T2D; however, genes directly involved in the pathogenesis of the disease are more intensively studied. Among these are genes involved in insulin resistance and insulin secretion. The heritability of insulin secretion is 67 % [Elbein et al., 1999]. Several studies of the insulin secretory capacity of glucose-tolerant individuals with predisposing ethnicity or a family history of T2D have indicated that β-cell dysfunction occurs in genetically predisposed individuals with normal glucose tolerance well before the onset of overt diabetes [Wajchenberg, 2007].

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**Box number 1.3. Heriability**

Heritability is the proportion of phenotype variation in a population that is attributable to genetic variation among individuals. Variation among individuals may be due to genetic and/or environment factors [Kempthorne 1957].
At present 17 genes are considered to be validated T2D susceptible genes. Four of these, PPAR, KCNJ11, TCF2, WFS1 have been identified using candidate gene approach [for references see Table 1.2] and only KCNJ11 and PPAR have been consistently replicated in GWA studies as well [Saxena et al., 2006]. TCF7L2 having the strongest diabetogenic effect has been identified by fine-mapping of linkage peak [Grant et al., 2006]. The odds ratio (OR) per allele for TCF7L2 is substantially higher than for the other identified variants. Thus, TCF7L2 is the most important T2D locus identified to date [reviewed in Frayling et al., 2007b]. It seems that the predominant effect of the majority of genes involved in T2D is to disrupt the pancreatic β-cell function rather than influencing insulin sensitivity [reviewed in Lindgren & McCarthy, 2008]. The remaining genes have been identified in GWA studies and subsequent meta-analyses. Many of the genes are involved in β-cell function and not in insulin action. The contribution of each common variant is modest (OR = 1.1 - 1.4), and identified variants explain only a fraction of the disease pathogenesis. The genes and/or regions shown to be associated with T2D are listed in (Table 1.2).

Table 1.2. Confirmed gene regions involved in T2D.

<table>
<thead>
<tr>
<th>SNP/ type of variant(s)</th>
<th>Gene Major/mi -nor allele</th>
<th>Discovery method</th>
<th>Function / Characteristics</th>
<th>Odds ratio (per allele)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs1801282/ Non-synonymus</td>
<td>PPAR C/G</td>
<td>Candidate -gene study</td>
<td>Transcription factor involved in adipocyte differentiation and function</td>
<td>1.14 (1.08-1.20)</td>
<td>Deeb et al. 1998; Scott et al. 2007; WTCCC 2007; Saxena et al. 2007</td>
</tr>
<tr>
<td>rs5215/ Non-synonymus</td>
<td>KCNJ11 T/C</td>
<td>Candidate -gene study</td>
<td>Subunit of the ATP-sensitive K⁺ channel, crucial for glucose- induced insulin secretion</td>
<td>1.14 (1.10-1.19)</td>
<td>Gloyn et al. 2003; Nielsen et al. 2003; Scott et al. 2007; WTCCC 2007, Saxena</td>
</tr>
<tr>
<td>SNP/ type of variant(s)</td>
<td>Gene Major/mi-nor allele</td>
<td>Discovery method</td>
<td>Function / Characteristics</td>
<td>Odds ratio (per allele)</td>
<td>References</td>
</tr>
<tr>
<td>------------------------</td>
<td>--------------------------</td>
<td>------------------</td>
<td>---------------------------</td>
<td>------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>rs7901695/ Intronic</td>
<td>TCF7L2 C/T</td>
<td>Fine-mapping of linkage peak</td>
<td>Transcription factor influencing on insulin and glucagon secretion</td>
<td>1.37 (1.31-1.43)</td>
<td>Grant et al. 2006; Scott et al. 2007; WTCCC 2007</td>
</tr>
<tr>
<td>rs4430796/ Intronic</td>
<td>TCF2 G/A</td>
<td>Candidate gene study</td>
<td>Transcription factor involved in pancreatic development</td>
<td>1.10 (1.07-1.14)</td>
<td>Gudmundsson et al. 2007; Winckler et al. 2007</td>
</tr>
<tr>
<td>rs10010131 / Intronic</td>
<td>WFS1 G/A</td>
<td>Candidate gene study</td>
<td>Critical for survival and function of pancreatic β-cell</td>
<td>1.11 (1.08-1.16)</td>
<td>Sandhu et al. 2007</td>
</tr>
<tr>
<td>rs5015480 / rs1111875/ Intergenic</td>
<td>HHEX-IDE C/T</td>
<td>GWA</td>
<td>HHEX, involved in pancreatic development; IDE, may affect insulin action or secretion</td>
<td>1.15 (1.10-1.19)</td>
<td>Sladek et al. 2007; Scott et al. 2007; Saxena et al. 2007; Zeggini et al. 2007</td>
</tr>
<tr>
<td>rs13266634 / Non-synonymous</td>
<td>SLC30A8 C/T</td>
<td>GWA</td>
<td>Zinc transporter expressed in secretory vesicles of β-cells, implicated in the final stages of insulin biosynthesis</td>
<td>1.15 (1.12-1.19)</td>
<td>Sladek et al. 2007; Scott et al. 2007; Saxena et al. 2007; Steinthorsdottir et al. 2007; Zeggini et al. 2007</td>
</tr>
<tr>
<td>rs10946398 / Intronic</td>
<td>CDKAL1 A/C</td>
<td>GWA</td>
<td>Possible effect on pancreatic β-cell development, regeneration and function</td>
<td>1.14 (1.11-1.17)</td>
<td>Scott et al. 2007; WTCCC 2007; Saxena et al. 2007; Zeggini et al. 2007</td>
</tr>
<tr>
<td>rs10811661 / rs64398/ Intergenic</td>
<td>CDKN2A-2B T/C</td>
<td>GWA</td>
<td>Possible effect in pancreatic β-cell development, and function</td>
<td>1.20 (1.14-1.25)</td>
<td>Scott et al. 2007, WTCCC 2007; Saxena et al. 2007; Zeggini et al. 2007</td>
</tr>
<tr>
<td>rs4402960/ Intronic</td>
<td>IGFBP2 G/C</td>
<td>GWA</td>
<td>Involved in translation of IGF2 involved in development growth and stimulation of insulin action</td>
<td>1.14 (1.11-1.18)</td>
<td>Scott et al. 2007; WTCCC 2007; Saxena et al. 2007; Zeggini et al. 2007</td>
</tr>
<tr>
<td>rs9939609/ Intronic</td>
<td>FTO T/A</td>
<td>GWA</td>
<td>Affects type 2 diabetes via obesity</td>
<td>1.17 (1.12-1.22)</td>
<td>Frayling et al. 2007a; Scuteri et al. 2007; Hinney et al. 2007; Scott et al. 2007</td>
</tr>
<tr>
<td>rs864745/ Intronic</td>
<td>JAZF1 T/C</td>
<td>Meta-analysis of GWA</td>
<td>The biological link to type 2 diabetes is unknown</td>
<td>1.10 (1.07-1.13)</td>
<td>Zeggini et al. 2008</td>
</tr>
<tr>
<td>rs12779790 /Intergenic</td>
<td>CDC123-CAMK1D A/G</td>
<td>Meta-analysis of GWA</td>
<td>The biological link to type 2 diabetes is unknown</td>
<td>1.10 (1.07-1.14)</td>
<td>Zeggini et al. 2008</td>
</tr>
<tr>
<td>rs7961581/ Intronic</td>
<td>TSPAN8-LGR5 T/C</td>
<td>Meta-analysis of GWA</td>
<td>The biological link to type 2 diabetes is unknown</td>
<td>1.09 (1.06-1.12)</td>
<td>Zeggini et al. 2008</td>
</tr>
<tr>
<td>rs7578597/ Non-synonymous</td>
<td>THADA T/C</td>
<td>Meta-analysis of GWA</td>
<td>The biological link to type 2 diabetes is unknown</td>
<td>1.15 (1.10-1.20)</td>
<td>Zeggini et al. 2008</td>
</tr>
<tr>
<td>rs4607103/ Intergenic</td>
<td>ADAMTS9 C/T</td>
<td>Meta-analysis of GWA</td>
<td>The biological link to type 2 diabetes is unknown</td>
<td>1.09 (1.06-1.12)</td>
<td>Zeggini et al. 2008</td>
</tr>
<tr>
<td>rs10923931 /Intronic</td>
<td>NOTCH2 G/T</td>
<td>Meta-analysis of GWA</td>
<td>The biological link to type 2 diabetes is unknown</td>
<td>1.13 (1.08-1.17)</td>
<td>Zeggini et al. 2008</td>
</tr>
</tbody>
</table>

Major/minor allele is taken from the genotyping in people from Northern and Western European.

Based on data from Lindgren & McCarthy 2008, and Zeggini et al. 2008. Odds ratios are based on the current available data. Only initial reference and initial/confirming GWA references.

WTCCC: The Welcome Trust Case Control Consortium
There has not been complete consensus in the GWA studies and it has not been possible to replicate several of the initial hits [McCarthy et al., 2008]. On the contrary, some of the validated associations, \textit{PPARG} and \textit{KCNJ11} were only paid attention in Phase I GWA studies because it was found in the Welcome Trust Case Control consortium (WTCCC) in 2007 [WTCCC, 2007]. The WTCCC is a collaboration of 24 leading human geneticists who analyse thousands of DNA samples from patients with different diseases with the aim to identify common genetic variation for each condition [WTCCC, 2007].

\subsection*{1.6.5 Genetic variants predisposing to obesity}

The linkage and candidate gene approaches have identified new genes and QTLs for obesity-related traits; however, only few have been convincingly confirmed. The latest update of the Human Obesity Gene Map, which covers the literature available at the end of October, 2005 reports 127 candidate genes that show associations with obesity-related traits [Rankinen et al., 2006]. Among those, findings for 12 genes, including \textit{UCP2} were replicated in 10 or more studies. Even though many replications of the findings are made, many other studies has shown no or even the opposite association and the function of the genes remains unclear.

Mutations in genes which are involved in appetite regulation can cause obesity. Rare monogenic recessive forms of human obesity have been identified in the genes that encode leptin, the leptin receptor (LEPR), prohormone convertase 1 and proopiomelanocortin (POMC). They all result in a phenotype with an excessive energy intake relative to energy expenditure. Syndromic forms of obesity also exist, at least 20 rare syndromes are caused by discrete genetic defects or chromosomal abnormalities and both autosomal and X-linked syndromes are characterised by obesity. Several GWA studies for both T2D and obesity have identified \textit{FTO} as a common obesity susceptible gene [Frayling et al. 2007a; Hinney et al. 2007] and \textit{FTO} is the first validated common obesity gene. The gene regions associated with obesity is shown in table 1.3.
Table 1.3. Confirmed gene regions involved in obesity, identified in GWA studies.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Location</th>
<th>Gene Major/Minor allele</th>
<th>Function / Characteristics</th>
<th>Odds ratio (per allele)</th>
<th>References Replication</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs7566605</td>
<td>Intergenic</td>
<td>INsIG2 G/C</td>
<td>Involved in adipocyte metabolism</td>
<td>1.22 (1.05-1.42)</td>
<td>Herbert et al. 2006; Liu et al. 2008</td>
</tr>
<tr>
<td>rs9939609</td>
<td>Intergenic</td>
<td>FTO G/C</td>
<td>Unknown function, influences on BMI</td>
<td>1.17 (1.12-1.22)</td>
<td>Frayling et al. 2007a; Scuteri et al. 2007; Hinney et al. 2007; Scott et al. 2007</td>
</tr>
<tr>
<td>rs6602024</td>
<td>Intronic</td>
<td>PFKP G/C</td>
<td>Subunit of phosphofructokinase, a critical enzyme of glycolysis</td>
<td>Not provided</td>
<td>Scuteri et al. 2007; Liu et al. 2008</td>
</tr>
<tr>
<td>rs6013029</td>
<td>Intronic</td>
<td>CTNNBL1 G/T</td>
<td>Unknown function, possible role in adipogenesis</td>
<td>1.42 (1.14-1.77)</td>
<td>Liu et al. 2008</td>
</tr>
<tr>
<td>rs1778231</td>
<td>Intergenic</td>
<td>MC4R T/C</td>
<td>Regulates appetite in combination with leptin</td>
<td>1.3 (1.20-1.41)</td>
<td>Loos et al., 2008</td>
</tr>
</tbody>
</table>

Odds ratios given for the first published GWA study, Only GWA references are included, ORs are calculated based on the current available data [reviewed in Frayling et al., 2007b].

Monogenic forms of obesity are rare; however, deficiency of the melanocortin-4 receptor (MC4R) is the most frequent cause of genetic obesity [Bell et al., 2005] and deficiency of the MC4R is present in 1-6% of obese individuals depending on ethnicity, with a higher prevalence in cases with increased severity and earlier age of onset. Loss of function mutations in MC4R cause severe familial forms of obesity [Vaisse et al., 1998, Yeo et al., 1998] and infrequent gain of function polymorphisms have been associated with protection against obesity [Geller et al., 2004; Stutzmann et al., 2007]. In a recent GWA study association of common variants near the MC4R with fat mass, weight, and risk of obesity has been established [Loos et al., 2008].
2 Study I: Analysis of association of polymorphisms rs1535435 and rs9494266 in AHI1 with type 2 diabetes

The Abelson helper integration site 1 (AHI1) has been associated with T2D in four different white populations in a GWA study of Diagen consortium including 500 cases and 497 controls; Eastern Finns, Ashkenazi Jews, Germans, and English individuals [Salonen et al., 2007]. The ten SNPs showing the lowest \( p \)-values in the GWA study, including SNPs located in AHI1, MYO10, and TTC7B, were selected for replication in a case-control setting.

The minor alleles of rs1535435 and rs9494266 located in AHI1 were associated with T2D in the GWA study, \( OR = 2.3, p = 2 \cdot 10^{-5} \) and \( OR = 2.3, p = 3 \cdot 10^{-5} \), respectively, and they were also associated with T2D in the replication case-control setting, \( OR = 3.6, p = 5 \cdot 10^{-5} \) and \( OR = 3.6, p = 2 \cdot 10^{-4} \), respectively. These two variants are present in the same haplotype and were the only variants that reached the statistical significance level of 0.05 after adjustment for multiple testing (Bonferroni correction). Since AHI1 is found on chromosome 6q23.3 in close proximity with regions that have previously shown suggestive linkage to T2D as well as fasting insulin and lipid concentrations, it was proposed to be a novel T2D susceptible gene [Xiang et al., 2004; Silander et al., 2004; Shtir et al., 2007; Ghosh et al., 2000; Duggirala et al., 2001].

**Figure 2.1.** LD plot showing the high LD between rs1535435 and rs9494266

AHI1 encodes the jouberin protein [Dixon-Salazar et al., 2004]. The exact function of jouberin has not yet been clarified; however, AHI1 is strongly expressed in the embryonic hindbrain and forebrain indicating that AHI1 is required for both cerebellum...
and cortical development in humans [Dixon-Salazar et al., 2004]. Similarly, it has been shown that the expression of AHI1 in mice and humans is highest in the most primitive haematopoietic cells and down-regulated during early differentiation. Perturbation in AHI1 expression may contribute to the development of specific types of leukaemia, e.g. elevated AHI1 mRNA levels are found in chronic myeloid leukaemia patients [Jiang et al., 2004]. HOXA9 is one of the important genes in the pathogenesis of chronic myeloid leukaemia, but it needs cooperative genes for the pathogenesis. AHI1 has been suggested to be one of these cooperative genes as it is located close to a known retroviral integration site [Jin et al., 2007]. AHI1 was first identified to be associated with the joubert syndrome, which is an autosomal recessive disorder characterised by hypotonia, ataxia, mental retardation, altered respiratory pattern, abnormal eye movement, agenesis of the cerebellar vermis, and brain malfunction known as the molar tooth sign [Joubert et al., 1969; Ferland et al., 2004]. Individuals carrying mutations in AHI1 may be at risk of developing both retinal dystrophy and renal cystic disease in joubert syndrome [Parisi et al., 2007]. Moreover, AHI1 was shown to contribute to schizophrenia in populations of Israeli-Arab and Icelandic origin [Ingason et al., 2007; Amann-Zalcenstein et al., 2006; Levi et al., 2005; Lerer et al., 2003].

The association with schizophrenia [Ingason et al., 2007; Amann-Zalcenstein et al., 2006; Levi et al., 2005; Lerer et al., 2003] and the known increased incidence of T2D in people diagnosed with schizophrenia [Brown et al., 2000] might suggest that AHI1 is involved in the pathogenesis of T2D.

### 2.1 Aim

The aim of this study was to replicate the association of the two variants in AHI1, rs1535435 and rs9494266, with T2D in a larger study population comprising 9,154 individuals and, furthermore, to explore the possible association of rs1535435 and rs9494266 with prediabetic quantitative traits.

### 2.2 Populations and methods

rs1535435 and rs9494266 were genotyped in 17,521 Danes. Participants from the population-based Inter99 cohort involving 6,162 individuals were characterised by an OGTT as having normal glucose tolerance (n = 4,567), impaired fasting glycaemia (IFG; n = 508), impaired glucose tolerance (IGT; n = 707) or screen-detected T2D (n = 256) and were investigated for an association between genotype and quantitative metabolic traits; 124 had known T2D and were excluded prior to these analyses. An additional 377 individuals recruited from a population-based sample of young, healthy Danish individuals at the Research Centre for Prevention and Health and 8,428 individuals recruited from the ADDITION study sampled through the Department of General Practice at University of Aarhus were enrolled to study metabolic traits.
The case-control study included all unrelated T2D patients and healthy glucose-tolerant control individuals from the Inter99 cohort (patients  \( n = 380 \), control individuals  \( n = 4,567 \)), the Danish ADDITION study (patients  \( n = 1,617 \); all screen-detected and untreated), and individuals recruited from the outpatient clinic at Steno Diabetes Center (patients  \( n = 2,107 \); all screen-detected and untreated). Detailed descriptions of the participants can be found in appendix 1. In brief, impaired glucose homeostasis (IFG, IGT and diabetes) was defined according to WHO criteria after a 75 g OGTT [Alberti et al., 1999]. All participants were of Danish nationality and were provided informed written consent before participation. The study was approved by the Ethical Committees of Copenhagen and Aarhus and was in accordance with the principles of the Helsinki Declaration II.

**Genotyping**

The  \( AHI1 \) variants rs1535435 and rs9494266 were genotyped using an allelic discrimination assay performed with a TaqMan®, ABI 7900 HT system (performed at KBiosciences, U.K.) with a success rate > 96.8%. Discordance was < 0.1% as judged from re-genotyping of 1,202 random duplicate samples. Further description of allelic discrimination can be found in appendix III.

**Statistical analyses**

Fisher's exact test and logistic regression analyses were used to analyse differences in allele and genotype frequencies between patients and controls; analyses were adjusted for age, sex, and BMI. A general linear model was used to test quantitative variables for genotype differences adjusting for age, sex, BMI, and diabetes status (0 vs. 1 variable) in analyses of the complete cohort. Meta-analyses of published data for the putative association between rs1535435 or rs9494266 and T2D were performed using a Mantel-Haenszel test to estimate the combined OR of these studies. Power was calculated using the Genetic Power Calculator (http://pngu.mgh.harvard.edu/~purcell/gpc/) [Purcell et al., 2003] where T2D prevalence was 10% and with a relative risk of 1.15 (as described in the replication sample in the original article) [Salonen et al., 2007]. The statistical analyses were performed using RGui version 2.7.1 (available at www.r-project.org). p-values were not adjusted for multiple hypothesis testing.

**2.3 Results**

rs1535435 and rs9494266 were genotyped in 17,521 Danish individuals previously used to replicate the associations of variants in  \( HHEX, CDKN2A/B, IGF2BP2 \) [Grarup et al., 2007], and  \( GCKR \) [Sparsø et al., 2008] with T2D. We performed a case-control study investigating the association between T2D and the two SNPs in 4,104 patients and 5,050 glucose-tolerant controls.
There were no differences in our allele frequencies of 8.4 % and 8.5 % and the originally published results [Salonen et al., 2007]. Furthermore, the data obeyed Hardy-Weinberg equilibrium (p > 0.05), D' and r2 values (D' = 0.99; r2 = 0.97) between rs1535435 and rs9494266 were in accordance with the data of Salonen et al., and the power to reject the null hypothesis was estimated to about 85 % assuming an additive model.

We found no significant differences our genotype distribution or MAF between T2D patients and glucose-tolerant controls for rs1535435 and rs9494266 in this case-control study with ORs (OR_add = 1.0 (95 % CI 0.9 -1.2); \( \beta_{add} = 0.7 \) and OR_add = 1.1 (0.9 - 1.2); \( \beta_{add} = 0.4 \)), respectively. The results of the case-control study are shown in Table 2.1.

### Table 2.1 Case-control study of type 2 diabetes in 4,104 type 2 diabetes patients and 5,050 glucose-tolerant controls.

<table>
<thead>
<tr>
<th>rs1535435</th>
<th>GG</th>
<th>GA</th>
<th>AA</th>
<th>OR_add</th>
<th>( \beta_{add} )</th>
<th>OR_add</th>
<th>( \beta_{add} )</th>
<th>OR_dom</th>
<th>( \beta_{dom} )</th>
<th>OR_rec</th>
<th>( \beta_{rec} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGT n (%)</td>
<td>4019</td>
<td>731</td>
<td>40</td>
<td>1.0</td>
<td>0.8</td>
<td>1.0</td>
<td>0.7</td>
<td>1.0</td>
<td>0.6</td>
<td>0.9</td>
<td>0.8</td>
</tr>
<tr>
<td>T2D n (%)</td>
<td>3228</td>
<td>594</td>
<td>25</td>
<td>(0.9-1.1)</td>
<td>(0.9-1.1)</td>
<td>(0.9-1.2)</td>
<td>(0.9-1.2)</td>
<td>(0.4-1.9)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Rs9494266</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGT n (%)</td>
</tr>
<tr>
<td>T2D n (%)</td>
</tr>
</tbody>
</table>

Allele association is not adjusted for age, sex, and BMI. Calculated p-values were adjusted for age, sex, and BMI assuming an additive (\( \beta_{add} \)), dominant (\( \beta_{dom} \)) or a recessive (\( \beta_{rec} \)) model.

Analyses examine the rs1535435 and rs9494266 polymorphisms for an association with T2D-related quantitative traits in Inter99 were performed in 6,038 participants. We found no strong association between rs1535435 and T2D-related traits. Assuming an additive model, the A-allele was nominally associated with an increased serum C-peptide and serum insulin concentrations at 120 minutes during an OGTT, \( \beta_{add} = 0.01 \) and \( \beta_{add} = 0.004 \), respectively (Table 2.2). Increased area under the curve (AUC) for serum insulin, plasma glucose, and serum C-peptide concentrations were also found, \( \beta_{add} = 0.05 \), \( \beta_{add} = 0.04 \), \( \beta_{add} = 0.03 \), respectively.
Table 2.2. Analysis of association for rs1535435 for T2D–related quantitative traits.

<table>
<thead>
<tr>
<th>Rs1535435</th>
<th>AA</th>
<th>AG</th>
<th>GG</th>
<th>Add</th>
<th>Dom</th>
<th>Res</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (men/women)</td>
<td>4,756 (2,337/2,419)</td>
<td>865 (4,63/402)</td>
<td>52 (28/24)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>46 ± 8</td>
<td>46 ± 8</td>
<td>47 ± 8</td>
<td>0.3</td>
<td>0.5</td>
<td>0.1</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.2 ± 5.0</td>
<td>26.2 ± 4.6</td>
<td>25.4 ± 4.3</td>
<td>0.8</td>
<td>0.8</td>
<td>0.9</td>
</tr>
<tr>
<td>Waist-to-hip ratio</td>
<td>0.86 ± 0.09</td>
<td>0.86 ± 0.09</td>
<td>0.86 ± 0.09</td>
<td>0.4</td>
<td>0.6</td>
<td>0.2</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>86 ± 13</td>
<td>87 ± 13</td>
<td>84 ± 13</td>
<td>0.2</td>
<td>0.4</td>
<td>0.09</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>172 ± 9.0</td>
<td>173 ± 9.0</td>
<td>171 ± 8</td>
<td>0.5</td>
<td>0.8</td>
<td>0.08</td>
</tr>
<tr>
<td>Plasma glucose (mmol/l)</td>
<td>5.5 ± 0.8</td>
<td>5.6 ± 0.9</td>
<td>5.4 ± 0.5</td>
<td>0.5</td>
<td>0.8</td>
<td>0.4</td>
</tr>
<tr>
<td>30-min during an OGTT</td>
<td>8.7 ± 1.9</td>
<td>8.8 ± 1.9</td>
<td>8.8 ± 1.5</td>
<td>0.4</td>
<td>0.4</td>
<td>0.7</td>
</tr>
<tr>
<td>120-min during an OGTT</td>
<td>6.2 ± 2.1</td>
<td>6.3 ± 2.3</td>
<td>6.1 ± 1.7</td>
<td>0.08</td>
<td>0.06</td>
<td>0.8</td>
</tr>
<tr>
<td>AUCglucose (mmol · min)</td>
<td>219 ± 135</td>
<td>229 ± 139</td>
<td>235 ± 120</td>
<td>0.04</td>
<td>0.04</td>
<td>0.4</td>
</tr>
<tr>
<td>Serum insulin (pmol/l)</td>
<td>42 ± 28</td>
<td>43 ± 28</td>
<td>38 ± 23</td>
<td>0.5</td>
<td>0.4</td>
<td>0.8</td>
</tr>
<tr>
<td>30-min during an OGTT</td>
<td>289 ± 178</td>
<td>303 ± 214</td>
<td>261 ± 181</td>
<td>0.4</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>120-min during an OGTT</td>
<td>215 ± 208</td>
<td>224 ± 224</td>
<td>264 ± 320</td>
<td>0.01</td>
<td>0.02</td>
<td>0.2</td>
</tr>
<tr>
<td>AUCinsulin (pmol · min)</td>
<td>22,706 ± 15,376</td>
<td>23,972 ± 18,119</td>
<td>23,529 ± 22,512</td>
<td>0.05</td>
<td>0.04</td>
<td>0.9</td>
</tr>
<tr>
<td>C-peptide (pmol/l)</td>
<td>594 ± 270</td>
<td>605 ± 279</td>
<td>569 ± 256</td>
<td>0.4</td>
<td>0.4</td>
<td>0.8</td>
</tr>
<tr>
<td>30-min during an OGTT</td>
<td>1,994 ± 710</td>
<td>2,037 ± 755</td>
<td>1,898 ± 719</td>
<td>0.4</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>120-min during an OGTT</td>
<td>2,301 ± 1,016</td>
<td>2,364 ± 1,010</td>
<td>2,485 ± 1,209</td>
<td>0.004</td>
<td>0.009</td>
<td>0.2</td>
</tr>
<tr>
<td>Incremental AUC (pmol/l)</td>
<td>160,611 ± 57,156</td>
<td>165,031 ± 60,784</td>
<td>166,450 ± 68,274</td>
<td>0.03</td>
<td>0.03</td>
<td>0.05</td>
</tr>
<tr>
<td>Fasting serum lipids (mmol/l)</td>
<td>5.5 ± 1.1</td>
<td>5.5 ± 1.0</td>
<td>5.4 ± 1.1</td>
<td>0.2</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td>1.4 ± 0.4</td>
<td>1.4 ± 0.4</td>
<td>1.4 ± 0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.8</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>1.3 ± 1.4</td>
<td>1.4 ± 1.2</td>
<td>1.3 ± 0.9</td>
<td>0.5</td>
<td>0.5</td>
<td>0.9</td>
</tr>
<tr>
<td>HOMA-IR (mmol/l · mmol/l)</td>
<td>10.5 ± 8.0</td>
<td>10.8 ± 8.0</td>
<td>9.4 ± 5.9</td>
<td>0.6</td>
<td>0.5</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Data are means ± SD. Inter 99, calculated p-values were adjusted for age, sex, and BMI. Individuals are both sexes: NGT (n = 4,522), IFG (n = 503), IGT (693), and screen-detected. Values were logarithmically transformed before analyses. Calculated p-values were adjusted for age, sex, and BMI assuming an additive (p_add), dominant (p_dom) or a recessive (p_rec) model.

We found no strong association between T2D-related traits and rs9494266. Carriers of the minor A-allele had significantly increased serum insulin and C-peptide levels at 120 min during an OGTTs, \( p_{add} = 0.1 \) and \( p_{add} = 0.004 \), respectively, assuming an additive model (Table 3). Furthermore, we found increased AUC for p-glucose, \( p_{add} = 0.1 \) (Table 2.3).


Table 2.3 Analysis of association for rs9494266 for T2D–related quantitative traits

<table>
<thead>
<tr>
<th>rs9494266</th>
<th>AA (4,689) (2303/2386)</th>
<th>AG (865) (465/400)</th>
<th>GG (47) (27/20)</th>
<th>Add</th>
<th>Dom</th>
<th>Res</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (men/women)</td>
<td>Age (years) 46 ± 8.0</td>
<td>BMI (kg/m²) 26.2 ± 4.5</td>
<td>Waist-to-hip ratio 0.85 ± 0.09</td>
<td>Waist circumference (cm) 86 ± 13</td>
<td>Height (cm) 172 ± 9.0</td>
<td>Plasma glucose (mmol/l) 5.5 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>30-min during an OGTT 8.7 ± 1.9</td>
<td>120-min during an OGTT 6.2 ± 2.1</td>
<td>AUCglucose (mmol · min) 218 ± 134</td>
<td>Fasting 42 ± 28</td>
<td>30-min during an OGTT 288 ± 175</td>
<td>120-min during an OGTT 214 ± 205</td>
</tr>
<tr>
<td></td>
<td>30-min during an OGTT 288 ± 175</td>
<td>120-min during an OGTT 214 ± 205</td>
<td>C-peptide (pmol/l) 594 ± 270</td>
<td>Fasting 594 ± 270</td>
<td>30-min during an OGTT 1994 ± 707</td>
<td>120-min during an OGTT 2,295 ± 1,011</td>
</tr>
<tr>
<td></td>
<td>30-min during an OGTT 1994 ± 707</td>
<td>120-min during an OGTT 2,295 ± 1,011</td>
<td>Fasting serum lipids Total Cholesterol 5.5 ± 1.1</td>
<td>10.5 ± 8</td>
<td>10.5 ± 8</td>
<td>9.5 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>30-min during an OGTT 1994 ± 707</td>
<td>120-min during an OGTT 2,295 ± 1,011</td>
<td>HDL-cholesterol 1.4 ± 0.4</td>
<td>10.9 ± 8.3</td>
<td>9.4 ± 6</td>
<td>9.5 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>30-min during an OGTT 1994 ± 707</td>
<td>120-min during an OGTT 2,295 ± 1,011</td>
<td>Triglyceride 1.3 ± 1.4</td>
<td>10.9 ± 8.3</td>
<td>9.4 ± 6</td>
<td>9.5 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>30-min during an OGTT 1994 ± 707</td>
<td>120-min during an OGTT 2,295 ± 1,011</td>
<td>HOMA-IR (mmol/l · mmol/l) 10.5 ± 8</td>
<td>10.9 ± 8.3</td>
<td>9.4 ± 6</td>
<td>9.5 ± 1.1</td>
</tr>
</tbody>
</table>

Data are means ± SD. Inter 99, calculated p-values were adjusted for age, sex, and BMI. Individuals are both sexes: NGT (n = 4,522), IFG (n = 503), IGT (693), and screen-detected. Values were logarithmically transformed before analyses. Calculated p-values were adjusted for age, sex, and BMI assuming an additive (p_{add}), dominant (p_{dom}) or a recessive (p_{rec}) model.

Meta-analysis

We performed meta-analyses for rs1535435 and rs9494266 combining all available data. At present, data for rs1535435 and rs9494266 investigating the putative association with T2D is only available from WTCCC [Zeggini et al., 2007], Diabetes Genetics Initiative (DGI) [Saxena et al., 2007] and our study. Only the Diagen consortium has shown an association with T2D; however, neither their data from were available for our meta-analysis nor were data for rs1515435 from the DGI study.

The meta-analyses did not support an association with T2D: OR_{add} = 0.97, (0.89 - 1.06); p = 0.6 and OR_{add} = 0.98 (0.91 - 1.06); p = 0.6), respectively (Figure 2.2).
2.4 Discussion

In the present study we investigated the putative association of rs1535435 and rs9494266 with T2D in a large case-control setting as well as a possible association with T2D-related quantitative traits in a population-based sample of Danes. Previously, these two SNPs were associated with T2D [Salonen et al., 2007]. We were not able to replicate this association in the present study; however, we found a nominal association of rs1535435 and rs9494266 with serum insulin and C-peptide concentrations 120 min post oral glucose load during an OGTT. Furthermore, nominal associations with decreased LDL, increased AUC plasma glucose and serum C-peptide concentrations were shown for the two SNPs, but considering the numbers of tests performed, the findings are likely to be false positives as they should be corrected for multiple hypothesis testing.

To date, the GWA study of Diagen consortium is the only GWA study of T2D which has shown a significant association at this locus; however, because of the strong p-values in the replication study and that 6q23.3 is in close proximity with regions that have
previously shown suggestive linkage to T2D as well as fasting serum insulin and serum lipid concentrations [Xiang et al., 2004; Silander et al., 2004; Shtir et al., 2007; Ghosh et al., 2000; Duggirala et al., 2001] we examined the putative associations of the two SNPs with T2D.

In the present study we were not able to replicate the initial findings of the GWA study of Diagen consortium in a case-control study of more than 9,000 individuals nor in a meta-analysis, which makes it likely that their findings were due to chance. This might be due to heterogeneity in their study population that consisted of four different white populations. Furthermore, the low power in the sample used in the GWA increases the likelihood for false positive findings and the $p$-values of $2 \cdot 10^{-5}$ and $3 \cdot 10^{-5}$ in the initial GWA are only considered borderline significant in a whole-genome context.

Another explanation for the contradictory results is that the two polymorphisms could be in high LD with a causative variant in the population used in the GWA of the Diagen consortium of Finns, Ashkenazi Jews, Germans, and UK individuals while this is not necessarily the case in our population due to differences in LD blocks between different populations. Internal replication efforts are often biased due to errors in phenotyping or genotyping and population stratification which might be an explanation for the results in the original publication. Therefore, independent validation is of high importance which is what we do with this study investigating more than 17,000 individuals.

In conclusion, the two previously associated variants rs1535435 and rs9494266 in the $AHI1$ are not associated with T2D or related quantitative metabolic traits in Danes.
Study II: Analysis of the UCP2 -866G>A polymorphism (rs659366) with obesity, type 2 diabetes, and related metabolic traits

Uncoupling protein 2 (UCP2) is ubiquitously expressed and there has been an ongoing debate about its function during the last couple of years. Four additional uncoupling proteins exist: UCP1, UCP3, UCP4, and UCP5. UCPs uncouple mitochondrial respiration from ATP production by letting $H^+$ flux into the mitochondrial matrix instead of passing through the ATP-synthase [Fleury et al., 1997; reviewed in Dalgaard et al., 2001], which is illustrated in figure 3.1. The proteins UCP2 and UCP3 are 58 % homologous with UCP1 and 72 % homologous with each other; however, their functions appear to be very different why their names are misleading. UCP1 is exclusively expressed in BAT of mammals, UCP3 predominantly in skeletal muscles, and UCP4 and UCP5 are expressed in the brain [reviewed in Graier et al., 2008].

**Figure 3.1.** Illustration of the function of UCP2; UCP2 is an alternative route for the protons which are pumped into the matrix by the electron transport chain (ETC) and thereby creating an electrochemical gradient, which normally pass through the ATP synthase. The ATP synthase uses the energy released from protons moving down the gradient to synthesise ATP.

UCP1 accounts for 10 % of the protein mass in the inner mitochondrial membrane whereas UCP2 and UCP3 exist in tiny concentrations, 0.01 - 0.1 %, in the mitochondrial membrane proteins [reviewed in Esteves & Brand, 2005]. Since UCP2 and UCP3 also are expressed in ectodermic fish and plants that do not require
thermogenesis, the proteins may have another function as well [reviewed in Graier et al., 2008].

It is well accepted that UCP2 has an uncoupling function involving the regulation of thermogenesis and energy metabolism [Fleury et al., 1997; reviewed in Graier et al., 2008]; however, it has been suggested that UCP2 is important for mitochondrial Ca\(^{2+}\) sequestration through the classical mitochondrial Ca\(^{2+}\) uniport [Trenker et al., 2007]. Furthermore, this Ca\(^{2+}\) uptake function of UCP2 and UCP3 might yield mitochondrial uncoupling indirectly. The uncoupling is suggested because Ca\(^{2+}\) acidifies the mitochondrial matrix and thereby increases the activity of the electron transport chain (ETC) without increasing the activity of the ATP synthesis. This reduces the H\(^{+}\) gradient which reduces the activity of the ATP synthase [reviewed in Graier et al., 2008]. An increase in the activity of ETC without a corresponding increase in ATP production is defined as uncoupling [Skulachev, 1998]. Moreover, results from studies of UCP2 in Chinese hamster ovary cells have postulated UCP2 to be a carrier of ions other than H\(^{+}\) [Mozo et al., 2006].

**UCP2 and fatty acid metabolism**

UCP2 might have a function in carrying excessive free fatty acids out of the mitochondria [reviewed in Graier et al., 2008]; however, most of the results have only been concerning UCP3. PPARs are important for the fatty acids metabolism and since PPARs regulate expression of UCP2 and UCP3 they might also play a role in fatty acid metabolism. Furthermore, the expression of UCP2 and UCP3 is up-regulated under conditions of high fatty acid availability, such as obesity [Millet et al., 1997], and high fat diet [Schrauwen et al., 2003], which also point towards a role of UCP2 in fatty acid metabolism. Furthermore, it has been shown that UCP2 and UCP3 are activated by fatty acids and blocked by nucleotides [Jezek, 1999; Brand & Esteves; 2005, Jezek, 2002]. Another postulate is that the free fatty acids enter the mitochondria as neutral protonated fatty acids by a so called “flip flop” mechanism, which is associated with a simultaneously H\(^{+}\) uniport once UCP2/UCP3 exports fatty acid anions [Jaburek et al., 2004].

**UCP2 protects against ROS damages**

There is increasing evidence that UCP2 and UCP3 also significantly serve to attenuate mitochondrial ROS production; however, the mechanism behind the function of UCP2 and UCP3 is not entirely resolved and the presence of any of the UCPs has shown a protective effect against damages linked to ROS [Mattiasson et al., 2003; Vincent et al., 2004]. It is suggested that the overload of the ETC and the following production of ROS stimulates UCP2 leading to uncoupling, thereby reducing the production of ROS [reviewed in Graier et al., 2008].

**UCP2 and insulin regulation**

Overexpression of UCP2 has been reported to inhibit glucose-stimulated insulin secretion in pancreatic islets from rats [Chan et al., 1999] and INS-1 β-cells
which proposes UCP2 to be a negative regulator for pancreatic insulin secretion. Knockout mice $Ucp2^{-/-}$ have increased circulating insulin levels and their isolated pancreatic islets secrete more insulin and show a higher ATP/ADP ratio in response to D-glucose [Zhang et al., 2001]. On a high-fat diet knock out mice have shown increased insulin secretion and decreased plasma triglyceride concentrations compared with wild type mice [Joseph et al., 2002]. $Ucp2$ was up-regulated in diabetic, obese ob/ob mice in which a lack of $Ucp2$ restored first-phase insulin secretion and reduced the level of hyperglycaemia [Zhang et al. 2001]. No effect of $Ucp2$ disruption on obesity was observed, even upon a high-fat diet [Arsenijevic et al., 2000]. The knock out mice have also shown that ablation of $ucp2$ results in slightly elevated plasma levels of inflammatory cytokines, e.g. tumor necrosis factor α (TNFα) or interferon γ (IFNγ) and persistent nuclear factor κB (NF-κB) activation, which enhances β-cell function and insulin secretion [Graier et al., 2008].

The $UCP2$ rs659366 and obesity

$UCP2$ is located on chromosome 11q13 and a frequent -866G>A variant, rs659366, has been identified in the gene [Esterbauer et al., 2001]. This variant has been shown to be located in the core promoter of $UCP2$ [Dalgaard et al., 2003] -a region with putative binding sites for two β-cell transcription factors. The minor allele A of the rs659366 has been shown to associate with a reduced risk of obesity among 596 and 791 white Europeans [Esterbauer et al., 2001], which has been replicated in an additional population of 39 obese non-diabetic white European [Krempler et al., 2002]. This agrees with the increased 24-hour energy expenditure shown in 83 obese Pima Indians [Kovacs et al., 2005] and increased resting-energy expenditure in 147 obese children [Le-Fur et al., 2004] and in the German population [Schäuble et al., 2003].

No association with obesity has been found in Spanish children and adolescents for the rs659366; however, the haplotype (-866G; rs659366)-(Del; 45bp)-(-55T; rs1800849) was significantly associated with obesity [Ochoa et al., 2007]. In 632 Japanese there was no association with obesity [Ji et al., 2004]. Neither in a Danish study that included 719 obese cases 816 controls were there any association with obesity or obesity-related intermediary phenotypes [Dalgaard et al., 2003] and no association with BMI was shown in 681 French Caucasians with T2D [Reis et al., 2004]. Among 465 healthy, British men the AA-genotype was more prevalent among obese participants [Dhamrait et al., 2004].

No consensus has been achieved regarding the association between the variant and adiposity. A range of studies reported no association with levels of BMI or waist-to-hip ratio [Dalgaard et al., 2003; Mancini et al. 2003; Sesti et al., 2003; D´Adamo et al., 2004; Ji et al., 2004; LeFur et al., 2004; Bulotta et al., 2005; Kovacs et al., 2005; Cha et al., 2007; Gable et al., 2007; Marvelle et al., 2008]. Assuming that a more subtle intermediary obesity-related phenotype is affected by the A-allele, a number of
interesting observations have been made; among French type 2 diabetic patients the \textit{UCP2} variant was associated with elevated triglyceride and total cholesterol concentrations and increased risk of dyslipidaemia [Reis \textit{et al}., 2004], and in line with this, decreased HDL-cholesterol levels were reported among Korean women [Cha \textit{et al}., 2007]. Contrary, a lack of association with lipid levels has also been reported [Mancini \textit{et al}., 2003; Sesti \textit{et al}., 2003; Dadamo \textit{et al}., 2004; Bulotta \textit{et al}., 2005]. Finally, 147 obese children homozygote of the A-allele had increased glucose oxidation rate and lower lipid oxidation rate [LeFur \textit{et al}., 2004].

\textbf{The \textit{UCP2} rs659366 and T2D}

Numerous studies have investigated the \textit{UCP2} rs659366 and its putative association with T2D; however, these studies have also been inconsistent. The minor A-allele has been associated with T2D in obese white Europeans in 201 type 2 diabetic patients and 291 controls [Krempler \textit{et al}., 2002] and in Italian women in a study that included 483 type 2 diabetic patients and 565 controls. In Italian women the association was suggested to be due to decreased insulin sensitivity [D´Adamo \textit{et al}., 2004]. The A-allele has also been shown to associate with modest risk of T2D in Northern Europeans among 131 diabetic patients and 118 controls [Wang \textit{et al}., 2004] and in agreement lower insulin secretion was associated with the A-allele of rs659366 in 791 white Europeans [Esterbauer \textit{et al}., 2001]; however, subsequent studies showed no association [Dalgaard \textit{et al}., 2003; Sesti \textit{et al}., 2003; Dadamo \textit{et al}., 2004; Ji \textit{et al}., 2004; Bulotta \textit{et al}., 2005]. Contrary, reduced risk was found among 746 type 2 diabetic patients and 327 controls in Caucasians from Italy [Bulotta \textit{et al}., 2005], and in Swedes among 1,659 type 2 diabetic patients and 724 controls [Lyssenko \textit{et al}., 2005].

An early onset of type 2 diabetes has been related to both the A-allele [Sasahara \textit{et al}., 2004, Gable \textit{et al}., 2007] and the G-allele [Lyssenko \textit{et al}., 2005]. In Japanese patients, A-allele carriers needed insulin therapy earlier than G-allele carriers and showed higher frequency of insulin therapy in 413 Japanese type 2 diabetic patients [Sasahara \textit{et al}., 2004] and in 681 French Caucasians with T2D [Reis \textit{et al}., 2004]. Also, a lower disposition index has been found although this may be induced by changes in insulin sensitivity rather than insulin secretory capacity [Krempler \textit{et al}., 2002; Sesti \textit{et al}., 2003]. Indeed, increased insulin resistance assessed by a hyperinsulinaemic-euglycaemic clamp or an intravenous glucose tolerance test among A-allele carriers have been reported in some [Krempler \textit{et al}., 2002; D´adamo \textit{et al}., 2004] but not all [Sesti \textit{et al}., 2003; Ji \textit{et al}., 2004, Ochoa \textit{et al}., 2007] studies. In 465 diabetic men the A-allele has been associated with increased oxidative stress [Dhamrait \textit{et al}., 2004].

\textbf{The \textit{UCP2} rs659366 and cardiovascular diseases}

In 3,122 French type 2 diabetic men the A-allele was associated with decreased risk of coronary artery disease [Cheurfa \textit{et al}., 2008]; however, it has also been associated with increased risk of hypertension [Ji \textit{et al}., 2004] and with increased dyslipidaemia.
among type 2 diabetic patients in 681 French Caucasian [Reis et al., 2004]. Other studies have found no association with lipids [Mancini et al., 20003; D’Aadamo et al., Bulotta, 2005] and, furthermore, a study showed decreased HDL [Cha et al. 2007].

**Expression of UCP2 in relation to UCP2 rs659366**

It is interesting to investigate if the associations of the A-allele are correlated with mRNA expression; however, the results of the expression are also inconsistent. Increased [Esterbauer et al., 2001; Krempler et al., 2002] and decreased [Wang et al. 2004] adipose UCP2 mRNA levels were found in adipose tissues in A-allele carriers.

**2.5 Aim**

By dissipating proton gradients, uncoupling respiration from oxidative phosphorylation and converting fuel to heat, UCP2 may play an important role in the regulation of human energy metabolism [Fleury et al., 1997. Moreover, UCP2 levels may be modulated by free fatty acids, and thus UCP2 may also be involved in fat and skeletal muscle lipid metabolism [Saleh et al., 2002]. The inconsistent studies have increased the importance to investigate the effect of the rs659366 variant in relation to the development of obesity and T2D in large scale study populations. The aim of this study was to investigate if the **UCP2** A-allele of rs659366 is associated with type 2 diabetes, obesity, or quantitative metabolic traits in large-scale studies of Danes.

**2.6 Populations and methods**

The **UCP2** rs659366 was genotyped in 16,173 Danes comprising 1) the population-based Inter99 sample of middle-aged Danes sampled at Research Centre for Prevention and Health (n = 6,163), 2) type 2 diabetic patients sampled through the outpatient clinic at Steno Diabetes Center (n = 1,961), 3) a population-based group of middle-aged glucose-tolerant individuals recruited from Steno Diabetes Center (n = 512), and 4) the ADDITION study group sampled through Department of General Practice at University of Aarhus (n = 7,220), and 5) population based sample of young healthy Danish Caucasians recruited from Research Centre for Prevention and Health (n = 317) who underwent a tolbutamide-modified intravenous glucose tolerance test. Detailed descriptions of study populations are available in appendix 1. Study groups 1 and 3 underwent a standard 75 g oral glucose tolerance test (OGTT). Methods for Biochemical and anthropometrical measurements are described in appendix 1.

**Genotyping**

The **UCP2** rs659366 polymorphism was genotyped using Taqman allelic discrimination (KBioscience, Herts, UK). Discordance between 965 random duplicate samples was 0.0 % and the genotyping success rate was 96.9 %. All genotype groups obeyed Hardy-Weinberg equilibrium. Further description can be found in appendix 3.
**Statistical analyses**

Fisher’s exact test was applied to examine differences in allele frequencies and logistic regression with adjustment for age and sex was applied to examine differences in genotype distributions between affected and unaffected individuals. A general linear model was used to test quantitative variables for differences between genotype groups among non-diabetic and untreated individuals. Meta-analysis of previously published data for the association between rs659366 and obesity as well as between rs659366 and T2D and test of homogeneity between studies were performed using a Mantel-Haenszel test and the combined OR of these studies were estimated. All analyses were performed using SPSS version 14.0 and RGui version 2.5.1. A p-value of less than 0.05 was considered to be significant, further description can be found in Appendix 2.

**2.7 Results**

The *UCP2* variant located in the promoter region was genotyped in 16,173 Danish individuals. We carried out case control studies of obesity (BMI < 25 kg/m$^2$ vs. BMI $\geq$ 30 kg/m$^2$) and WHO-defined type 2 diabetes. Furthermore, we investigated the variant for association with metabolic quantitative traits.

To investigate if the A-allele in the rs659366 is associated with obesity we performed a case control study of individuals with a BMI below 25 as controls and individuals with a BMI above 30 as cases. The case-control study of obesity was performed separately for the Inter99 and ADDITION study samples. There was a nominal higher prevalence of the A-allele carriers in the group of cases with a BMI above 30 kg/m$^2$; however, the case-control study in the ADDITION study was negative, see table 3.1. Additional exploratory association studies of dyslipidaemia and hypertension were all negative.
## Table 3.1. Case control study of obesity in 4,703 middle-aged individuals and in 4,022 individuals from the ADDITION.

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 4,703)</th>
<th>Cases (n = 4,022)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Middle-aged individuals</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI &lt; 25 kg/m²</td>
<td>3,153 (1,242/1,911)</td>
<td>1,550 (784/766)</td>
</tr>
<tr>
<td>Genotype distribution</td>
<td>1,133/1,499/521</td>
<td>584/756/14</td>
</tr>
<tr>
<td>MAF (95% CI)</td>
<td>40.3 (36/48/17)</td>
<td>37.9 (38/49/14)</td>
</tr>
<tr>
<td>p_AF</td>
<td>0.91 (0.83 - 0.99)</td>
<td>0.03</td>
</tr>
<tr>
<td>p_GD</td>
<td></td>
<td>0.03</td>
</tr>
<tr>
<td>OR (95% CI)</td>
<td></td>
<td>0.99 (0.91 - 1.09)</td>
</tr>
<tr>
<td>ADDITION (n = 4,022)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>1,567 (697/870)</td>
<td>2,455 (1,251/1,204)</td>
</tr>
<tr>
<td>Genotype distribution</td>
<td>534/799/51</td>
<td>874/1183/398</td>
</tr>
<tr>
<td>MAF (95% CI)</td>
<td>40.4 (34/51/15)</td>
<td>40.3 (36/48/16)</td>
</tr>
<tr>
<td>p_AF</td>
<td>0.99 (0.91 - 1.09)</td>
<td>0.9</td>
</tr>
<tr>
<td>p_GD</td>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td>OR (95% CI)</td>
<td></td>
<td>0.99 (0.91 - 1.09)</td>
</tr>
</tbody>
</table>

Data are number of individuals with each genotype (% below) and frequencies of the minor allele (MAF).

The case control study of T2D was performed using 3,214 glucose tolerant controls and 4,904 T2D cases. There were no differences in minor allele frequencies (MAFs) or genotype distributions between cases and controls (Table 3.2).
Table 3.2. Case control study of T2D in 4,904 T2D cases and 3,214 glucose tolerant controls

<table>
<thead>
<tr>
<th>Genotype distribution</th>
<th>MAF (95% CI)</th>
<th>p_AF</th>
<th>p_GD</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Middle-aged individuals (n = 8,118)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose-tolerant individuals</td>
<td>3,214 (1,886/1,328)</td>
<td>1,158/1,546/510 (36/48/16)</td>
<td>39.9 (38.7-41.1)</td>
<td>1 (0.94 - 1.06)</td>
</tr>
<tr>
<td>T2D patients</td>
<td>4,904 (2,275/2,629)</td>
<td>1,806/2,307/791 (37/47/17)</td>
<td>39.9 (38.7-40.6)</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Data are number of individuals with each genotype (% below) and frequencies of the minor A-allele (MAF).

An analysis examining the possible association between the rs659366 variant and quantitative metabolic traits was conducted in a total of 5,781 treatment-naïve individuals from the population-based Inter99 study sample. The analysis was performed by applying a general linear model assuming a recessive model and calculated p-values were adjusted for age, sex and BMI. Significant associations with decreased plasma fasting glucose and serum insulin at 120-min during an OGTT were found. Furthermore an improved insulin sensitivity expressed as HOMA-IR was observed, see table 3.3.
Table 3.3: Analyses of anthropometric and metabolic characteristics of 5,781 middle-aged Danes from Inter99.

<table>
<thead>
<tr>
<th>Rs659366</th>
<th>GG (men/woman)</th>
<th>GA (men/woman)</th>
<th>AA (men/woman)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>2,164 (1,087/1,077)</td>
<td>2,694 (1,331/1,363)</td>
<td>923 (451/472)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>46 ± 8</td>
<td>46 ± 8</td>
<td>46 ± 8</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.2 ± 4.4</td>
<td>26.2 ± 4.6</td>
<td>26.1 ± 4.6</td>
</tr>
</tbody>
</table>

Plasma glucose (mmol/l)

<table>
<thead>
<tr>
<th></th>
<th>Fasting</th>
<th>30-min post-OGTT</th>
<th>120-min post-OGTT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5.6 ± 0.9</td>
<td>5.5 ± 0.7</td>
<td>5.5 ± 0.7</td>
</tr>
<tr>
<td>Rs659366</td>
<td>9.0 ± 0.9</td>
<td>9.1 ± 0.7</td>
<td>9.1 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>8.8 ± 2.0</td>
<td>8.6 ± 1.8</td>
<td>8.7 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>6.3 ± 2.2</td>
<td>6.2 ± 2.1</td>
<td>6.2 ± 2.0</td>
</tr>
<tr>
<td>Fasting (mmol/l)</td>
<td>8.0 ± 2.0</td>
<td>8.1 ± 1.8</td>
<td>8.2 ± 1.8</td>
</tr>
<tr>
<td>30-min post-OGTT</td>
<td>6.3 ± 2.2</td>
<td>6.2 ± 2.1</td>
<td>6.2 ± 2.0</td>
</tr>
<tr>
<td>120-min post-OGTT</td>
<td>220 ± 204</td>
<td>219 ± 224</td>
<td>201 ± 184</td>
</tr>
<tr>
<td>Serum insulin (pmol/l)</td>
<td>10.9 ± 8.1</td>
<td>10.4 ± 8.0</td>
<td>10.2 ± 7.6</td>
</tr>
</tbody>
</table>

Moreover, meta-analysis investigating association of rs659366 with obesity and T2D were performed. Available data and data from present studies were included. For obesity 9 studies were included inclusive the present studies of obesity (Steno/Inter99 and ADDITION) [Esterbauer et al., 2001; Dalgaard et al., 2003, Schäuble et al., 2003; Mancini et al., 2003; Le Fur et al., 2004; Ochoa et al., 2007]. For the meta-analysis for T2D 6 studies were included inclusive present study (ADDITION) [Krempler et al., 2002; Dadamo et al., 2004; Ji et al., 2004; Bulotta et al., 2005; Rai et al., 2007]. The meta-analysis showed a borderline protective association with obesity for the AA-genotype, OR = 0.89 (0.80 – 1.0), p = 0.04) (Figure 3.4). The meta-analysis for T2D included studies that were too heterogeneity to compare them; however, they showed no association with T2D, OR = 0.94 (0.84 - 1.05), p = 0.3 (Figure 3.4).
2.8 Discussion

In the present study we were not able to demonstrate any association of the A-allele of *UCP2* rs659366 with T2D in 8,118 middle-aged Danes; however, middle-aged A-allele carriers had decreased fasting glucose levels, decreased insulin release, and improved insulin sensitivity expressed as HOMA-IR. These traits indicate that the variant may have a protective effect against T2D.

In our case control study of obesity (BMI < 25 kg/m² vs. BMI ≥ 30 kg/m²) in middle aged individuals we found an association with of the G-allele with obesity; however, we found no association with obesity in the ADDITION study. We were not able to deduce that the G-allele is associated with obesity; however, it might have an effect on obesity as other studies proposed due to our nominal finding in the middle-aged individuals. The observed effect of increased insulin sensitivity of the A-allele carriers do not agree with the decreased insulin sensitivity found in Italian women of A-allele carriers [D’Adamo et al., 2004] and the lower insulin secretion, which was observed
associated with the A-allele [Esterbaur et al., 2001]. When combining available data of association for rs659366 with obesity in a meta-analysis, it showed a protective association with obesity. A meta-analysis for rs659366 with T2D showed no association.

Our results do not clarify whether the rs659366 contributes to obesity and T2D, why additional studies are needed to clarify the effect of the variant. The 95 % CI in the present study indicate however that an association of the A allele carriers with T2D would be modest. An indication that the variant does not contribute to the development of T2D is that the GWA study performed of the WTCCC, which also included the UCP2 rs659366 did not report any association of rs659366 with T2D. False positives are a general problem in association studies, but also missed true findings can be a problem. Furthermore, the different findings might not only be due to false positives or false negatives, but can also be due to differences in ethnicity. In different populations and especially in different ethnic groups, different patterns in LD blocks exist. This could be a possible explanation for the contradicting results. For example it is reported that the A-allele frequency is higher in Japanese compared to Caucasians, which might influence the results [Sasahara et al., 2004].

A study of 3,782 women with different ethnicities investigated 14 SNPs (including rs659366) spanning the UCP2 and UCP3 loci [Hsu et al., 2008]. No single-SNP association with type 2 diabetes was observed following correction for multiple testing. Haplotype analysis indicated an association with increased risk for type 2 diabetes among 968 Caucasian women; this effect was further accentuated by overweight although no association with BMI was observed. The four-SNP haplotype in the study was in high LD with the rs659366 A-allele, suggesting that an yet unidentified variation covered by the haplotype-spanned area may be responsible for the observed relationships of the variant with metabolic variables.

In conclusion, the A-allele of the rs659466 was not associated with obesity or T2D in white Danes; however, the A-allele was associated with decreased fasting glucose levels, decreased insulin release, and improved insulin sensitivity expressed as HOMA-IR.
3 Study III: Investigation of the putative impact of *LMNA* rs4641 on RNA splicing and lamin A and C expression in relation to type 2 diabetes

**Background**

The lamin gene (*LMNA*), which is located on chromosome 1q21.2 [Lin & Worman, 1993; Wydner et al., 1996] encodes four lamins by alternative splicing: A, C, AΔ10, and C2 [Worman & Courvalin, 2002]. The fibrous proteins, lamin A and C are co-expressed and form a polymer integrated in the nuclear lamina [Worman & Courvalin, 2002]; a membrane located just below the inner nuclear membrane consisting of lamins and nuclear lamin-associated membrane proteins. Lamins have a scaffolding function for several nuclear processes such as transcription, chromatin organisation, and DNA replication [Verstraeten et al., 2007]. Lamin A and C are shown to be very important physiological since mice lacking lamin A but not lamin C develop normally to term; however, after two-three weeks their growth were reduced and after four weeks their growth had ceased [Sullivan et al., 1999].

![Figure 4.1 Nuclear lamina is shown as yellow and blue strings inside the nuclear membrane.](image)

Different types of lamins exist; A-type lamins which include lamins A and C, and B-type lamins which include lamin B. A-type lamins are expressed in terminally differentiated cell but are largely absent from embryonic and adult stem cells whereas B-type lamins are encoded by *LMNB1* and *LMBN2* and are expressed in all cells [Rankin & Ellard, 2006]. A-type and B-type lamins differ in that B-type lamins remain associated with membrane vesicles during mitosis while A-type lamins do not.

Located just upstream of the alternative splicing site in exon 10 of *LMNA* is the silent nucleotides substitution rs4641 (1908C>T). The genetic variant changes the third nucleotide of codon 566 (His566His) in exon 10 and it is hypothesized to influence the alternative splicing of the *LMNA* and thereby the ratio of lamin A to lamin C [Linn &
Worman, 1993]. The SNP is interesting as the T-allele was nominal associated with T2D [Wegner et al., 2007].

Laminopathies

Multiple diseases and syndromes are caused by mutations in LMNA which often are referred to as laminopathies; however, the precise mechanism by which defective lamin A and C cause these diseases remains unclear. Laminopathies include monogenic syndromes such as muscular dystrophy, Hutchinson-Gilford progeria syndromes (figure 4.2), familial partial lipodystrophy, and altered fat distribution [reviewed in Rankin & Ellard, 2006].

Mutations causing familial partial lipodystrophy are located in exon 8; but mutations in other areas in the gene have also been identified [Vigouroux et al., 2000]. The most common mutation in the extremely rare condition Hutchinson-Gilford progeria syndrome with aspects of greatly accelerated aging is a single base pair substitution, 1824C>T within exon 11. This creates an abnormal splice donor site leading to expression of a truncated unfunctional lamin A [Cao & Hegele, 2003; reviewed in Rankin & Ellard, 2006].

Type 2 diabetes linkage studies

The chromosome 1q21-23 region has been suggested to harbour potential T2D susceptibility loci by Pima Indian sib-pair analyses [Hanson et al., 1998] and in Utah Caucasians [Elbein et al., 1999], which were replicated among French Whites [Vionnet et al., 2000], a UK population [Wiltshire et al., 2001; Frayling et al., 2003], and in Hong Kong Chinese [Ng et al., 2004]. An additional study of Pima Indians deduced that LMNA rs4641 does not contribute to the linkage of T2D on chromosome 1q [Wolford et al., 2001]. Furthermore, the region is linked to the metabolic syndrome in Hispanic families [Langefeld et al., 2004].

Association studies of T2D and obesity

The most frequently studied variant in LMNA is rs4641 - at least in relation to type 2 diabetes and metabolic syndrome [Duesing et al., 2008; Mesa et al., 2007; Hegele et al., 2000; Hegele et al., 2007; Murase et al., 2002; Steinle et al., 2004; Weyer et al., 2001, Owen et al., 2007 Wegner et al., 2007]. The T-allele of rs4641 was nominal associated with T2D in a study of 1,324 diabetic patients and 4,386 glucose tolerant individuals and elevated fasting plasma glucose in the population-based Inter99 of 5,395 [Wegner et al., 2007].
Several studies have shown an association between the rs4641 T-allele and T2D-related traits in ethnically diverse populations [Steinle et al., 2004; Hegele et al., 2000; Hegele et al., 2007; Wegner et al., 2007]. However, two of these studies investigated UK individuals and did not find an association with T2D [Mesa et al., 2007; Owen et al., 2008]. One of the studies investigating UK individuals included two case-control studies of 834 and 1,044 participants, respectively and additionally a study investigating quantitative traits of 1,572 individuals [Mesa et al., 2007]. The other UK study included 5,046 [Owen et al., 2008]. The two UK studies agreed with a study of 3,091 French Europeans, which did not find an association with T2D. A meta-analysis comprising 15,591 individuals from four published studies [Duesing et al., 2008; Mesa et al., 2007; Owen et al., 2007; Wegner et al., 2007] has been performed investigating the association of the rs4641 T-allele with T2D. This meta-analysis did not support a major effect of the rs4641 on T2D susceptibility [Duesing et al., 2008].

The rs4641 T-allele has previously been shown to associate with obesity in a small study comprising 186 non-diabetic Canadian Inuit [Hegele et al., 2001]. Moreover, results of 306 non-diabetic individuals indicated that the rs4641 was associated with a modest but significant variation in plasma leptin concentrations, leptin-to-BMI ratio, BMI, percent body mass, and waist-to-hip ratio in a non-diabetic Oji-Cree population. In particular, homozygous carriers of the T-allele had significantly higher mean plasma leptin and a tendency towards obesity [Hegele et al., 2000]. Subsequently, these findings have been replicated in 186 Canadian Inuit individuals. This study showed that T-allele carriers had significantly higher weight, BMI, waist circumference, waist-to-hip-ratio, sub-scapular skin fold thickness, and sub-scapular to triceps skin fold ratio [Hegele et al., 2001]. Increased concentrations for plasma leptin were also found, but it was not significant possible due to the smaller sample size. In a Japanese study of 171 non-diabetic individuals and 164 type 2 diabetic patients the T-allele was significantly associated with hyperinsulinaemia, hypertriglyceridaemia, high concentrations of LDL cholesterol, and low concentrations of HDL cholesterol, which all are traits of the metabolic syndrome; however, the T-allele was not associated with T2D [Murase et al., 2002]. Furthermore, the T-allele was associated with the metabolic syndrome and dyslipidaemia in an Amish population of 971 individuals [Steinle et al., 2004]. Additionally, a modest increase in risk of diabetic nephropathy and cerebral vascular disease in T-allele carriers of 166 Japanese diabetic men was found [Liang et al., 2005].

**Expression studies**

A previous study investigated the possible effect of the ratio of lamin A to C mRNA levels in subcutaneous adipose tissue in obesity and T2D in Caucasians. The study of T2D was performed in 28 diabetic patients and 27 non-diabetic individuals and the study of obesity included 52 individuals who were stratified according to four BMI intervals (18.5 < BMI ≤ 25 (n = 15); 25 < BMI ≤ 30 (n = 13); 30 < BMI ≤ 40 (n = 14); BMI > 40 (n = 14) to investigate the ratio of lamin A to C mRNA levels in the four
different BMI intervals [Miranda et al., 2007]. Among 54 T2D patients no differences in lamin A and C mRNA levels were found between patients in the four BMI intervals. Nevertheless, there were significantly higher levels of both lamin A and C mRNA in T2D patients compared to non-diabetic individuals independently of BMI and the lamin A/C ratio was lower in type 2 diabetic patients.

In subcutaneous adipose tissue from obese individuals (BMI ≥ 40) there was also a tendency toward higher levels of lamin A and lamin C expression compared with individuals in the three lower BMI intervals; although only lamin C reached the statistical significance level [Miranda et al., 2008]. Nevertheless, the lamin A/C ratio was lower in obese individuals (BMI ≥ 40) [Miranda et al., 2008].

**Alternative splicing**

By alternative splicing exons of the primary gene transcript pre-mRNA are separated from introns at splice sites and reconnected. Subsequently translation takes place and specific and unique sequences of amino acids are specified. In this way different proteins are synthesised from the same gene. It is performed by five small nuclear ribonucleoproteins (U1, U2, U4, U5, and U6) and more than 60 polypeptides.

Different elements are involved in regulation of alternative splicing: Cis-elements, which are present on the same strand as the gene they regulate and trans-elements that can regulate genes distant from the gene. These elements are either enhancers stimulating splicing or silencers repressing splicing. It is essential that cis-elements are present in the coding sequences of genes to allow the splicing machinery to distinguish between genuine and pseudo-exons and to modulate the selection of alternative splice site. Enhancers are more prevalent than silencers; they might be present in most if not all exons, including constitutive exons.

![Alternative splicing diagram](image)

**Figure 4.3.** Alternative splicing. Boxes represent exons, black horizontal lines represent introns. Grey boxes are constitutive exons. Red, yellow, green and blue boxes are different multiple cassette exons of which only one of them or few are further delivered to the mRNA. The lines in top of the multiple cassette exons shows exons further spliced to mRNA.

In higher eukaryotes the requirement for accurate splicing is accompanied by exon-intron junctions that are defined by weakly conserved intronic cis elements, the 5´ splice site, 3´ splice site and branch A site. The classical splice sites include the nearly 100 % conserved nucleotides GU at the 5´ end and an AG dinucleotide at the 3´ end, a
polypyrimidine tract, and a branch point A situated close to the 3´ splice site, which attacks the G in the intronic 5´ end when splicing starts to form a 2´,5´-phosphodiester linkage. Then the 3´ end of the upstream exon (G) captures the 3´ end of the intron by forming phosphodiester bond again so that the exons are joined together, leaving a free intron in a lariat form. It is indicated that about 60 % of genes are represented by two or more transcripts [reviewed in Cartegni et al., 2002]. SR proteins are also important for the alternative splicing: they are proteins that act as binding sites for serine and arginine rich proteins and participate in different steps of splicing. SR proteins bind to exon skipping enhancers through their RNA-binding domain, and promote exon definition by recruiting splicesomal components via different proteins-protein interactions. Multiple classes of exon skipping enhancers have been described [Fairbrother et al., 2002; reviewed in Cartegni et al., 2002]. The splice sites and branch site do not provide sufficient information for the recognition of the spliceosome. Exon skipping enhancers are additional intron and exon sequences necessary for efficient and/or accurate splicing of many higher eukaryotic pre-mRNA. The cis-acting elements are such exonic splicing enhancers that are found in purine-rich context. Exon skipping enhancers are mostly found in exons which typically have weak splice sites and require ESE for exon inclusion [Liu et al., 1998].

Many diseases are caused by mutations which alter an amino acid in the encoded protein. But many diseases are also caused by exonic mutations that affect pre-mRNA splicing. Nonsense, missense and even translationally silent mutations can inactivate genes by inducing the splicing machinery to skip the mutant exons [Wang, 1996].

**Alternative splicing of LMNA**

Alternative splicing within the exon 10 of human LMNA gives rise to pre-lamin A (pre-cursor of lamin A) mRNA and lamin C mRNA. Lamin A and C are identical through exon 1 to 9; however, lamin A contains only the first 5´ 90 bases of exon 10 and, additionally, exons 11 and 12, whereas lamin C contains the whole exon 10 (of 111 nucleotides), but lacks exons 11 and 12. The rs4641 is located in the splice site in exon 10 [Lin & Worman.1993] (Figure 4.1).
3.1 Aim

The aim of the present study is to investigate the impact of rs4641 on \textit{LMNA} splicing of exon 10 by exon trapping and by investigating the lamin A-to-C mRNA ratio in human subcutaneous adipose tissue and skeletal muscle in Danish monozygotic (MZ) and dizygotic (DZ) twins. Moreover, we will examine if there is an association of the mRNA expression levels or the rs4641 genotype with pre-diabetic quantitative phenotypes. The primary hypothesis for the association studies are that rs4641 is associated with fasting glucose and obesity-related traits in two metabolically well-characterised populations of Danish twins due to a changed ratio of lamin A to C.

3.2 Populations and methods

3.2.1 Methods for gene expression

The present study of gene expression of \textit{LMNA} is an extension of two studies initiated by Pernille Poulsen in 1994 and 1997 and clinical data were already available [Poulsen \textit{et al.}, 1997; Poulsen \textit{et al.}, 2005]. Likewise, the mRNA had been purified from tissues. At Steno Diabetes Center the cDNA synthesis was performed by Lise Wegner and I performed the measurement of mRNA expression.

3.2.2 Quantitative PCR

Gene expression of lamin A and lamin C was measured by quantitative PCR using SYBR Green (Appendix III) and the housekeeping gene cyclophilin A (Appendix III) was measured using the TaqMan® real-time PCR technique in cDNA from subcutaneous adipose tissue and skeletal muscle biopsies. The conventional PCR technique is used to amplify DNA sequences [Mullis \textit{et al.}, 1986]. The method can be divided into three steps; 1) separation of the two DNA strands by heat denaturation,
2) annealing of polynucleotides primers, and 3) elongation of the new complementary DNA strand. The procedure enables the ability of *Thermus aquaticus* (Taq) polymerase to replicate DNA at temperatures which exceeds the optima of eukaryotic DNA polymerase. This property is essential since heating to approximately 95ºC is required to denaturate the DNA double strand [Busting, 2004]. Quantitative-PCR (Q-PCR) measures the quantification of mRNA or DNA sequences of interest as it is possible to monitor the PCR amplification cycle by cycle [Higuchi *et al.*, 1993]. By this method measurements of accumulation of a product during the exponential stages of the PCR is obtained compared to conventional PCR which only measures the end point of the product.

For measuring lamin A and C mRNA expression SYBR Green was used and ABI PRISM 7900HT Sequence Detection System (TaqMan ®) was used to measure cyclophilin A. [ABIa]. A primer set flanking the region of interest is used for the two methods. SYBR Green is a minor groove DNA-binding dye and binds non-specifically to all DNA also to primer-dimers whereas a probe, which was used to measure cyclophilin A is more

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Figure 4.5. Q-PCR using SYBR Green. SYBR Green fluoresces when it binds to double stranded DNA. When DNA is denaturated the dye is not bound and it does not fluoresce (green circles). When the primers anneal to DNA and double stranded DNA is synthesized, the dye binds to the synthesised DNA and fluoresces.
specific and its use is a more accurate method. SYBR Green was used to measure lamin A and C mRNA as we had to distinguish between lamin A and lamin C and since there were not enough nucleotides to place a probe between the primers, we used the non specific dye, SYBR Green.

SYBR Green dye binds to double stranded DNA and increases the intensity of fluorescent emission. The fluorescent dye makes it possible to detect the amount of DNA by lasers and detectors during each cycle by the PCR apparatus as the amount of fluorescent dye increases proportionally with the increase in double stranded DNA (Figure 4.5).

TaqMan ® technique uses a sequence specific RNA or DNA-based probe to quantify DNA containing the probe sequence. This allows multiplexing, which is assaying for several genes in the same reaction by using specific probes with different-coloured labels, provided that all genes are amplified with similar efficiency. Because SYBR Green and ABI PRISM 7900HT Sequence Detection System were used for measuring mRNA of different genes it was not possible to multiplex in this present study.

The probe used in the TaqMan® real-time PCR technique is designed to bind between the primers to the minor grove or the DNA strand. Thereby a short probe is still able to bind relatively strongly to the DNA. The 5´end of the probe is labelled with a fluorescent reporter dye and the 3´end with a quencher dye which absorbs the emitted light. DNA is initially denaturated at 95 °C. Hereafter the temperature is lowered to 60 °C allowing the primers and probe to anneal to the DNA string. During the annealing phase, the primers and the probe bind to the single stranded DNA and the amplification starts. When the primers are extended, the strand extension complexes run into the probes, which will be displaced by the Taq DNA polymerase, the reporter group will be cleaved from the probe and is the capable of emitting light which is detected by the real time PCR-apparatus (Figure 4.6).
Fluorescence detected by the PCR apparatus is plotted against the PCR cycle number. After a number of cycles the reaction enters the exponential phase where the amount of DNA is doubled in each cycle. The cycle number at which the reporter due fluorescence intensity increases above a threshold value, is termed $C_t$. This value reflects the quantity of the initial target gene cDNA in the sample. The reaction is completed after 40 cycles [Bustin, 2004].

In this project the absolute quantification method is used; however, it is also possible to use a relative method in which differences in $C_t$ values between the target gene and the endogenous control are calculated.

For the absolute quantification method a standard curve with known amount of cDNA on the PCR plate and the quantity of the target gene can be estimated from the standard curve.

**Optimization of Q PCR**

The $C_t$ values have to be approximately between 15 and 30. If cDNA concentrations are too high it may disturb the amplification and low cDNA concentrations are also associated with an error due to the fact that initial errors will be more profound in high $C_t$ values. Using a dissociation curves it was tested if the primers produced primer dimers. Primer concentrations of 150 nM, 300 nM, 600 nM, and 900 nM were tested and we decided to use a concentration of 150 nM. If the concentration of primers was too low the $C_t$ value would represent the primer concentration instead of the cDNA concentration. However, this does not seem to be a problem in this case as the $C_t$ values changed as the cDNA values changed. It is important not to use higher primer concentrations that are too high as it increases the amount of primer dimers.

**Figure 4.6.** TaqMan ® Q-PCR method. The quencher prevents the detection signal from the reporter dye. When Taq polymerase reaches the reporter part of the probe the reporter part is cleaved off and when the reporter part and quencher are separated the fluorescence light is emitted and is measured [ABIb]
It was tested how large concentrations of cDNA was needed in order to obtain \( C_T \) values in the range between 15 and 30. It was decided to use 2 µL 5 ng/uL cDNA from subcutaneous abdominal fat biopsies and 1 µL of the skeletal muscles of 10 ul/L. Since the amplification of cDNA is exponential, a difference of 1 \( C_T \) values reflects a double amount of initial cDNA in the sample. A maximally acceptable difference between \( C_T \) values of sample duplicates was defined as 50 % [ABI1]. Protocol for the Q-PCR can be found in Appendix III.

### 3.2.3 Study populations

Two study populations of twins were used: Population 1 consisting of monozygotic (MZ) and same-sex dizygotic (DZ) twins and population 2 consisting of MZ and same-sex DZ young and elderly twins.

Participants were identified through the Danish Twin Register. Population 1 is population-based and consists of 586 elderly Danish twins of (67 ± 4.9 years) of whom 389 were glucose-tolerant, 118 had impaired fasting glucose, and 80 had T2D [Poulsen et al., 1999; Poulsen et al., 2002]. Among the type 2 diabetic patients 32 had known T2D and 48 had previously unknown T2D. They were examined in 1994/1995 \((n = 606)\) and in 2004/05 \((n = \sim 400)\). The individuals were selected randomly among same sex, MZ, and DZ twin pairs born in Zealand, Funen, and Jutland.

Population 2 consists of 194 MZ and DZ twins from two different age groups: elderly (62 ± 1.9 years) and young (28 ± 1.9 years) without known T2D born in Funen County. The individuals were selected as a random sample of same sex MZ and DZ
twin pairs. Among elderly ($n = 86$) 64 were NGT, 19 had IGT, and 3 had previously unknown T2D. Among the young twins ($n = 110$) 108 were NGT and 2 had IGT.

The exclusion criteria for the two populations were:

- Either twin from the pair not willing to participate
- Information of pre- or post maturity (birth before or later than three weeks from term)
- Known diabetes, serious heart, liver, or kidney disease
- Medication with influence on glucose or lipid metabolism including oral contraception which could not be withdrawn
- Pregnancy or lactation

Zygosity status of the twins was determined by serological testing. In our analysis of quantitative traits only treatment-naive individuals were included. The study was approved by the ethics comities and conducted according to the principles of the Helsinki Declaration.

**Study population examination**

Both populations underwent anthropometric measurements of height, weight, waist circumference, and hip circumference. All individuals underwent a 75 g OGTT. Blood samples were withdrawn from participants who had fasted for 12 hours. Afterwards the glucose load was ingested and peripheral venous blood was drawn 30 and 120 min later for population 1 and 30, 60, and 120 min after for population 2. Furthermore, a questionnaire about medication was given and information about the causes of their possible disease, about their familial predisposition and their birth weight data from which birth records were available was obtained. In addition, various fasting serum and plasma samples, triglycerides, total cholesterol, HDL, LDL, blood for DNA extraction were withdrawn. For population 1 biopsies were taken from abdominal subcutaneous adipose tissue.

In population 2 further measurements of VO$_2$ max was estimated by an indirectly measure of the maximal oxygen uptake per kg body weight from a maximal bicycle load. To assess insulin sensitivity the individuals underwent a 2-hour-euglycemic, hyperinsulinaemic clamp. Two biopsies were obtained from the vastus lateralis before and after the hyperinsulinaemic euglycaemic clamp. First phase insulin response during an intravenous glucose tolerance test and pancreatic $\beta$-cell function were estimated during an IVGTT.

From the clamp glucose disposal rate (Rd), hepatic glucose production (HGP), glucose- and fatty acids oxidation were obtained. By indirect calorimetry glucose oxidation (GOX) and fat oxidation (FOX) rates were estimated. Body compositions of population 2 were also measured by DXA scan with measures of total and regional body fat percentages. All physical examination were conducted by Dr. Pernille Poulsen at Odense University Hospital. Further description can be found in Appendix I.
3.2.4 Statistical analysis

Test of normality
By plotting of the variable in a residual plot they were all examined for normality. If the test for normality failed, data were transformed by natural logarithm to yield a normal distribution.

T-test
A t-test was performed to test if there were any differences in mRNA expression of lamin A and lamin C between the two age-groups in population 2.

Paired t-test
A paired t-test was used to compare if insulin stimulation influences lamin A and lamin C mRNA expression. Samples were compared before and after the clamp.

Proc mix models
Phenotypes were compared using SAS (version 9.1; SAS institute) proc MIXED models. A mixed analysis of variance (ANOVA) (referred to as proc MIXED in SAS) was used either to calculate adjusted mean values or to create linear models. As mentioned, MZ twins share their entire genome and DZ twins share half of their segregating genes. Thus observation on twin pairs can not be considered independent which is a requirement when performing an ordinary ANOVA. Furthermore, the effect of being a member of a twin pair may be different for MZ and DZ twins. A feature of the mixed ANOVA is that it makes possible adjustment for pair and zygosity status. Covariates, a variables that are possibly predictive of the outcome under study, are sex, age, BMI, zygosity, rs4641.

When using the mixed ANOVA model, it is furthermore assumed that the association between explanatory variables and response variables are linear. Another assumption is that the included response variables are independent. However, this will seldom bee true in a biological model, and some degree of interrelation may therefore be accepted. In each mixed ANOVA, residuals for the response variable were plotted versus the predicted values. The residual plot was subsequently analysed for normality. If the residuals were not normally distributed, the response variable was log transformed. In analyses where groups were compared, it was ensured, that the within group residual were equal. It was decided to adjust for sex and BMI.

3.2.5 Genotyping
Genomic DNA was extracted from blood using conventional methods. The rs4641 was genotyped using allelic discrimination performed with an ABI 7900 system (KBioscience, UK). The overall genotyping success rate was > 97 % and the error rate was 0 %. The method is described in Appendix III.
3.2.6 Methods for exon trapping

Exon trapping was used to investigate the alternative splicing of LMNA. This is a method developed to trap exons with the intention to identify new exons and was originally called exon amplification [Buckler et al., 1991]; however, in this case the purpose was to identify the effect of rs4641 on alternative splicing of LMNA. Detailed descriptions of transfection, isolation of RNA, cDNA, PCR synthesis, and primer sequences are found in Appendix XX. Before starting the analyses we investigated the splicing by the program ESEfinder 3.0., on the internet [http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home].
To investigate alternative splicing by exon trapping the aim was to amplify and insert two inserts, one containing CC (wt) and one containing TT of the rs4641 into the specialised vector, pSPL3, shown in figure 4.9. The pSPL3 vector contains an origin of
replication and APr marker for growth in E. coli, an SV40 segment provided for replication and transcription in COS-7 cells, HIV-1 tat splicing signals, and a multiple cloning site. The splicing at the splice donor and splice acceptor sites of the HIV-1 tat gene is slow, which may enhance their joining with cloned splice sites and they are compatible with splice sites from unrelated genes. The multiple cloning sites of pSPL3 are flanked by functional splice donor and acceptor sites. The pSPL3 derivatives are propagated in E. coli and the DNA is isolated (Appendix III).

The purpose was to transfect the pSPL3 containing the two different inserts into COS-7, African-green-monkey kidney cells transformed by a derivative of SV40 and permit a high level of replication and transcription from pSPL3. Splicing should occur between pSPL3 and insert sequences when the cloned DNA contains a sequence in the right orientation. If the rs4641 enhanced splicing, splicing would occur at the splice site in exon 10 more often in the construct having CC (wt) in the insert.

The cells should do alternative splicing of the constructs and the splicing should be inspected by isolating RNA (Appendix III) from the cells and subsequently synthesising cDNA and PCR amplification using vector-derived primers. If the variant caused alternative splicing different lengths of spliced products would be visible on a gel. It

**Figure 4.9.** pSPL3.
would then be possible to identify the nature of the alternative splicing by sequencing the PCR products.

**Procedure**

The pSPL3 was transformed into competent cells to amplify the plasmid, an overnight culture and glycerol stock were made with subsequent mini-preps and midi-preps using Charge switch prep-kit from Invitrogen (Appendix III). To evaluate the sequence of the plasmid it was digested with NheI, EcoRI, and HindI (Appendix III). Primers were designed for a polymerase chain reaction (PCR) to amplify the two different variants of rs4641 homozygous and wt DNA. The amplified DNA had to contain intron 9 with branch A, as it is involved in splicing, exon 10, a part of intron 10, but not branch A in intron 10. Furthermore, BamHI and XhoI sites were included in the multiple cloning site of pSPL3 and since these were not located in the *LMNA* sequence, these were used as digestion sites for the cloning transformation and the sites for these were added to the PCR-primers (Appendix III). For restriction digestion BamHI and XhoI need only two extra nucleotides surrounding the digestion site why to additional nucleotides were added at the primers.

![Figure 4.10. Overview of the sequence which the primers amplify. Two sets of primers are placed in the sequence, indicated by blue. The grey is the sequence for branch point. The pink is rs4641. The dark red tying is the exon 10.](image-url)

To prepare the pSPL3 and the insert for the transformation they were all digested with BamHI and XhoI and the digested DNA was loaded onto a gel to separate linearised plasmid from uncut DNA using Qiaquick gel extraction kit (Appendix III). The pSPL3 was further extracted using phenol chloroform extraction (Appendix III). Following, the inserts were ligated with pSPL3 using the rapid-ligation kit from Roche (Appendix III) and further transformed into competent bacteria, spread out and placed in 37 degrees overnight.
Plasmid DNA from colonies on the plates was examined either by digestion or by colony-PCR (Appendix III). Control digestion was performed by inoculation a few colonies to LB medium and growing them overnight, shaking and by 37 ° C. Mini-preps (Appendix III) were made and these were digested with BamHI and XhoI, loaded on a gel and the sizes of the bands were inspected. The bands had to be 764 nucleotides to confirm an insert in the pSPL3. Colony-PCR was used to screen a larger number of colonies for having a possible insert in a short time. A number of colonies were inoculated in water and were further used for a PCR with primers located in the pSPL3 plasmid.

When having difficulties of cloning inserts into pSPL3 or other plasmids, TOPO-cloning (Appendix III) is an alternative method (Appendix III). It is based in the use of a specialised vector, the TOPO-vector, which is used as a transit vector. Taq polymerase is used for amplifying PCR products intended for TOPO-cloning as it adds single deoxyadenosines (A´s) to the ends of the 3´ ends of the PCR-products. The linearised vector has a single overhanging deoxythymidine (T) residue and ligation of taq-PCR product is done without ligase but with the use for an activated form of topoisomerase. The TOPO-plasmid is shown in figure 4.11.

Figure 4.11. pCR 2.1-TOPO.

Ligating inserts excised from the TOPO-vector into pSPL3 should be easier than ligating PCR amplified inserts directly into the pSPL3. This is due to more exact restriction enzyme digestion of the insert when it is located in a vector, because the restriction enzymes are more precise when there are more bases surrounding the restriction site (Figure 4.12).
When the insert was ligated into the TOPO vector it had two possible directions. To examine if the direction was right, miniApreps of the colonies were performed. The miniApreps were then digested with XhoI and a combination of Pst1 and Xba1. If the insert had sense direction the XhoI restriction would result in a 800 bp section and if it was antisense it would result in a 50 bp section. By the restriction with Pst1 and Xba1 it would result in a 100 bp section for sense direction and in a 800 bp section for the antisense direction. If the product had the right direction, it would further be transfected into COS-7 cells.

3.3 Results

3.3.1 Results of association studies

Influence of rs4641 on anthropometry and body composition

We examined the rs4641 in relation to anthropometric traits in population 1 and in young and elderly twins from population 2.

Neither in population 1 of 534 treatment-naïve twins nor in population 2 of 189 twins did we find any differences in any of the anthropometric measurements between genotype groups (Table 1).
Table 4.1. Anthropometric measures of population 1 (n=534) and population 2 (n=189)

<table>
<thead>
<tr>
<th>rs4641</th>
<th>CC</th>
<th>CT</th>
<th>TT</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Population 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n (men/women)</td>
<td>283 (135/148)</td>
<td>218 (107/111)</td>
<td>33 (20/13)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>67 ± 5</td>
<td>66 ± 5</td>
<td>66 ± 5</td>
<td>0.40</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.8 ± 4.00</td>
<td>26.1 ± 4.5</td>
<td>24.9 ± 4.3</td>
<td></td>
</tr>
<tr>
<td>Waist-to-hip ratio</td>
<td>0.87 ± 0.10</td>
<td>0.88 ± 0.10</td>
<td>0.86 ± 0.07</td>
<td>0.20</td>
</tr>
<tr>
<td><strong>Population 2 young individuals</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n (men/women)</td>
<td>63 (33/30)</td>
<td>37 (21/16)</td>
<td>6 (4/2)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>28 ± 2</td>
<td>28 ± 2</td>
<td>27 ± 1</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.7 ± 2.6</td>
<td>24.5 ± 3.1</td>
<td>27.4 ± 5.3</td>
<td>0.1</td>
</tr>
<tr>
<td>Total fat (%)</td>
<td>21.9 ± 7.1</td>
<td>22.2 ± 7.0</td>
<td>23.7 ± 8.3</td>
<td>0.7</td>
</tr>
<tr>
<td>Trunk fat (%)</td>
<td>18.3 ± 6.6</td>
<td>18.6 ± 6.5</td>
<td>20.9 ± 7.8</td>
<td>0.5</td>
</tr>
<tr>
<td>Leg fat (%)</td>
<td>25.9 ± 9.7</td>
<td>26.4 ± 9.9</td>
<td>26.9 ± 10.7</td>
<td>0.6</td>
</tr>
<tr>
<td>VO₂ max (ml · kg⁻¹ · FFM · min⁻¹)</td>
<td>39.3 ± 7.7</td>
<td>39.7 ± 6.1</td>
<td>39.5 ± 15.6</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Population 2 elderly individuals</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n (men/women)</td>
<td>40 (17/23)</td>
<td>38 (15/23)</td>
<td>5 (5/0)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>63 ± 2</td>
<td>61 ± 2</td>
<td>59 ± 0</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.1 ± 3.9</td>
<td>27.0 ± 5.0</td>
<td>25.9 ± 3.8</td>
<td>0.1</td>
</tr>
<tr>
<td>Total fat (%)</td>
<td>27.6 ± 10.1</td>
<td>29.1 ± 9.1</td>
<td>20.2 ± 5.5</td>
<td>0.09</td>
</tr>
<tr>
<td>Trunk fat (%)</td>
<td>24.8 ± 11.2</td>
<td>25.7 ± 9.5</td>
<td>19.4 ± 6.9</td>
<td>0.3</td>
</tr>
<tr>
<td>Leg fat (%)</td>
<td>31.3 ± 11.2</td>
<td>33.5 ± 10.6</td>
<td>20.2 ± 5.2</td>
<td>0.6</td>
</tr>
<tr>
<td>VO₂ max (ml · kg⁻¹ · FFM · min⁻¹)</td>
<td>25.9 ± 7.05</td>
<td>25.8 ± 6.5</td>
<td>34.8 ± 5.0</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Data are means ± SD. p-values were adjusted for age, sex, and BMI. Obesity measures were not adjusted for BMI.

Influence of rs4641 on glucose tolerance and insulin secretion during an OGTT in population 1

We further investigated the rs4641 with the putative association with glucose tolerance and with insulin secretion. In population 1 (n=534) we were not able to demonstrate any differences in glucose tolerance between the three genotype groups (Table 2). Carriers of the minor T-allele had a borderline increased insulin release at 30-min during an OGTT. The incremental 30-min AUC for insulin and for C-peptide were increased among T-allele carriers. The incremental area under the curve is the area under the curve at a certain time minus the area under the curve at fasting. Additionally, T-allele carriers had significantly higher insulinogenic index. The insulinogenic index measures β-cell function during an OGTT and is defined as the ratio between AUC for insulin at 30-min divided by in AUC for glucose at 30-min.
Table 4.2. Population 1. Glucose tolerance and insulin secretion during an OGTT in relation to rs4641.

<table>
<thead>
<tr>
<th>rs4641</th>
<th>CC</th>
<th>CT</th>
<th>TT</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Population 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n (men/women)</td>
<td>283 (135/148)</td>
<td>218 (107/111)</td>
<td>33 (20/13)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>67 ± 5</td>
<td>66 ± 5</td>
<td>66 ± 5</td>
<td></td>
</tr>
<tr>
<td>Plasma glucose (mmol/l)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting</td>
<td>5.8 ± 0.9</td>
<td>5.9 ± 0.9</td>
<td>5.6 ± 0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>30-min</td>
<td>9.4 ± 1.7</td>
<td>9.4 ± 2.0</td>
<td>8.7 ± 1.8</td>
<td>0.3</td>
</tr>
<tr>
<td>120-min</td>
<td>7.2 ± 2.8</td>
<td>7.2 ± 2.8</td>
<td>6.2 ± 1.7</td>
<td>0.2</td>
</tr>
<tr>
<td>Inc AUC 30-min</td>
<td>53.7 ± 18.9</td>
<td>52.5 ± 22.2</td>
<td>46.1 ± 22.0</td>
<td>0.2</td>
</tr>
<tr>
<td>Inc AUC 120-min</td>
<td>276.1 ± 143.6</td>
<td>271.1 ± 149.9</td>
<td>208.2 ± 124.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Plasma insulin (pmol/l)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting</td>
<td>44 ± 25</td>
<td>45 ± 27</td>
<td>40 ± 26</td>
<td>1.0</td>
</tr>
<tr>
<td>30-min</td>
<td>321 ± 244</td>
<td>286 ± 183</td>
<td>348 ± 279</td>
<td>0.05</td>
</tr>
<tr>
<td>120-min</td>
<td>291 ± 261</td>
<td>314 ± 314</td>
<td>249 ± 183</td>
<td>0.7</td>
</tr>
<tr>
<td>Inc AUC 30-min</td>
<td>4,153 ± 34,13</td>
<td>3,618 ± 2497</td>
<td>4,615 ± 3,991</td>
<td>0.03</td>
</tr>
<tr>
<td>Inc AUC120-min</td>
<td>27,710 ± 21,263</td>
<td>26,564 ± 20,293</td>
<td>27,869 ± 19,976</td>
<td>0.50</td>
</tr>
<tr>
<td>Insulinogenic index</td>
<td>86 ± 79</td>
<td>82 ± 100</td>
<td>118 ± 107</td>
<td>0.006</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.7 ± 1.1</td>
<td>1.8 ± 1.2</td>
<td>1 ± 1</td>
<td>1.00</td>
</tr>
<tr>
<td>HOMA-IS</td>
<td>57.8 ± 32.9</td>
<td>55.9 ± 27.8</td>
<td>53 ± 26</td>
<td>0.50</td>
</tr>
<tr>
<td>C-peptide (pmol/l)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting</td>
<td>683.5 ± 287.1</td>
<td>689.7 ± 331.1</td>
<td>619.4 ± 259.3</td>
<td>0.60</td>
</tr>
<tr>
<td>30-min</td>
<td>2,180.5 ± 917.0</td>
<td>2,066.4 ± 856.7</td>
<td>2,322.9 ± 1093.4</td>
<td>0.04</td>
</tr>
<tr>
<td>120-min</td>
<td>3,153.6 ± 1226.4</td>
<td>3,236.5 ± 1471.7</td>
<td>2,884.0 ± 1243.9</td>
<td>0.90</td>
</tr>
<tr>
<td>Inc AUC 30-min</td>
<td>22,484.1 ± 11,800.8</td>
<td>20,650.7 ± 10220.8</td>
<td>25,555.0 ± 14,648.4</td>
<td>0.06</td>
</tr>
<tr>
<td>Inc AUC 120-min</td>
<td>200,902.6 ± 75,114.8</td>
<td>197,208.8 ± 82949.1</td>
<td>204,132.7 ± 87,273.8</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Data are means ± SD. p-values were adjusted for age, sex, and BMI. Insulin, and values derived from insulin were logarithmically transformed before analyses. Inc incremental; AUC area under the curve.
Influence of rs4641 on insulin secretion during an IVGTT in population 2

In young individuals from population 2, the T-allele carriers had increased fasting plasma insulin; however, we did not demonstrate the same effect in the elderly twins.

Table 4.3. Population 2, young (n = 83) and elderly twins. Insulin secretion during an IVGTT in relation to rs4641

<table>
<thead>
<tr>
<th>Rs4641</th>
<th>CC</th>
<th>CT</th>
<th>TT</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Population 2 young twins</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n (men/women)</td>
<td>63 (33/30)</td>
<td>37 (21/16)</td>
<td>6 (4/2)</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>28 ± 2</td>
<td>28 ± 2</td>
<td>27 ± 1</td>
<td></td>
</tr>
<tr>
<td><strong>Plasma Insulin (pmol/l)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting</td>
<td>31 ± 10</td>
<td>34 ± 20</td>
<td>57 ± 20</td>
<td>0.006</td>
</tr>
<tr>
<td>IncAUC 10-min</td>
<td>2,836 ± 1903</td>
<td>2,557 ± 1799</td>
<td>3,395 ± 2,011</td>
<td>0.6</td>
</tr>
<tr>
<td><strong>Population 2 elderly twins</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n (men/women)</td>
<td>40 (17/23)</td>
<td>38 (15/23)</td>
<td>5 (5/0)</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>63 ± 2</td>
<td>61 ± 2</td>
<td>59 ± 0</td>
<td></td>
</tr>
<tr>
<td><strong>Plasma Insulin (pmol/l)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting</td>
<td>33 ± 18</td>
<td>29 ± 22</td>
<td>28 ± 22</td>
<td>0.2</td>
</tr>
<tr>
<td>IncAUC 10-min</td>
<td>2,059 ± 1821</td>
<td>1,829 ± 1267</td>
<td>1,061 ± 396</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Data are means ± SD. p-values were adjusted for sex and BMI. Insulin and values derived from insulin were logarithmically transformed before analyses. Inc: incremental. AUC: area under the curve.

Influence of rs4641 and peripheral insulin sensitivity of study population 2

The study group 2 was examined for hepatic and peripheral insulin sensitivity and differences in genotype distribution for the different traits were analysed. The young T-allele carriers were more insulin resistant in the liver at the basal level. After the clamp the differences disappeared. We calculated the difference in hepatic glucose production at base line and after the clamp and compared the differences between genotypes groups; however, we were not able to demonstrate any significant differences neither for the young twins nor for the elderly twins.
Table 4.4. Hepatic and peripheral insulin action in young (n=106) and elderly (n=84) twins. IVGTT in relation to LMNA rs4641 genotype

<table>
<thead>
<tr>
<th>Rs4641</th>
<th>CC</th>
<th>CT</th>
<th>TT</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Population 2 – young twins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N(men/women)</td>
<td>63 (33/30)</td>
<td>37 (21/16)</td>
<td>6 (4/2)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>28 ± 2</td>
<td>28 ± 2</td>
<td>27 ± 1</td>
<td></td>
</tr>
<tr>
<td>Basal (mg · kg⁻¹ · min⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatic glucose production (HGP)</td>
<td>3.1 ± 0.6</td>
<td>3.0 ± 0.5</td>
<td>2.8 ± 0.4</td>
<td>0.7</td>
</tr>
<tr>
<td>Glucose disposal rate (Rd)</td>
<td>3.1 ± 0.5</td>
<td>3.0 ± 0.5</td>
<td>2.9 ± 0.5</td>
<td>0.6</td>
</tr>
<tr>
<td>Glucose oxidation</td>
<td>3.1 ± 0.6</td>
<td>3.0 ± 1.4</td>
<td>0.4 ± 1.2</td>
<td>0.40</td>
</tr>
<tr>
<td>Non-oxidative glucose metabolism</td>
<td>1.1 ± 0.9</td>
<td>0.7 ± 1.0</td>
<td>1.0 ± 0.5</td>
<td>0.2</td>
</tr>
<tr>
<td>Hepatic insulin resistance index</td>
<td>102.9 ± 37.0</td>
<td>116.6 ± 52.9</td>
<td>185.9 ± 42.6</td>
<td>0.005</td>
</tr>
<tr>
<td>Clamp (mg · kg⁻¹ · min⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatic glucose production (HGP)</td>
<td>1.6 ± 0.5</td>
<td>1.4 ± 0.4</td>
<td>1.2 ± 0.4</td>
<td>0.03</td>
</tr>
<tr>
<td>Glucose disposal rate (Rd)</td>
<td>11.9 ± 2.9</td>
<td>11.8 ± 3.4</td>
<td>9.5 ± 3.9</td>
<td>0.9</td>
</tr>
<tr>
<td>Glucose oxidation</td>
<td>4.8 ± 1.3</td>
<td>4.8 ± 1.4</td>
<td>3.6 ± 0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>Non-oxidative glucose metabolism</td>
<td>7.1 ± 2.7</td>
<td>7.1 ± 2.9</td>
<td>5.9 ± 3.3</td>
<td>0.9</td>
</tr>
<tr>
<td>Disposition index -10⁻⁸</td>
<td>22.9 ± 9.0</td>
<td>28.8 ± 7.7</td>
<td>5.9 ± 4.6</td>
<td>0.9</td>
</tr>
<tr>
<td>HGPbasal/HGpclamp</td>
<td>1.6 ± 0.6</td>
<td>1.6 ± 0.6</td>
<td>1.6 ± 0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>Population 2 – elderly twins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N(men/women)</td>
<td>40 (17/23)</td>
<td>38 (15/23)</td>
<td>5 (5/0)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>63 ± 2</td>
<td>61 ± 2</td>
<td>59 ± 0</td>
<td></td>
</tr>
<tr>
<td>Basal (mg · kg⁻¹ · min⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatic glucose production (HGP)</td>
<td>3.2 ± 0.5</td>
<td>3.0 ± 0.3</td>
<td>2.7 ± 0.2</td>
<td>0.05</td>
</tr>
<tr>
<td>Glucose disposal rate (Rd)</td>
<td>3.2 ± 0.4</td>
<td>3.1 ± 0.4</td>
<td>2.7 ± 0.1</td>
<td>0.05</td>
</tr>
<tr>
<td>Glucose oxidation</td>
<td>2.0 ± 1.1</td>
<td>1.6 ± 0.8</td>
<td>1.5 ± 0.5</td>
<td>0.1</td>
</tr>
<tr>
<td>Non-oxidative glucose metabolism</td>
<td>1.2 ± 1.1</td>
<td>1.5 ± 0.9</td>
<td>1.2 ± 0.5</td>
<td>0.3</td>
</tr>
<tr>
<td>Hepatic insulin resistance index</td>
<td>124.9 ± 107.4</td>
<td>120.12 ± 79.0</td>
<td>68.8 ± 49.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Clamp (mg · kg⁻¹ · min⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatic glucose production (HGP)</td>
<td>1.7 ± 0.8</td>
<td>1.59 ± 0.6</td>
<td>1.3 ± 0.4</td>
<td>0.2</td>
</tr>
<tr>
<td>Glucose disposal rate (Rd)</td>
<td>10.1 ± 3.8</td>
<td>10.0 ± 2.7</td>
<td>8.4 ± 4.2</td>
<td>0.9</td>
</tr>
<tr>
<td>Glucose oxidation</td>
<td>4.4 ± 1.5</td>
<td>4.0 ± 1.0</td>
<td>2.5 ± 0.6</td>
<td>0.1</td>
</tr>
<tr>
<td>Non-oxidative glucose metabolism</td>
<td>5.8 ± 3.7</td>
<td>6.1 ± 2.5</td>
<td>5.8 ± 3.9</td>
<td>0.6</td>
</tr>
<tr>
<td>Disposition index -10⁻⁸</td>
<td>12.0 ± 9.0</td>
<td>2.8 ± 7.7</td>
<td>5.9 ± 4.6</td>
<td>0.9</td>
</tr>
<tr>
<td>HGPbasal/HGpclamp</td>
<td>1.5 ± 0.8</td>
<td>1.4 ± 0.6</td>
<td>1.37 ± 0.5</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Data are means ± SD. p-values are adjusted for sex and BMI. Values derived from insulin were logarithmically transformed before analyses.

Anthropometry and metabolism in dizygotic twins discordant for T-allele dosage of rs4641

Investigating DZ twins discordant for allele dosage allows the adjustment for common gene variants (approximately 50 %) and common environmental factors. Twin pairs discordant for T-allele dosage were identified in both populations and compared. In discordant DZ pairs from population 1 (n = 63 pairs) the T-allele was associated with increased fasting plasma glucose and increased plasma glucose at 120-min during an OGTT. The T-allele was also associated with decreased fasting C-peptide and decreased C-peptide incremental area under the curve 120-min during an OGTT.
### Table 4.5. Dizygotic twins discordant for T-allele dosage of rs4641 in population 1

<table>
<thead>
<tr>
<th>Population 1</th>
<th>0-1 T-alleles</th>
<th>1-2 T-alleles</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (men/women)</td>
<td>63 (35/28)</td>
<td>63 (35/28)</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>67 ± 5</td>
<td>66 ± 5</td>
<td>0.9</td>
</tr>
<tr>
<td>BMI</td>
<td>25.57 ± 3.7</td>
<td>25.64 ± 4.1</td>
<td></td>
</tr>
<tr>
<td>Plasma glucose (mmol/l)</td>
<td></td>
<td></td>
<td>0.006</td>
</tr>
<tr>
<td>Fasting</td>
<td>5.62 ± 0.6</td>
<td>6.13 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>30-min</td>
<td>9.12 ± 1.4</td>
<td>9.63 ± 2.5</td>
<td>0.2</td>
</tr>
<tr>
<td>120-min</td>
<td>6.50 ± 1.5</td>
<td>8.12 ± 4.6</td>
<td>0.01</td>
</tr>
<tr>
<td>Inc AUC 30-min</td>
<td>52.64 ± 18.69</td>
<td>52.43 ± 22.9</td>
<td>1.0</td>
</tr>
<tr>
<td>Inc AUC 120-min</td>
<td>298.93 ± 213.8</td>
<td>250.29 ± 90.7</td>
<td>0.1</td>
</tr>
<tr>
<td>Plasma insulin (pmol/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting</td>
<td>43.00 ± 24.1</td>
<td>42.51 ± 26.1</td>
<td>0.9</td>
</tr>
<tr>
<td>30-min</td>
<td>342.27 ± 291.6</td>
<td>276.41 ± 238.2</td>
<td>0.1</td>
</tr>
<tr>
<td>120-min</td>
<td>297.20 ± 285.2</td>
<td>249.44 ± 193.0</td>
<td>0.1</td>
</tr>
<tr>
<td>Inc AUC 30-min</td>
<td>4,489.52 ± 4,158.3</td>
<td>3,508.57 ± 3,382.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Inc AUC 120-min</td>
<td>29186.95 ± 24887.5</td>
<td>23346.43 ± 18462.6</td>
<td>0.06</td>
</tr>
<tr>
<td>C-peptide</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting</td>
<td>673.76 ± 296.715</td>
<td>634.75 ± 296.5</td>
<td>0.4</td>
</tr>
<tr>
<td>30-min</td>
<td>2121.12 ± 1061.4</td>
<td>1,942.86 ± 1,044.0</td>
<td>0.1</td>
</tr>
<tr>
<td>120-min</td>
<td>3,195.69 ± 1321.4</td>
<td>2,847.70 ± 1264.3</td>
<td>0.04</td>
</tr>
<tr>
<td>Inc AUC 30-min</td>
<td>23075.32 ± 13916.5</td>
<td>19,621.7 ± 13,261.4</td>
<td>0.03</td>
</tr>
<tr>
<td>Inc AUC 120-min</td>
<td>88914.6</td>
<td>83303.7</td>
<td></td>
</tr>
<tr>
<td>Insulinogenic index</td>
<td>95.8 ± 112.1</td>
<td>81.8 ± 95.4</td>
<td>0.4</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.7 ± 0.9</td>
<td>1.7 ± 1.3</td>
<td>0.4</td>
</tr>
<tr>
<td>HOMA-IS</td>
<td>60.2 ± 38.1</td>
<td>51.8 ± 30.6</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Data are means ± SD. The twins who had 0-1 T-alleles were compared with twins who had 1-2 T alleles.

In population 2 there was no association investigating DZ twins discordant for allele dosage for the rs4641 in 14 pairs.

### Table 4.6. Dizygotic twins discordant for T-allele dosage of rs4641 in population 1

<table>
<thead>
<tr>
<th>Population 2</th>
<th>0-1 T-alleles</th>
<th>1-2 T-alleles</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (men/women)</td>
<td>14 (6/8)</td>
<td>25 (6/8)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>44.9 ± 17.8</td>
<td>44.9 ± 17.8</td>
<td>0.05</td>
</tr>
<tr>
<td>BMI</td>
<td>23.5 ± 3.0</td>
<td>25.6 ± 3.7</td>
<td></td>
</tr>
<tr>
<td>Plasma glucose (mmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting</td>
<td>5.3 ± 0.7</td>
<td>5.2 ± 0.3</td>
<td>0.7</td>
</tr>
<tr>
<td>30-min</td>
<td>8.4 ± 1.6</td>
<td>8.1 ± 1.3</td>
<td>0.4</td>
</tr>
<tr>
<td>60-min</td>
<td>6.5 ± 1.0</td>
<td>6.0 ± 1.2</td>
<td>0.05</td>
</tr>
<tr>
<td>Plasma insulin (pmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting</td>
<td>25.55 ± 19.6</td>
<td>32.02 ± 20.3</td>
<td>0.2</td>
</tr>
<tr>
<td>Inc AUC 10-min</td>
<td>83.15 ± 8.7</td>
<td>88.84 ± 16.6</td>
<td>0.2</td>
</tr>
<tr>
<td>Basal (mg · kg⁻¹ · min⁻¹)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatic glucose production</td>
<td>3.12 ± 0.3</td>
<td>3.08 ± 0.5</td>
<td>0.7</td>
</tr>
<tr>
<td>Glucose disposal rate (Rd)</td>
<td>3.13 ± 0.28</td>
<td>3.04 ± 0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Glucose oxidation</td>
<td>1.89 ± 0.79</td>
<td>2.29 ± 1.05</td>
<td>0.3</td>
</tr>
<tr>
<td>Non-oxidative glucose metabolism</td>
<td>1.23 ± 0.8</td>
<td>0.75 ± 1.0</td>
<td>0.2</td>
</tr>
<tr>
<td>Clamp (mg · kg⁻¹ · min⁻¹)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatic glucose production</td>
<td>1.49 ± 0.92</td>
<td>1.65 ± 0.48</td>
<td>0.4</td>
</tr>
<tr>
<td>Glucose disposal rate (Rd)</td>
<td>12.00 ± 2.8</td>
<td>11.43 ± 2.3</td>
<td>0.5</td>
</tr>
<tr>
<td>Glucose oxidation</td>
<td>4.78 ± 1.4</td>
<td>4.88 ± 1.4</td>
<td>0.8</td>
</tr>
<tr>
<td>Non-oxidative glucose metabolism</td>
<td>7.22 ± 2.4</td>
<td>6.54 ± 2.2</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Data are means ± SD. The twins who had 0-1 T-alleles were compared with twins who had 1-2 T alleles.
3.3.2 Results of gene expression

The LMNA mRNA levels were investigated in adipose tissue of twins from population 1 and in skeletal muscle of twins from population 2 to examine if there were any differences between genotype groups in gene expression. Furthermore, the aim was to investigate if differences in levels of lamin A, lamin C, and the ratio between lamin A and C were associated with any pre-diabetic traits.

There were no differences between the genotype groups in mRNA levels of lamin A, lamin C, and lamin A to C ratio in population 1. In young twins from population 2 there were no differences in lamin A, lamin C, and the ratio between lamin A and C before the clamp between the genotype groups; however, after the clamp there was a significant increase in lamin A for the T-allele carriers, but there was no difference in lamin C and lamin A to C ratio after the clamp between the genotype groups. In elderly twins from population 2 there were no differences between the genotype groups except for the lamin A to C ratio which was increased after the clamp (Table 4.7).

Table 4.7. mRNA expression of lamin A and lamins C and the ratio lamin A/C in relation to rs4641

<table>
<thead>
<tr>
<th>Population I</th>
<th>C/C</th>
<th>C/T</th>
<th>T/T</th>
<th>p&lt;sub&gt;add&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>73.5 ± 5.2</td>
<td>73.5 ± 5.2</td>
<td>73.5 ± 5.2</td>
<td>0.7</td>
</tr>
<tr>
<td>Lamin A (n = 122)</td>
<td>1.0 ± 0.9</td>
<td>0.9 ± 1.0</td>
<td>0.8 ± 0.3</td>
<td>0.8</td>
</tr>
<tr>
<td>Lamin C (n = 127)</td>
<td>1.1 ± 0.8</td>
<td>1.1 ± 1.0</td>
<td>1.0 ± 0.33</td>
<td>0.8</td>
</tr>
<tr>
<td>Lamin A/C (n = 124)</td>
<td>0.5 ± 0.26</td>
<td>1.1 ± 3.5</td>
<td>0.6 ± 0.3</td>
<td>0.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Population 2 young twins</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>28 ± 2</td>
<td>28 ± 2</td>
<td>27 ± 1</td>
<td>0.3</td>
</tr>
<tr>
<td>Lamin A&lt;sub&gt;basal&lt;/sub&gt; (n = 23)</td>
<td>1.9 ± 2.0</td>
<td>0.8 ± 1.1</td>
<td>2.2 ± 3.0</td>
<td>0.03</td>
</tr>
<tr>
<td>Lamin C&lt;sub&gt;basal&lt;/sub&gt; (n = 39)</td>
<td>0.8 ± 0.5</td>
<td>0.6 ± 0.4</td>
<td>0.4 ± 0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>Lamin A/C&lt;sub&gt;basal&lt;/sub&gt; (n = 2)</td>
<td>2.1 ± 0.7</td>
<td>3.1 ± 2.7</td>
<td>0.4 ± -</td>
<td>0.7</td>
</tr>
<tr>
<td>Lamin A&lt;sub&gt;post&lt;/sub&gt; (n = 26)</td>
<td>2.5 ± 1.8</td>
<td>1.3 ± 1.2</td>
<td>2.9 ± 2.6</td>
<td></td>
</tr>
<tr>
<td>Lamin C&lt;sub&gt;post&lt;/sub&gt; (n = 42)</td>
<td>0.9 ± 0.5</td>
<td>0.5 ± 0.3</td>
<td>0.6 ± 0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>Lamin A/C&lt;sub&gt;post&lt;/sub&gt; (n = 25)</td>
<td>2.2 ± 2.1</td>
<td>2.2 ± 2.1</td>
<td>3.8 ± 0.7</td>
<td>0.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Population 2 elderly twins</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>63 ± 2</td>
<td>61 ± 2</td>
<td>59 ± 0</td>
<td></td>
</tr>
<tr>
<td>Lamin A&lt;sub&gt;basal&lt;/sub&gt; (n = 10)</td>
<td>3.4 ± 5.3</td>
<td>3.8 ± 4.0</td>
<td>5.9 ± -</td>
<td></td>
</tr>
<tr>
<td>Lamin C&lt;sub&gt;basal&lt;/sub&gt; (n = 18)</td>
<td>8.2 ± 9.3</td>
<td>5.4 ± 3.0</td>
<td>3.5 ± 4.7</td>
<td></td>
</tr>
<tr>
<td>Lamin A/C&lt;sub&gt;basal&lt;/sub&gt; (n = 21)</td>
<td>0.9 ± 0.3</td>
<td>0.9 ± 0.3</td>
<td>0.6 ± 0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Lamin A&lt;sub&gt;post&lt;/sub&gt; (n = 13)</td>
<td>6.6 ± 9.7</td>
<td>8.8 ± 6.7</td>
<td>6.7 ± -</td>
<td>0.3</td>
</tr>
<tr>
<td>Lamin C&lt;sub&gt;post&lt;/sub&gt; (n = 26)</td>
<td>7.9 ± 7.2</td>
<td>10.3 ± 10.3</td>
<td>7.9 ± 8.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Lamin A/C&lt;sub&gt;post&lt;/sub&gt; (n = 18)</td>
<td>0.9 ± 0.4</td>
<td>1.7 ± 0.6</td>
<td>1.4 ± -</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Data are mean ± SD. The level of mRNA LMNA expression is normalized to the mRNA expression of cyclophilin A. The p-values are calculated using a dominant model. Some of the standard deviations were not possible to calculate as too few people had the CC-genotype.

To investigate if there were any differences in gene expression between young and elderly individuals they were compared with a t-test (Table 4.8). All the p-values were significant and the expression for lamin A and lamin C before and after the clamp was increased in elderly twins compared with the young twins; however, the ratios of lamin A to C before and after the clamp were decreased (Table 4.8).
Table 4.8. mRNA expression between young and elderly twins in population 2 before and after a hyperinsulinaemic euglycaemic clamp independently of genotype of rs4641.

<table>
<thead>
<tr>
<th></th>
<th>Lamin A_{basal}</th>
<th>Lamin C_{basal}</th>
<th>Lamin A/C_{basal}</th>
<th>Lamin A_{post}</th>
<th>Lamin C_{post}</th>
<th>Lamin A/C_{post}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pop 2 young (n = 20)</td>
<td>1.53 ± 1.7</td>
<td>0.70 ± 0.4</td>
<td>2.4 ± 2.0</td>
<td>1.79 ± 1.6</td>
<td>0.73 ± 0.5</td>
<td>2.7 ± 2.3</td>
</tr>
<tr>
<td>Pop 2 elderly (n = 13)</td>
<td>3.67 ± 4.1</td>
<td>6.6 ± 7.3</td>
<td>0.86 ± 0.3</td>
<td>7.45 ± 7.9</td>
<td>8.47 ± 8.1</td>
<td>1.13 ± 0.6</td>
</tr>
<tr>
<td>P</td>
<td>0.033</td>
<td>2.16 · 10^{-6}</td>
<td>5.8 · 10^{-4}</td>
<td>1.4 · 10^{-3}</td>
<td>2.5 · 10^{-6}</td>
<td>9.3 · 10^{-3}</td>
</tr>
</tbody>
</table>

Data are mean ± SD of mRNA expression normalized to the mRNA expression of cyclophilin A.

Figure 4.12. Data are mean ± SD of mRNA expression normalized to the mRNA expression of cyclophilin A. The level of mRNA lamin A and lamin C expression are normalized to the mRNA expression of cyclophilin A. mRNA expression for young compared to elderly twins at baseline and post hyperinsulinaemic euglycaemic clamp. E means elderly twins and Y means young twins.

Also we investigated if the mRNA expression of lamin A, C, and lamin A/C ratio was influenced by insulin stimulation with a paired T-test comparing the data before and after the clamp (Table 4.9). There were no differences before and after the clamp in population 1 or in population 2.

Table 4.9. mRNA expression in population 2 of lamin A, C, and A/C ratio at baseline and after a hyperinsulinaemic euglycaemic clamp compared with a paired t-test

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th>P</th>
<th>Elderly</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lamin A_{basal}</td>
<td>1.23 ± 1.40</td>
<td></td>
<td>103.2 ± 30.2</td>
<td></td>
</tr>
<tr>
<td>Lamin A_{post}</td>
<td>1.7 ± 2.00</td>
<td>0.17</td>
<td>800 ± 1121</td>
<td>0.53</td>
</tr>
<tr>
<td>Lamin C</td>
<td>0.77 ± 0.48</td>
<td>15.5 ± 27.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lamin C_{post}</td>
<td>0.62 ± 0.37</td>
<td>0.26</td>
<td>7.6 ± 4.5</td>
<td>0.31</td>
</tr>
<tr>
<td>Lamin A/C_{basal}</td>
<td>2.54 ± 2.29</td>
<td></td>
<td>0.8 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Lamin A/C_{post}</td>
<td>2.81 ± 2.83</td>
<td>0.70</td>
<td>1.3 ± 0.8</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Data are mean ± SD. Expression of mRNA for lamin A and lamin C is normalized to the mRNA expression of cyclophilin A. Data are mRNA expression for baseline and post hyperinsulinaemic euglycaemic clamp.
3.3.3 Results of exon trapping experiments

By investigating the influence of rs4641 on splicing of LMNA by ESEfinder 3.0 it was shown that the splicing changed when T was included for the rs4641 in the sequence investigated compared to when the C (wt) was included. The score for the wt for the 5SSU2 protein was higher than for the genotype homozygous for the T-allele indicating that splicing is more enhanced in C-allele carriers. Thus higher levels of lamin A is expected in the CC genotype.
Minipreps of the pSPL3 were done and the digestion of the restriction with NheI, EcoRI, and HindI are shown on gel number 1. There are three replicated minipreps all digested with EcoRI and three with NheI and three with HindI. The restrictions elucidated that the plasmid truly was pSPL3.

Gel number 4.1
1. Digested by HindI and EcoRI (fragments at 3514, 1805, and 712 were expected)
2. Digested with NheI and EcoRI (fragments of 5107 and 924 were expected)
3. Digested with NheI and EcoRI
4. Digested with HindI and EcoRI
5. Digested with HindI and EcoRI
6. Digested with NheI and EcoRI. The marker in the right is 1KB+. They all have the expected sizes. Lane number 3 and number 6 are not digested completely.

The cloning procedure was repeated numerous times; however, when examining the clones obtained by digestion with BamHI and XhoI none of the colonies had any insert (gel number 2). When repeating the cloning, different attempts were made to ligate the inserts with the plasmids: New-PCR products were used, different concentrations of inserts and pSPL3, Fermentas ligation kit used instead of Roche rapid ligation kit. To be sure that the multiple cloning site had the described sequence, it was sequenced by MWG Biotech and it showed that the sequence was as expected.
Afterwards new primers were designed containing new restriction sites PstI and XhoI. By using the new primers genomic DNA was amplified. The PCR-product was inserted into the TOPO-vector and by digestion of mini-preps of the TOPO-clones it turned out that two of the clones contained the insert of 764 nucleotides. The mini-preps were digested with EcoRI as the TOPO-vector contained EcoR1 restriction sites surrounding the PCR-product. The inserts were contained in number 2 and 5.

**Gel number 4.2.** From the left: two wt, two homozygous for the T-allele, and 1kb + marker. Wt and homozygous are digested with BamH1 and Xho1

Gel number 4.3. Number 3 and 5 clones have inserts of 764 bp. The marker to the left is 1kb plus.
The insert in the TOPO-vector have two possible directions. To examine the direction of the insets restrictions of the mini-pres was done with XhoI and a combination of PstI and XbaI. If the insert is in sense direction the XhoI restriction will result in a 800 bp section and if it is antisense it will result in a 50 bp section. By the restriction with PstI and XbaI it will for sense direction result in a 100 bp section and for the antisense direction in an 800 bp section.

Clone number 3: The first lane has a band at about 800 indicating that the insert has sense direction. The two bands in lane number two has two double bands at about 1000 and 3000 bp this indicates that the insert is a mix between two clones of which only one of them has the insert. Clone number 5: The large bands of restriction of clone number 5 indicate that the clone does not have an insert after all.

I tried to clone the insert in clone number 3 into pSLP3 by digesting with PstI and XhoI followed by ligation and transformation. However, this cloning failed. Thus despite several approaches, it was impossible to clone an inset into pPSL3.

When the constructs are finally made they should be transfected into the COS-7 cells and the remaining part of the exon trapping procedure may be carried out as described in the method section.

---

**Gel number 4.4. Analysing the direction of The inserts.**

1: 1 kb+ DNA ladder
2: number 3 digested with XhoI
3: number 3 digested with PstI and XbaI
4: number 5 digested with XhoI
5: number 5 digested with PstI and XbaI
3.4 Discussion

In this study we examined the influence of rs4641 on different T2D-related traits and the splicing of LMNA. Furthermore, we investigated if rs4641 influenced expression of LMNA and if there was any correlation between the gene expression and the traits associated with rs4641.

We found no association with any of the anthropometric traits in the population 1 or 2 and rs4641 genotype. By investigating glucose tolerance and insulin secretion during an OGTT we found that carriers of the minor T-allele in population 1 had a borderline increased plasma insulin secretion and plasma C-peptide at 30-min during an OGTT, and the incremental area under the curve for plasma insulin at 30-min during an OGTT was increased among T-allele carriers; however, considering the fact that these traits were not included in our primary hypothesis these significant values would not survive correction for multiple testing.

The TA-allele carriers in population 1 had an increased insulinogenic index, which is a measure of the β-cell function suggesting that the T-allele carriers in population 1 had an increased β-cell function compared with non-T-allele carriers. For this index the CT-genotype individuals had a slightly higher value; however, taken the large standard deviation in consideration for the three genotypes there is an increased index for the T-allele carriers. The p-value is 0.006 and might not survive a correction for multiple testing.

The twins from population 2 underwent an IVGTT. Before the IVGTT the young T-allele carriers had increased fasting serum insulin level; however, we were not able to demonstrate this association in the elderly twins. In addition, in population 2 the young T-allele carriers were more insulin resistant in the liver before the 2-hour euglycaemic hyperinsulinaemic clamp and after the clamp they had lower hepatic glucose production. The increased hepatic insulin resistance is a pre-diabetic trait while decreased hepatic glucose production is not; however, the elderly T-allele carriers also had borderline lower hepatic glucose production before the clamp.

By investigation of discordant DZ pairs from population 1 (n = 63 pairs) we found that the T-allele was associated with increased fasting plasma glucose and increased plasma glucose at 120-min during an OGTT in accordance with the finding of Wegner et al. [2007]. The T-allele was also associated with decreased fasting C-peptide and decreased C-peptide incremental area under the curve at 120-min during an OGTT. We were not able to demonstrate the same effect in population two. It is still unknown if the young twins from population 2 will develop diabetes and pre-diabetic traits later in life and they might still be too young to show any pre-diabetic traits. The elderly twins in population 2 are selected for not having T2D why these twins are healthier than the general population and these individuals do not represent the Danish population.
This investigation of discordant DZ pairs is a very powerful test and the elevated plasma glucose among T-allele carriers is in accordance with earlier results [Wegner et al., 2007]. However, we were not able to find an association of rs4641 with fasting plasma glucose as it was demonstrated in the Inter99 cohort [Wegner et al., 2007] when the twins were investigated as non-related individuals. This might be due to lower power in the study compared to the study in Inter99 which included many more individuals.

We have not been able to calculate power in the study as the participants are twins and therefore strongly related and why a normal power calculation can not be used; however, the study only included 746 individuals, which does not gives a high power. We did not find any convincing association with any pre-diabetic traits in the elderly twins in population 2 which might be due to strict inclusion criteria as they are selected for not having T2D. We investigated if the variants in high LD with rs4641 were investigates in other GWA studies and if they had been associated with any disease or metabolic traits. This was not the case.

In further studies it would be interesting to investigate the association with fasting plasma glucose in a larger sample trying to replicate the finding in the Inter99 cohort. Furthermore, it would be interesting to investigate determinants of tissue LMNA expression including fat percentage, VO_{2} max, birth weight, zygosity and sex. This could be done by multiple regression analyses with a stepwise elimination of insignificant co-variables until obtaining the final reduced models.

**Expression studies**

For the expression studies only very few individuals passed our inclusion criteria which were: Replicates had to be available for both A, C, and cyclophilin A and for population 2 furthermore samples had to be available both before and after the euglycaemic hyperinsulinaemic clamp. The replicates were excluded if the C_{T} values differed more than 40 %. Because of large variation we priori decided only to include values ± 2 SD´s from the mean. This resulted in very low power of the study as most of the samples were excluded; however; the calculations were carried out anyway.

We investigated the putative effect of rs4641 on the mRNA levels of LMNA. We found no differences in mRNA expression between the genotypes in population 1. In young twins from population 2 we found a significant increased lamin A mRNA level for the T-allele carriers after the clamp. Furthermore, there was an increased lamin A/C ratio for the elderly twins after the clamp. By comparing the expression of lamin A and C between young and elderly twins we found significant increased expression in elderly twins; however, the ratio of lamin A to C was decreased in elderly twins. The data show very low p-values probably due to errokes in the study and as very few individuals are included in the study, the data are not due to true differences between the young and elderly twins.
Because of the very few individuals included in the expression study of mRNA of lamin A, lamin C and cyclophilin A the association is not convincing. We had to distinguish between lamin A and lamin C we only had very few nucleotides at which we could place the primers why we had to design the assay ourselves and because of this assay design there was only room for 2 µL cDNA in each sample. Accuracy of pipetting only 2 µL is difficult to achieve and the huge variation within the samples might be due to inaccuracy in pipetting. It might also be due insufficient optimising of the PCR reaction. It is optimal to run triplets as recommended by the kit and instrument manufacturer (ABI). We only ran duplicates in order to save reagents and due to limited access to mRNA samples.

If it was possible to design sequence specific probes for the two isoforms it would be of interest to multiplex the PCR. Then the three genes could be measured in one reaction only which get a quite accurate ratio, although the duplicates or triplicates still would have to pass our quality requirements. Furthermore, by multiplex only needed to run 1/3 of the reactions had to be done. Nevertheless, this was not possible in this study because the nucleotide difference between the two isoforms did not leave us with enough nucleotides to design isoform-specific probes and primers. To rerun the samples it would be preferable to redesign the assay in which larger volumes of cDNA are used.

**Exon trapping**

Unfortunately, we did not manage to get any results of the exon trapping experiments. It would have given us important knowledge of the splicing of *LMNA*. By knowing if the rs4641 influences alternative splicing and changes the ratio of lamin A to C it is possible to hypothesize if it is due to changed ratio of the lamin A to C that rs4641 is associated with T2D and other quantitative traits.

By ESEfinder (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi) it is possible to evaluate the splicing of a given gene. ESEfinder estimate putative binding sites for splicosomes for a sequence of interest. By investigating the splicing of *LMNA* by ESEfinder we found that splicing was more enhanced in the C-allele compared with in T-allele. Increased splicing in the C-allele is predicted to result in higher levels of the lamin A isoform and a higher A to C ratio. At the time I received the pSPL3 from David Calabrese, PRA, Maloney Lab, Pulmonary & CCM, UCHSC, they had not performed any experiments with the plasmid yet; however, after we finished our experiments we were informed that they also had problems working with the plasmid and were not able to clone any inserts into it. It is not possible to buy the plasmid as it is not on the market anymore, maybe because it is very difficult to use; however, it would be interesting to get another plasmid and try to carry out the exon trapping again or investigating the rs4641 influence on alternative splicing with a different method, e.g., by constructing minigene constructs containing C and T-variants of rs4641. A minigene is a genomic fragment that includes alternative exon or exons and the surrounding introns as well as flanking constitutively spliced exons. The minigene is
cloned into a eukaryotic expression vector which is further transfected into a specific cellular environment. Thus the transfected minigene should contain all necessary RNA-elements to show the same alternative splicing patterns as the corresponding endogenous alternatively spliced gene [Zhang et al., 2007]. The splicing pattern is then examined by splicing/isoform-specific Q-PCR.

In conclusion, there is no association of rs4641 any of pre-diabetic quantitative phenotypes, lamin A and C expression. It was not possible to detect if rs4641 influenced the alternative splicing of LMNA. Further studies are needed with more individuals to clarify the association of the rs4641 with T2D and related traits.
4 Discussion and perspectives

Type 2 diabetes and obesity are conditions in which many factors are involved and they are both growing public health problems with a tremendous impact on morbidity and mortality. The aetiologies of T2D and obesity are complex involving both genetic and environmental factors. Identification of genetic variants and understanding of gene-environment interactions in the development of T2D and obesity would give an advantage in prevention and treatment on a society level but especially for each individual as it enables personalized prevention and treatment. It is of high priority to prevent T2D and obesity instead of desperate and countless attempts of treatment. Beside this, increased knowledge within the field will provide possibilities to discover new drug targets hopefully leading to new therapeutic opportunities as well as classification and prediction would be much more efficient.

The aim of the thesis was to contribute to the identification of genetic variants involved in the pathogenesis of T2D and obesity and T2D related traits. Variants in \( AHI1 \) and \( UCP2 \) have been investigated by genetic-epidemiological studies and a variant in \( LMNA \) has been investigated both by genetic and epidemiological studies as well as by functional studies.

GWA studies are relatively new strategies to identify genetic variants predisposing to common metabolic diseases. The studies have already led to identification of several variants associated with type 2 diabetes and obesity and some of them have already been replicated in different populations including our Danish populations. At the time identification of a genetic variant predisposing to T2D or a phenotype trait is performed, validation is needed by replication in a different study population. This is one of the major difficulties in genetic epidemiology due to e.g. differences in ethnicity. Different study populations have different LD blocks and this can be the reason why some findings are not possible to replicate. Another reason why replication is difficult can simply be due to false positives which might be because of insufficient power in the study or because of multiple testing or false negatives. Moreover, functional studies are of great importance in order to justify the suggested biological mechanism underlying the association.

Technical capabilities to produce very large amounts of genomic data have evolved rapidly in the past decade and more powerful genome studies will be leading in the future. Probably whole genome sequencing will be the new attempts to identify new genetic variants. Today GWA studies investigate genetic variants with a frequency above 5 % while future GWA studies most likely will begin to focus on genetic variants with a frequency below 5 % as well. To detect the true causative disease susceptibility variant, the whole genome sequencing approach covering both coding and non-coding variants including common and rare variants seems an attractive solution; in fact, the ’1000 Genome Project’ was launched in January 2008 [Siva, 2008]. The aim of this international mega-effort is to sequence the genome of thousand people from around
the world. It will capture variants with a frequency of 1% or more across most of the genome. Even though the project does not cover the entire genome it is expected to contribute considerably to fully understand the genetic basis of type 2 diabetes and its related complex diseases.

In conclusion rs1535435 and rs9494266 in AHI1 are not associated with type 2 diabetes or any pre-diabetic traits. The rs659366 in UCP2 is not associated with obesity or T2D in our populations; however, we found increased insulin sensitivity for the A-allele carriers. Additional studies are needed to clarify the association of rs659366 with T2D and obesity. The rs4641 in LMNA was not associated with any pre-diabetic traits. Furthermore, it was not possible to determine the influence of the rs4641 on alternative splicing by exon trapping or by expression studies why additional studies are needed to determine the function of rs4641.
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6 Appendix List

Appendix I: Study populations

Appendix II: Statistical methods

Appendix III: Methods and protocols
Appendix I: Study populations

Biochemical and anthropometric measurements

In all study groups, height and weight were measured in light indoor clothing and without shoes, and BMI was calculated as weight in kg divided by height in meters squared (kg/m\(^2\)). Waist circumference (cm) was measure in the upright position midway between the iliac crest and the lower costal margin, and hip circumference (cm) was measured at its maximum.

Serum LDL-cholesterol was calculated as total serum cholesterol minus serum HDL-cholesterol minus serum triglyceride/2.2. Serum VLDL-cholesterol was calculated as serum triglyceride divided by 5 [Friedewald et al., 1972].

Measurements of fasting plasma glucose and fasting serum insulin can be used to calculate the validated homeostasis model assessment of insulin resistance (HOMA-IR) index which is \((\text{fasting plasma glucose mmol/l} \times \text{fasting serum insulin pmol/l})/22.5\) [Matthews et al., 1985]

Inter99

The Inter99 is a population-based sample of unrelated Danish Whites. It is collected from the Research Centre for Prevention and Health at Glostrup University Hospital. Individuals living in the South-Western part of Copenhagen County in December 1998, born in 1939-1940, 1944-45, 1949-50, 1954-55, 1959-60, 1964-65, and 1969-1970 \((n = 61,301)\) were drawn from the Danish Population register (CPR). From this study population an age- and sex-stratified random sample called Inter99 \((n = 13,016)\) were examined further, some were excluded, due to e.g. death, alcoholism, drug abuse, leaving 6,784 individuals for analysis. The median age for this population was 45.1 years (range 30-60), with 48.7 % being men and 51.3 % women.

All participants filled out questionnaire concerning lifestyle such as smoking habits, food- and alcohol intake, physical activity and possibilities to change lifestyle; educational- and working status; information about family occurrence of chronic disease such as T2D and cardiovascular disease; health status such as medication and use of the health care system [Jørgensen et al., 2003]. Information about lifestyles, such s smoking and physical activity was reported. All the participants in the study were Danish Whites by self-report. Informed written consent was obtained from all participants, and studies were conducted in accordance with the Helsinki Declaration II and were approved by the ethics committee of Copenhagen.

All physical examination were conducted by medical staff at Steno Diabetes Center and Research Centre for Prevention and Health. Blood pressure after 5 minutes of lying down, height without shoes, weight, and waist and hip circumference were measured. Fasting blood samples were drawn for assessment of total serum cholesterol, HDL-cholesterol and triglyceride levels, whereas VLDL-cholesterol and
LDL-cholesterol levels were calculated by Friedewald's equation. An oral glucose tolerance test (OGTT) was performed, giving the participants, without known diabetes, 57 g anhydrous glucose dissolved in 250 ml of water. Plasma glucose, serum insulin and C-peptide were measured fasting and after 30 and 120 minutes.

**Methods for measurements of clinical and biochemical variables**

- **Lipid concentrations:** Total cholesterol was measured by the Cholesterol CHOD-PAP method, HDL-cholesterol levels were measured using an enzymatic technique (Boehringer, Mannheim, Germany) and triglycerides levels were measured using the Triglyceride GPO-PAP method.
- **Glucose concentration:** Plasma glucose concentrations were measured automatically using hexokinase 1GP-DH technique (Granutest, Merck, Damstadt, Germany).
- **Insulin concentrations:** Serum insulin levels were measured by enzyme-linked-immuno-adsorbant-assay (ELISA) excluding intact pro-insulin (Dako insulin kit K6219, Dako diagnostics Ltd, Ely, UK).

Classification of the glucose tolerance status was done according to the criteria set by WHO in 1999. Individuals with self reported diabetes were classified as known diabetics. Individuals nor reporting having diabetes and who had fasting plasma glucose ≥ 7.0 mmol/L or 120 min plasma glucose ≥ 11.1 mmol/l were diagnosed as having screen detected diabetes. Individuals were diagnosed with IGT when having fasting plasma glucose < 7.0 mmol/l and 120 min plasma glucose ≥ 7.8 mmol/l but < 11.1. Those with fasting plasma glucose ≥ 6.1 mmol/l but < 7.0 mmol/l and 120 min plasma glucose < 7.8 mmol/l were diagnosed with impaired fasting glycemia (IGF). Normal glucose tolerance (NGT) was defined as fasting plasma glucose < 6.1 mmol/l and 120 min plasma glucose < 7.8.

Based on measures of plasma glucose levels, the glucose tolerance status were successfully determined for 6,410 participants, where 4,735 (2,209 men and 2,526 women) were NGT, 520 (380 men, 140 women) were IFG, 751 (369 men and 382 women) were IGT, 256 (167 men and 98 women) were diagnosed with screen detected diabetes, and 139 (72 men and 67 women) had known T2D [Glümer et al., 2003]

**PASSION**

This cohort includes 8,539 individuals and contains the Inter 99 cohort and other small cohorts.

P: Probands: n = 135 (67/68) with T2D; Age: 60 ± 13 years; Average BMI: 29.4 ± 5.3 kg/m².

A: Asso-control study. T2D patients (n = 557) sampled from Steno Diabetes Center with matched controls for age, sex, and BMI; n = 287 (476/368); Age: 58 ± 12 years; Average BMI: 27.9 ± 5.2.
S: Sopson; cohort with microalbuminuria selected from the outpatient clinic at Steno Diabetes Center [Gæde et al., 2003] \( n = 160 \) (119/41); Age: 55 ± 7; Average BMI: 29.9 ± 4.4 kg/m\(^2\).

S: Sixty-year-old study: A population-based sample of 60 years old glucose tolerant individuals; \( n = 695 \) (325/370) from Copenhagen [Drivsholm et al., 2001]; Age: 61 ± 1 years; Average BMI: 26.8 ± 4 kg/m\(^2\)

I: Inter99; \( n = 6,514 \) (3,169/3,345); Age: 46 ± 8; Average BMI: 26.3 ± 5 kg/m\(^2\)

O: Oluf Pedersen research samples. \( n = 102 \) (56/46); Age: 54 ± 9; Average BMI: 29.3 ± 4.9 kg/m\(^2\)

N: Nation wide collection of T2D patients. \( n = 86 \) (62/26). All hospitals and doctors in Denmark were requested to send blood samples along with patients data such as BMI to Steno Diabetes Center. Age: 58 ± 11 years; Average BMI: 31.1 ± 5.3 kg/m\(^2\).

**ADDITION**

The ADDITION study (Anglo-Danish-Dutch Study of Intensive Treatment and Complication Prevention in Type 2 Diabetic Patients Identified by Screening in Primary Care) includes participants that were recruited via a step-wise strategy based on a mailed risk questionnaire followed by measurements of random blood glucose, HbA1C, fasting plasma glucose, and oral glucose tolerance test to diagnose diabetes. Patients from Denmark are included in the present study. In Denmark, approximately 120,000 persons aged 40 to 69 years were invited by mail. They were asked to score their risk for diabetes. Of those at high risk 28,266 consulted their general practitioner.

Based on HbA\(_{1C}\) and random plasma glucose measurements their fasting plasma glucose was determined in 11,646. All 10,672 participants have undergone a thorough phenotype characterisation including standardised questionnaire and interview (age, sex, socio-economic status, smoking habits, alcohol and food intake, physical activity, information about family history in relation to diseases predisposing to CVD, and detailed information about health status in relation to medical treatment and/or symptoms) and physical examination (standardised measurements of height, weight, waist- and hip circumferences, blood pressure, resting ECG, lung function). Among the 10,672 participants with extensive base-line data more than 4,000 are obese, more than 5,000 have hypertension, and 1,250 have verified type 2 diabetes. An additional 600 participants had type 2 diabetes at follow-up. In this cohort total serum cholesterol and serum HDL-cholesterol is measured by enzymatic test using the Hitachi 071 system (Roche Diagnostic GmbH, Mannheim, Germany [Lauritzen et al., 2000]

**Young healthy Danish Caucasians**

Blood samples were drawn after 12 hour overnight fast. Serum triglyceride, total cholesterol, and HDL-cholesterol were analysed using standard methods (Boehringer...
Mannheim, GmbH Diagnostic, Germany). Serum insulin levels were determined by Elisa excluding des (31,32) and intact proinsulin by applying the Dako insulin kit with an overnight incubation (code No. K6219; Dako Diagnostic, Ltd, Ely, UK [Clausen et al., 1997].

Fasting serum leptin levels were measured by means of human leptin RIA kit (Linco, St Charles, MO, USA) [Echwald et al., 1999]. Body fat content (kg) and fat percent were estimated using a bio-impedance technique.

Lean mass was calculated as body weight (kg) minus fat free mass (kg). Data on birth weight and birth length were obtained from the Danish midwife original records. The ponderal index was calculated as (birth weight (kg) / (birth length (m)³) [Clausen et al., 1997].

**Diabetic patients from Steno Diabetes Center and Healthy individuals from Steno Diabetes Center**

These two cohorts are sampled at Steno Diabetes Center and consist of 521 glucose tolerant controls and 2,111 type 2 diabetic patients.

Blood samples were drawn after a 12 hour overnight fast. Serum triglyceride, total serum cholesterol and HDL-cholesterol were analysed using enzymatic colorimetric methods (GPO-PAP and CHOD-PAP; Roche Molecular Biochemicals, Mannheim, Germany).

**Twin populations**

Two twin populations were used in this thesis; they were identified through The Danish Twin Register. Population 1 (n = 586) consists of MZ twins and same sex DZ twins and population 2 (n = 194) consists of MZ and same sex DZ twin young and elderly twins.

**Analysis of plasma glucose and insulin**

Plasma was made by centrifugation of blood samples for population 1 and 2 and plasma glucose and insulin was analysed.

**Euglyceamic, hyperinsulinaemic clamp and intravenous glucose tolerance test**

In the clamp period, plasma insulin concentration is initially raised and subsequently kept constant for 120 min by a continuous infusion of insulin. During the insulin infusion, plasma insulin concentration is raised to 400-500 pmol/L. The plasma glucose concentration is kept constant at 5 mmol/l by a continuous infusion of variable amounts of glucose solution. Blood glucose is monitored every 5-10 min and the values are used to regulate the rate of glucose infusion. At steady-state, the rate of glucose appearance (from infusion and HGP) equals insulin-stimulated glucose
disposal rate (RD clamp). Thus RD clamp constitutes an estimate of insulin-stimulated cellular glucose uptake when it is assumed that urinary glucose excretion is negligible. In the present study Rd clamp is expressed as mg glucose per kg FFM per min. The infused glucose solution is enriched with a tracer (3-\textsuperscript{3}H-glucose) which makes assessment of HGP possible.

The clamp was carried out at subjects who had fasted for 12 hours. A bolus of 3-\textsuperscript{3}H-glucose (22 µCi) was given at 0 min followed by a 0.22µCI infusion throughout the basal, IVGTT, and clamp periods. Steady-state was assumed in the last 30 min of the basal period. Basal insulin sensitivity was assessed by collection of blood samples every 10 min. Subsequently, the IVGTT was carried out by injection of a bolus of 18% glucose solution (0.3 g · kg\textsuperscript{-1} bodyweight with an upper limit of 25 glucose). Blood samples for plasma glucose and insulin measurements were drawn during the IVGTT period at 0, 2, 4, 6, 8, 10, 15, 20, 30 min. After IVGTT, a primed-continuous insulin infusion (40 mU m\textsuperscript{-2} · min\textsuperscript{-1}) was initiated and continued for 120 min. Plasma glucose was monitored every 5-10 min by means of Glucose Analyzer 2 (Beckman Instruments). Steady-state was defined as the last 30 min of the clamp period when tracer equilibrium was anticipated. Collection of blood samples and monitoring of plasma glucose was performed as in the basal period [Poulsen et al., 2005].

**Indirect calorimetry**

Indirect calorimetry was performed during two (basal and insulin-stimulated) 30 min steady state periods using a Deltrac computerized flow through a canopy gas analyser system (Deltratrac, Datex, Helsinki, Finland). Gas exchange was determined. After an equilibrium period of 10 min, the average gas exchange was recorded. Urine was collected after the clamp period. The O\textsubscript{2} and CO\textsubscript{2} exchange rates and urinary carbamide content were used to calculate to estimate insulin stimulated glucose oxidation (GOX) and fat oxidation (FOX) rates.

**Insulin sensitivity**

R\textsubscript{d} clamp: During the steady-state period of the euglycaemic, hyperinsulinaemic clamp the rate of glucose infusion equals the rate of glucose disposal, R\textsubscript{d}.

Steele’s equation for not non-steady state was used even though steady-state was sought to be obtained.

\[
R_a = \frac{R^* - pV_D \frac{dS_A}{dt}}{S_A}
\]

\[
R_d = R_a - p \cdot V_D \frac{dc}{dt}
\]
HGP: The HGP was calculated within the basal and clamp steady state periods using the following equation:

\[ \text{HGP} = R_a - R_{inf} \]

\( R_{inf} \) is the rate of glucose infusion.

HGP is suppressed during the clamp and therefore the more negative the differences between HGP during and before the clamp, the more insulin sensitive is the liver. The difference \( \Delta \text{HGP} \) is expressed in absolute values.

\[ \Delta \text{HGP} = |\text{HGP clamp} - \text{HGP basal}| \]

\textbf{β-cell function:}

\( \Phi_1_{\text{IVGTT}} \): First phase insulin response in relation to plasma glucose concentration, \( \Phi_1_{\text{IVGTT}} \) was calculated as the area under the curve (AUC) for insulin divided by AUC for glucose during the initial 10 min of the IVGTT.

\( \text{Di1}_{\text{IVGTT}} \): The first phase insulin disposition index is the insulin secretory capacity relative to insulin sensitivity. \( \text{Di1}_{\text{IVGTT}} \) was used as measure of β-cell function in this thesis, it was calculated as:

\[ \text{Di1}_{\text{IVGTT}} = \Phi_1_{\text{IVGTT}} \cdot R_{inf} \]

\textbf{Glucose and fat turnover rates}

\( \text{GOX} \): The glucose oxidation rate (GOX) was calculated by indirect calorimetry in the clamp steady-state period.

\[ \text{GOX} = 4.55 \text{VCO}_2 \text{L} \cdot \text{min}^{-1} - 3.21 \text{VO}_2 - 2.87 \text{n g} \cdot \text{min}^{-1}, \]

\( n \) is urinary nitrogen calculated from the carbamide concentration in the collected urine.

\( \text{FOX} \): The fat oxidation rate, calculated by indirect calorimetry analogous to GOX.

\[ \text{FOX} = 1.67 \text{VCO}_2 \text{L} \cdot \text{min}^{-1} - 1.67 \text{VO}_2 - 1.92 \text{n g} \cdot \text{min}^{-1}, \]

\( n \) is urinary nitrogen calculated from the carbamide concentration in the collected urine.

\textbf{Physical fitness}

\( \text{VO2max} \) was calculated from the maximal work load on ergometer bicycle, \( \text{watt}_{\text{max}} \) and the body weight.

\[ \text{VO2max} = \frac{\text{watt}_{\text{max}} \cdot 3.5 \cdot \text{weight in kg}}{\text{weight in kg}} \]
Appendix II: Statistical methods

All statistical analyses are performed using RGui version 2.51. A \( p \)-value of less than 0.05 is considered to be statistical significant.

Case-control studies

In case-control studies the difference in allele frequencies and genotype distributions between cases and controls are compared by Fisher’s exact test using a 2 x 2 contingency table where the total number of observation are classified by two factors, the number of alleles (\( A_1 \) and \( A_2 \)) in affected and unaffected.

<table>
<thead>
<tr>
<th></th>
<th>Affected</th>
<th>Unaffected</th>
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<tbody>
<tr>
<td>(A_1)</td>
<td>A</td>
<td>B</td>
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<td>(A_2)</td>
<td>C</td>
<td>D</td>
</tr>
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</table>

\(A1\) and \(A2\): Alleles; \(a, b, c, d\): numbers of alleles.

A \( p \)-value is calculated by logistic regression to determine if allele frequencies differ between affected and unaffected by adding either an additive, dominant of recessive model. The \( p \)-value can be adjusted for the effect of sex, age, and BMI where appropriate. Estimates from the logistic regression are given as OR with 95% confidence interval.

The null hypothesis does not include genetic contribution and states that the probability of being affected (\( p \)) is explained solely as an intercept (\( \alpha \)) and independent contributions of the estimated variables considered; in this study sex, age, and BMI (\( \beta_1 \) sex, \( \beta_2 \) age, \( \beta_3 \) BMI). In an alternative hypothesis, an additive contribution (\( \beta_4 X_{GM} \)) is added to the null hypothesis. \( GM \) refers to the genetic model applied e.g. additive, dominant or recessive and \( X \) is a chosen design variable here a dummy variable indicating the genotype. In his study \( X_{GM} \) for an additive model is defined as 0, 1 or 2 for wild type, heterozygous and homozygous individuals, respectively, while in the recessive model as 0, 0, 1, respectively. If data is explained significantly better by the alternative hypothesis, then \( \beta_4 X_{GM} \neq 0 \) and a genetic impact is assumed. The \( p \)-value is obtained using likelihood functions and \( \chi^2 \)-testing.

Null hypothesis: \( \log \left( \frac{p}{1-p} \right) = \alpha + \beta_1 \) sex + \( \beta_2 \) age + \( \beta_3 \) BMI

Alternative hypothesis: \( \log \left( \frac{p}{1-p} \right) = \alpha + \beta_4 X_{GM} + \beta_1 \) sex + \( \beta_2 \) age + \( \beta_3 \) BMI
Associations studies of quantitative traits

Differences in anthropometrics and quantitative traits between genotype groups are evaluated using a general linear model assuming an additive, dominant or recessive model and the calculated p-values are adjusted for age, sex, and BMI when appropriate. Before statistical analysis some traits are transformed either logarithmically or cubically in order to fit data to a normal distribution. A general linear model is used in analysis of a quantitative trait to test for a genetic effect on the trait studies. In model 0 the value of quantitative trait \( y \) is explained by an intercept \( (\alpha) \) and the independent contribution of the estimated variables \( (\beta_1 \text{sex}, \beta_2 \text{age}, \beta_3 \text{BMI}) \) while in Model 1 a genetic component \( \beta_4 X_{GM} \) is included. An ANOVA test based on F-statistics is used to compare the two models and hence describe which model explains data better.

Model 0: \[ y = \alpha + \beta_1 \text{sex} + \beta_2 \text{age} + \beta_3 \text{BMI} \]

Model 1: \[ y = \alpha + \beta_1 \text{sex} + \beta_2 \text{age} + \beta_3 \text{BMI} + \beta_4 X_{GM} \]
Appendix III: Methods and protocols

TaqMan allelic discrimination

Two probes are designed for binding specifically to the wt allele and one binding to the minor allele between the two primers. Allele calling will show if the DNA is heterozygous (1/2), homozygous (1/1), or homozygous (2/2). Each probe contains 5´reporter dye and 3´quencher dye. When the probe is intact the quencher dye suppressed the reporter fluorescence. During the PCR, forward and reverse primers hybridise to a specific sequence of the target DNA. The TaqMan probe hybridises to a target sequence within the PCR-product. By cleavage of reporter dye and quencher by the 5´nuclease activity of the TaqDNA polymerase they are separated and it results in increased fluorescence of the reporter dye. During PCR, forward and reverse primers hybridize to a specific

If Fam is covalently linked to the 5´end of the probe for detection of allele 1 (illustrated as red signal) and if VIC is covalently linked to the 5´end of the probe for detection of allele 2 (illustrated with yellow signal) it is illustrated in the table how the signals will be.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Allele</th>
<th>Signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heterozygous</td>
<td>(1/2)</td>
<td><img src="image" alt="Red and Yellow Signal" /></td>
</tr>
<tr>
<td>Homozygous</td>
<td>(1/1)</td>
<td><img src="image" alt="Red Signal" /></td>
</tr>
<tr>
<td>Homozygous</td>
<td>(2/2)</td>
<td><img src="image" alt="Yellow Signal" /></td>
</tr>
</tbody>
</table>

*Table A.1 Allele calling.*

Protocols for gene expression

RNA extraction and purification

RNA was extracted and purified from skeletal muscle tissue using Trizol method from Invitrogen. This procedure was carried out by technicians at Steno Diabetes Center.

cDNA synthesis

cDNA synthesis is series of complex enzymatic steps copying mRNA into double-stranded cDNA.
**Protocol Q-PCR using SYBR Green**

cDNA for a standard curve is prepared by pooling of several cDNA samples. The cDNA for the standard curve is from the same tissue origin as the samples to be analysed. The following dilutions are made from the stock solution: 1, 1/2, 1/4, 1/8, and 1/16.

2 µl 5 ng/µL cDNA from subcutaneous abdominal fat biopsies and 1 µl of the skeletal muscles of 10 ul/l) is added to 384-well plate. Each sample is applied in duplicates. The Q-PCR was carried out on ABI Prism 7900 HT (Applied Biosystem).

The sequences of the primers:

LTD 390 5’ TCT GAG TCA CCT GGA CCA CCA 3’
LTD 391 5’ ATC TCA GTG GTA TTT GTG AGA 3’

**Protocols for Exon trapping**

The pSPL3 was received from

Maloney Lab
University of Colorado, HSC
Pulmonary & CCM

**Primer design**

The specificity of the PCR product is highly dependent on the primers because the primers ought to hybridise well to its target. When designing primers the overall GC % should be the same in the two primers and approximately equal to the template DNA. Primers should not contain self-complementary sequences that can form hairpin structures or sequence that can hybridise between the primers and form primer dimers.

Specific mismatches in the 5´end of the primer do not prevent annealing of the primers or subsequent polymerase extension of the strand, and they allow one to introduce restriction sites as was done for the exon trapping.

**Optimisation of PCR**

To obtain a specific PCR product it is necessary to optimise the reaction. Optimisation is often done by changing the concentration of MgCl₂ and the annealing temperature. The hybridisation becomes more efficient when the annealing temperature is increased. The same can be accomplished by changing the MgCl₂ concentration. Finally, cycling conditions can also be varied as well as the template DNA concentration, primer concentration, polymerase concentration, or additives like DMSO and betain can be used.
LTD 394 and LTD 395 were the first set of primers using BamHI and XhoI restriction enzymes and LTD 398 and LTD 399 were the second set of primers using XhoI and PstI.

LTD 394: 5’- CTC TCG AGA AGG GGT GGA AGT TAG ACA GTG AGA -3’
LTD 395: 5’- CTG GAT CCG AGG GGC GGT CAG GAA TGA -3’
LTD 398: 5’- GAC TCT CGA GAA GGG GTG GA A GTT AGA CAG TGA GA -3’
LTD 399: 5’- GAC TCT GCA GGA GGG GCG GTC AGG AAT GA -3’

The amplified PCR product was 764 nucleotides.

**PCR protocol**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>19.3 µL</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1.5 µL</td>
</tr>
<tr>
<td>PCR buffer</td>
<td>2.5 µL</td>
</tr>
<tr>
<td>Primer 394</td>
<td>0.25 µL</td>
</tr>
<tr>
<td>Primer 395</td>
<td>0.25 µL</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>0.2 µL</td>
</tr>
<tr>
<td>DNA template 100 mg/µL</td>
<td>1 µL</td>
</tr>
</tbody>
</table>

**PCR program:**

3 min 95ºC

35 timer:

30 sec 95º C denaturating

30 sec 63.9º C annealing

1 min 72º C elongation

5 min 72 ºC elongation

**Transformation**

1. 5 ul (plasmid pslpl3) was transformed into 100 ul competent cells (DH5α).
2. On ice for 5 min.
3. Heat shock 45 sec at 42ºC in water.
4. 1 ml soc is added.
5. 1 hour at 37 ºC vigorous shaking (200 rpm). Spread out on LB-ampicillin plates.
6. Left in incubator at 37ºC overnight.

The transformation is also carried out with a control plasmid.

**Controls:**

control plasmid (yes 2), plasmid non-cut, restricted plasmid but not ligated,
When colonies appear on the plates selected colonies were spread out separately and inoculated in LB-medium and incubated overnight in at 37 °C, vigorous shaking (200 rpm).

**Protocol for mini prep preparation**

1. 5 ml overnight bacterial LB culture was harvested by centrifugation at room temperature for 15 minutes.

2. The cell pellet was resuspended in 300 µl resuspension buffer containing RNase A. Pipetting up and down to completely resuspend the pellet.

3. 300 µl lysis buffer was added, inverting the capped tube 6 times.

4. Incubated at room temperature for 4 minutes.

5. The tube were vortexed containing the chargeSwitch® magnetic beads to fully resuspend and evenly distribute the beads in the storage buffer.

6. 300 µl chilled precipitation buffer was added and mixed by inverting the capped tube until a white, free-flowing precipitate was formed.

7. The tube was centrifuged 10 minutes at 4°C.

8. The supernatant was transferred to a new micro centrifuge.

9. 40 µl of ChargeSwitch® magnetic beads was added to the lysate.

10. Gently mixed by pipetting up and down.

11. Incubated at room temperature for 1 minute.

12. Placed on the magnetic rack for 2 minute.

13. The supernatant was discarded.

14. The tube was removed from the rack.

15. 1 ml wash buffer was added to the tubes and pipeted up and down.

16. Placed in the rack again for 2 minutes.

17. The supernatant was removed.

18. The tube was removed from the rack.

19. 50 µl elution buffer was added and pipeted up and down 10 times.
Protocol for midi-preps preparation

A single colony was inoculated in a starter culture of 5 ml LB medium containing AMP. Incubated for 7 h at 37°C with vigorous shaking (300 rpm).

50 ml of the starter culture is diluted 1/1000 into LB containing amp. Grown at 37°C for 15–16 h with vigorous shaking (approx. 250 rpm).

The bacteria were harvested by centrifugation at 6000 x g for 15 min at 4°C. All traces of supernatant were removed by inverting the open centrifuge tube until all medium has been drained.

The bacterial pellet was resuspended in 6 ml buffer P1.

6 ml buffer P2 was added, mixed by vigorously inverting the tube. Incubated at room temperature (15–25°C) for 5 min. the QIAfilter Cartridge was prepared during the incubation. The cap was screwed onto the outlet nozzle of the QIAfilter Midi or QIAfilter Maxi cartridge. The QIAfilter cartridge was placed into a convenient tube.

6 ml chilled buffer P3 was added and mixed thoroughly by vigorously inverting 4–6 times.

The lysate was poured into the barrel of the QIAfilter Cartridge. Incubated at room temperature for 10 min.

A hispeed midi tip was equilibrated by applying 4 ml buffer QBT and allow the column to empty by gravity flow.

The cap is removed from the QIAfilter outlet nozzle and the plunger is inserted into the QIAfilter maxi cartridge. The cell lysate was filtered into the previously equilibrated hispeed tip.

The cleared lysate was allowed to enter the resin by gravity flow.

The hispeed midi tip was washed with 20 ml buffer QC.

DNA was eluted in 5ml buffer QF in a 10 ml tube.

DNA was precipitated by adding room temperature 3.5 ml isopropanol.

The plunger was removed during the incubation from a 20 ml syringe and attached to the QIAprecipitator midi module onto the outlet nozzle.

The QIAprecipitator was placed over a waste bottle and the eluate/isopropanol was transferred to a 20 ml syringe, and the plunger was inserted.

The QIAprecipitator was removed from the 20 ml syringe and the plunger was pulled out. The QIAprecipitator was re-attached and 2 ml 70 % ethanol was added to the syringe. The DNA was washed by inserting the plunger and pressing the ethanol through the QIAprecipitator using constant pressure.

The QIAprecipitator was removed from the 20 ml syringe the plunger was inserted and the membrane was dried by pressing air through the QIAprecipitator. This step was repeated.
The outlet nozzle of the QIAprecipitator was dried with absorbent paper to prevent ethanol carryover.

The plunger from a new 5 ml syringe was removed and the QIAprecipitator was attached onto the outlet nozzle. The QIAprecipitator was held over a 1.5 ml collection tube. 1 ml of Buffer TE was added to the 5 ml syringe. The plunger was inserted and eluted DNA was pressed into the collection tube using constant pressure.

The QIAprecipitator was removed from the 5 ml syringe, the plunger was pulled out and the QIAprecipitator was reattached to the 5 ml syringe.

The eluate from step 19 was transferred to the 5 ml syringe and eluted for a second time into the same 1.5 ml tube.

**Restriction enzyme digestion**

The volumes differed; however, generally the following protocol was used, digested for 3 hours at 37°C.

<table>
<thead>
<tr>
<th>ddH₂O</th>
<th>Up to 50 µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 x buffer</td>
<td>5</td>
</tr>
<tr>
<td>100 x BSA</td>
<td>5</td>
</tr>
<tr>
<td>Enzyme</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>DNA</td>
<td>1 µg</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>50 µl</strong></td>
</tr>
</tbody>
</table>

**QIAquick Gel Extraction Kit Protocol**

1. The DNA fragment was excised.
2. The gel slice was weighed. 3 volumes of Buffer QG to 1 volume of gel was added.
3. Incubated at 50°C for 10 min and vortexed every 2-3 min during the incubation.
4. 1 gel volume of isopropanol was added to the sample and mixed.
5. The sample was applied to QIAquick column, and centrifuged for 1 min.
6. Flow-through was discarded.
7. 0.5 ml of buffer QG was added to QIAquick column and centrifuged for 1 min.
8. 0.75 ml was added of Buffer PE to QIAquick column and centrifuged for 1 min.
9. The flow-through was discarded and centrifuged for 1 min 17,900 x g.
10. Place QIAquick column into a clean 1.5 ml micro centrifuge tube.
11. 30 µl of buffer EB was added and centrifuged the column for 1 min.
Phenol-chloroform extraction by Klenow Fill-In Kit

1. 150 µl of 1× STE buffer was added
2. 50 µl of 10× STE buffer was added
3. 500 µl of phenol–chloroform was to each reaction.
4. Vortex, then micro centrifuged for 2 minutes room temperature at maximum speed.
5. the upper aqueous layer was transferred to a fresh tube.
6. The step was repeated.
7. 500 µl chloroform was added, vortex.
8. Centrifuged for 2 min at room temperature at max speed.
9. Upper aqueous layer was transferred to a fresh tube.
10. 1 ml Ethanol was added.
11. 30 minutes at −20°C
12. Micro centrifuged for 10 minutes at 4°C.
13. Supernatants was discarded.
14. Pellet was washed with ice-cold 70% (v/v) ethanol.
15. Dried.
16. DNA was resuspended in 25 µl of TE buffer.

For colony PCR the following primers were used:

Colonies were selected for analysis and inoculated in 10 µl water. PCR was run with following primers and 2 µl of the solution.

LTD 396:5'- GGC ACC ATG CTC CTT GGG ATG TTGA3'
LTD 397:5'- CAC TCT ATT TTG TGC ATC AGA TGA3'

Rapid Ligation kit, Roche

Control for ligation was plasmid alone.

1. Vector DNA and insert DNA was diluted in 1 x conc. DNA dilution buffer to a final volume of 10 µl.
1. T4 DNA ligation buffer was mixed.
2. 10 µl T4 DNA ligation buffer (vial 1) was added to the reaction, mixed.
3. 1 µl T4 DNA ligase was added, mixed.
4. In incubated for 5 min at 15-25°C.
**TOPO cloning**

Following were mixed:

3.2 µl PCR fragment

0.8 µl salt solution

0.8 µl TOPO vector 4.1

Incubated for 30 minutes at room temperature. Afterward the reaction was placed on ice.