The genetics of type 2 diabetes

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The Genetics of Type 2 Diabetes

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List of abbreviations

2hrG	2 Hour Glucose
A	Additive effects
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
C	Shared environmental influences
DCS	Diabetes Care System West-Friesland
df	Degrees of Freedom
DZF	dizygotic female
DZM	dizygotic male
E	Unique environmental influences
$FADH_2$	Flavin Adenine dinucleotide reduced
FPG	Fasting Plasma Glucose
GDM	Gestational Diabetes Mellitus
GWAS	Genome Wide Association Study
HAART	Highly Active Anti Retroviral Therapy
HWE	Hardy Weinberg Equilibrium
IFG	Impaired Fasting Glucose
IGT	Impaired Glucose Tolerant
LD	Linkage Disequilibrium
MAF	Minor Allele Frequency
MIDD	Maternally Inherited Diabetes and Deafness
MODY	Maturity Onset Diabetes of the Young
mtDNA	mitochondrial DNA
mtSSB	Mitochondrial Single-Stranded DNA-Binding protein
MZF	monozygotic female
MZM	monozygotic male
NADH	Nicotinamide Adenine Dinucleotide (oxidized form)
NGT	Normal Glucose Tolerance
NHS	New Hoorn Study
OGTT	Oral Glucose Tolerance Test
OR	Odds Ratio
QC	Quality Control
SNP	Single Nucleotide Polymorphism
TCA	Tricaboxylic acid
TWINKLE	Replicative mitochondrial Helicase
VDCC	Voltage Dependent Calcium Channel
WHO	World Health Organization

Chapter 1

Introduction

Glucose homeostasis

Glucose levels are tightly regulated in the human body. Even after food-intake or fasting, glucose levels remain relatively stable. The major organs involved in regulating glucose levels are the pancreas, liver, skeletal muscle and adipose tissue. In the pancreas beta-cells are present, which are part of the Islets of Langerhans. Triggered by increased glucose levels these beta-cells will secrete insulin. The main target tissues of insulin action are liver, skeletal muscle and adipose tissue. Insulin promotes uptake of glucose from the periphery in adipoytes and muscle. The liver is involved in gluconeogenesis in which glucose is synthesized from C-3 compounds such as lactate, glycerol and pyruvate, derived from amino acids and by glycolysis. The rate limiting step of gluconeogenesis is the activity of the enzyme phosphoenolpyruvate carboxykinase (PEPCK). In the presence of high insulin levels, PEPCK expression is suppressed, yielding a decrease in gluconeogenesis. In addition, in the liver and muscle insulin regulates the balance between glycogenesis, which forms glycogen from glucose and glycogenolysis, yielding glucose from glycogen breakdown. Glycogen is a polysaccharide chain, which is an efficient form of glucose storage. In the presence of high insulin and low glucagon levels (the latter produced by pancreatic alpha-cells within the islets of Langerhans), glucose is converted into glycogen, whereas in situations with low insulin and high glucagon concentrations, glycogen is degraded into glucose. Furthermore, insulin increases glucose uptake predominantly by skeletal muscle and adipose tissue resulting in a drop of blood glucose levels. After uptake, excess of glucose is either stored as glycogen (muscle) or converted into triglyceride (adipocytes). In addition it may be degraded into lactate via a process called glycolysis and further into CO_2 via the tricarboxylic acid cycle and oxidative phosphorylation when metabolic energy is needed. These degradations are coupled to ATP synthesis as described in more detail later in this introduction. Moreover, insulin inhibits lipolysis and increases triglyceride synthesis in the adipose tissue. These processes are reviewed in references 1-3.
Taken together, increased glucose levels will result in insulin secretion by beta-cells leading to decreased gluconeogenesis by the liver and increased glucose uptake by skeletal muscle and adipose tissue. In addition glycogenesis is

increased while glycogenolysis is decreased. Together, this will result in a drop in glucose levels and subsequently decreased insulin secretion preserving the physiological glucose levels around 5 mmol/L. This is important because high levels of glucose (hyperglycaemia) cause damage to blood vessels and nerves by chemical modification of proteins, while low levels of glucose (hypoglycaemia) can results in dysfunction of the brain leading to coma and death (1-3).

Diabetes

Diabetes Mellitus is a common disease and its prevalence is rapidly increasing. It is expected that the global burden of diabetes mellitus is 300 million people in 2025, while this was 150 million in 2000 (4). The disease is divided in several subtypes; type 1 diabetes and type 2 diabetes being the most common forms. Both subtypes are characterized by chronic hyperglycaemia defined as having fasting plasma glucose (FPG) concentrations above 7.0 mmol/L (4-6). The prevalence of especially type 2 diabetes exhibits an explosive growth in societies with a western life style.

Long term hyperglycaemia can lead to several complications like micro-vascular complications in the eye and kidney and neurologic complications. In addition, type 2 diabetes is strongly associated with an increased risk for cardiovascular disease due to macro-vascular complications. The latter is predominantly responsible for the increased mortality (6-9). Next to type 1 diabetes and type 2 diabetes several other specific subgroups of diabetes exist like Maturity Onset Diabetes of the Young (MODY) and Maternally Inherited Diabetes and Deafness (MIDD). Both are high penetrance, monogenic forms of the disease, caused by single nucleotide polymorphisms (SNP). Another diabetes subtype is Gestational Diabetes Mellitus (GDM), which occurs in pregnant women. Women who suffered from GDM are more likely to develop type 2 diabetes later in life (6). Latent Autoimmune Diabetes in Adults (LADA) is yet another subtype of diabetes mellitus. This syndrome associates with autoimmunity against glutamic acid decarboxylase. The disease is often diagnosed as type 2 diabetes because of its late onset (10).

Type 1 diabetes and type 2 diabetes

Type 1 diabetes is characterized by an absolute insulin deficiency, most commonly caused by auto-immune destruction of insulin producing beta-cells in the pancreas (6). The disease has an early onset with a peak at 12 years of age. Patients are fully dependent on exogenous insulin, administered by injections (11). There are genetic risk factors, determining the vulnerability to develop type 1 diabetes. HLA haplotypes account for ~50% of the familial clustering of type 1 diabetes. Genome wide association studies (described in more detail later in this introduction) have now increased this number to 12 loci (12;13).

Type 2 diabetes is a disease with a gradual onset, mostly later in life. In most cases the disease manifests above 40 years of age with a peak at 60 years. Type 2 diabetes patients show a relative rather than an absolute insulin deficiency in combination with an insulin resistant state in which target tissues have an impaired reaction on insulin. When the beta-cells cannot produce sufficient amounts of insulin to correct for the increased demand, hyperglycaemia will develop.

Patients are initially treated by diet and life style interventions, aimed at reducing the level of insulin resistance by weight loss and enhanced muscle glucose uptake, independent of insulin, by physical exercise. When this approach fails pharmaceutical treatment is initiated. Commonly used drugs include metformin, which suppresses hepatic glucose production and sulfonylurea which triggers additional insulin secretion by pancreatic beta-cells independent of glucose. Gradually, the severity of the disease progresses which can lead to insulin dependency (6;14).

Several risk factors for type 2 diabetes are known, like ageing, overweight, low levels of physical activity and genetic variation. The increased prevalence of type 2 diabetes is most likely caused by western life style, which means overfeeding and too little exercise. Which genes are involved in the onset of type 2 diabetes, is not completely clear (14;15). Because type 2 diabetes is a very heterogeneous disease it is difficult to make a good estimation of the heritability, but a Danish twin study has resulted in an estimation up to 72%, depending on the applied model (16). In theory, genetic risk factors may directly affect the risk for type 2 diabetes, e.g. by modulating the capacity of the beta cells to secrete insulin or by inducing insulin

resistance. Genetic variation may also indirectly affect the risk for type 2 diabetes, e.g. by affecting the satiety feeling after food intake as excessive food intake enhances the risk for diabetes. This distinction is important when interpreting data from genetic studies

Genetics of type 2 diabetes

In the last decades efforts were made to identify type 2 diabetes genes, using the candidate gene approach and linkage studies in which the transmittance of an allele or locus is examined in families with multiple cases of type 2 diabetes. When applying candidate gene studies, the genes to be examined are selected based on data on their direct or indirect involvement in glucose homeostasis. Genetic variation, most commonly single nucleotide polymorphisms (SNPs), in these genes is measured in a sample of subjects with and without type 2 diabetes. Such sample could be a population based study, which is a random selection of subjects from a population. The allele frequency of a SNP is than compared between healthy participants and type 2 diabetes patients in the selected population. More commonly case-control studies are used, which are selected groups of control and type 2 diabetes subjects. Matching can be performed e.g. for age and BMI as these are major additional determinants for type 2 diabetes susceptibility. The allele frequency of the SNP in the type 2 diabetes group is compared to the control group. If the allele frequency is enriched in the type 2 diabetes group, the allele is assumed to predispose for type 2 diabetes susceptibility. The benefit of a case-control study compared to a population based study is that it is a better controlled experiment. This approach is well powered for common SNPs with a Minor Allele Frequency (MAF) above 5% even if the penetrance is low, as long as the selected population is of suitable size. A different approach is the linkage study. In linkage studies SNPs or loci are measured in family pedigrees and the transmittance of a risk allele or risk loci to type 2 diabetes family members is analyzed. The latter approach is more applicable for rare variants with high penetrance (minor allele frequency (MAF) below 1%), but is underpowered for SNPs with a low penetrance. Both approaches require replication of novel findings in additional populations. It is important that a replication study is of suitable size in order to obtain sufficient

statistical power. Differences in for instance ethnicity of participants in the studies could result in a false negative replication. Therefore, a replication study should be well designed.

The linkage studies were quite successful in identifying the genes for the MODY subtypes and for MIDD, which were found to be monogenetic diseases, caused by SNPs with a high penetrance. These approaches had, however, very little success in identifying the genes for common type 2 diabetes. Only three type 2 diabetes genes were identified via case-control studies: Peroxisome Proliferator-activated Receptor Gamma (*PPARG*), Calpain10 (*CAPN10*) and Potassium inwardly-rectifying channel, subfamily J, member 11 (*KCNJ11*). The effect of these genes on the risk for type 2 diabetes in the general population was found to be small (17-21). The only gene which was associated with type 2 diabetes with a higher impact was Transcription Factor 7-like2 (*TFC7L2*) (OR ~1.35), which was found in an Icelandic population and widely replicated in various ethnic populations. This gene was identified by extensive analysis of a genomic region which showed evidence for linkage with type 2 diabetes in previous studies and therefore its identification was not the result from a classical candidate gene study (21;22).

More progression in identifying type 2 diabetes susceptibility genes was made when genome wide association studies (GWAS) were developed. Using dense arrays with up to 500,000 SNPs a large proportion of genetic variation in the human genome was covered. Because the GWAS approach covers up to 500,000 SNPs randomly spread through the human genome, the outcome is not biased by candidate gene hypothesis. However, the statistical drawback of testing 500,000 SNPs is that the p-value has to be corrected for 500,000 tests. Using Linkage Disequilibrium (LD) even more SNPs can be assessed. If there is no recombination hotspot between SNPs, they will not be separated by recombination during meiosis and therefore be in high LD and inherited together. So, a group of SNPs which are in between two recombination hotspots will be in high LD. Such group of SNPs is called an LD-block. Using this knowledge one SNP can be predicted by genotyping of several others. Therefore, association of a SNP can be tested without directly genotyping the SNP. This strategy is called imputation. Data of linkage between SNPs is available on the HapMap database (www.HAPMAP.org) (23-25). Using

this strategy it is currently possible to test the association of up to 2,000,000 SNPs with type 2 diabetes from GWAS data. The p-value has to be corrected for 2,000,000 tests when this strategy is applied and only a p-value smaller then $2.5x10^{-8}$ can be considered as statistical significant. In order to reach such small p-values very large study samples have to be used consisting of up to 50,000 participants and even more.

GWAS allows the identification of common SNPs that have a relatively low effect on type 2 diabetes susceptibility. However, it does not allow the detecting of rare and low frequency SNPs (MAF < 5%) with a modest effect on type 2 diabetes susceptibility. These studies are underpowered to detect such associations. Moreover, the coverage of low frequency and rare variation on GWAS genotyping chips is relatively low and much variation will therefore be missed.

After several GWAS and a meta-analysis of three of these, the list of known type 2 diabetes loci was expanded to 20, all with marginal effect sizes (OR 1.05 – 1.35) (21;26-32). Most of the newly identified SNPs are not located within the introns or exons of a gene and the pathogenic mechanism of associated SNPs is often unknown. If there is a suggested mechanism, it is mostly involved in decreased insulin secretion via an impaired beta-cell function. Remarkably, only one gene was identified as an insulin resistance gene (*PPARG*). This gene controls fatty acid metabolism and by that, indirectly insulin resistance. Insulin resistance was considered a major candidate pathway to be involved in the pathogenesis of type 2 diabetes (table 1).

The development of GWAS, during the preparation of this thesis, has dramatically changed the approach of genetic association studies. Therefore, my PhD-project starts with ‘old-fashioned’ candidate gene studies and is expanded with GWAS data, which came available after completion of our association studies.

Table 1. Confirmed type 2 diabetes susceptibility loci

Gene (nearest)	Strategy	Mechanism	Location
PPARG	Candidate gene	Insulin sensitivity/ lipid metabolism	Exon
CAPN10	Linkage	Glucose transport	Intron
KCNJ11	Candidate gene	beta-cell function	Exon
TCF7L2	Candidate region	beta-cell function	Intron
CDKAL1	GWAS	beta-cell function	Intron
CDKN2A/B	GWAS	beta-cell function	Intergenic
HHEX/IDE	GWAS	beta-cell function	Intergenic
SLC30A8	GWAS	beta-cell function	Exon
IGF2BP2	GWAS	beta-cell function	Intron
WFS1	Candidate gene	Unknown	Intron
TCF2	GWAS	Unknown	Intron
FTO	GWAS	Obesity	Intron
NOTCH2	GWAS	Unknown	Intron
ADAMTS9	GWAS	Unknown	Intergenic
THADA	GWAS	Unknown	Exon
TSPAN8/LGR5	GWAS	Unknown	Intergenic
CDC123/CAMK1D	GWAS	Unknown	Intergenic
JAZF1	GWAS	Unknown	Intron
KCNQ1	GWAS	beta-cell function	Intron
MTNR1B	GWAS	beta-cell function	Intron

Confirmed type 2 diabetes loci. OR ranging between 1.05 and 1.35.

Adapted from Ridderstrale M et al, Mol.Cell Endocrinol (2009) (21).

Scope of the thesis

The aim of this thesis was to elucidate the role of genetic variation in type 2 diabetes susceptibility and related traits, in particular in genes involved in mitochondrial function. The experimental part of this thesis is divided in three parts.

1. **Nuclear encoded mitochondrial proteins**: In the first part (chapters 2 and 3) we have examined the relation between SNPs in nuclear encoded mitochondrial candidate genes and the risk for type 2 diabetes. We selected these mitochondrial targeted genes because we hypothesized that mitochondrial function is associated with type 2 diabetes, as described in this section.
2. **Mitochondrial DNA content and type 2 diabetes:** In this part (chapter 4) we have investigated whether mitochondrial DNA content is associated with the risk for type 2 diabetes.
3. **Genes regulating fasting plasma glucose concentrations**: In this part (chapter 5) we have analyzed the association of four combined fasting plasma glucose genes with glucose levels and type 2 diabetes susceptibility.

The main topic is genetics of type 2 diabetes. The first two parts describe the role of mitochondria in type 2 diabetes; with a distinct focus on candidate genes (part 1) and mitochondrial DNA content (part 2). The third part does not focus on mitochondria, but describes cytosolic targeted genes influencing FPG levels.

Finally the results obtained during this PhD-project are summarized and discussed in chapter 6.

Part1: Nuclear encoded mitochondrial proteins

Mitochondria and energy homeostasis

Mitochondria are the organelles in cells which are responsible for most of the adenosine triphosphate (ATP) production. Furthermore, they play a role in oxidative removal of fatty acids and of metabolites from amino acids. They are also involved in apoptosis. Because mitochondria have a crucial role in ATP production, cells requiring high levels of ATP like cardiac and skeletal muscle, neurons and beta-cells show a high mitochondrial density. Mitochondria have their own genome, which encodes for only a small fraction of mitochondrial components. It is estimated that a mitochondrion contains ~1500 different proteins. Only 13 of these are encoded by the mitochondrial genome, the remaining are nuclear encoded. In addition, the mitochondrial genome encodes for 2 rRNAs and 20 tRNAs (33;34). The mitochondrial genome is described in more detail in part 2 of this introduction.

Oxidative Phosphorylation

Cells take up glucose by different glucose transporters. Only muscle and adipocytes contain Glut4 transporters which are translocated to the cell membrane in response to insulin. In the cytosol glucose is metabolized into pyruvate via glycolysis. Pyruvate is transported into the mitochondria where it is further converted into CO_2 by the Tricaboxylic acid cycle (TCA cycle). This cycle yields hydrogens in form of Nicotinamide Adenine Dinucleotide (oxidized form) (NADH) and Flavin Adenine Dinucleotide reduced ($FADH_2$). These hydrogens are oxidized to H_2O and the energy that is released by this oxidation is converted into chemical energy in form of ATP. This conversion is performed by the respiratory chain, which consists of 5 enzyme complexes, embedded in the mitochondrial inner membrane. The electrons flowing from H to O during the oxidation of NADH and $FADH_2$ enter the respiratory chain at complex 1 in case of NADH and at complex 2 in case of $FADH_2$ oxidation. At complex 4 the electrons are transferred onto oxygen. The flow of electrons through the respiratory chain is coupled to a proton pumping outwards the mitochondrial matrix by complex I, III and IV. This results in formation of a proton gradient with excess of protons at the outside of the inner

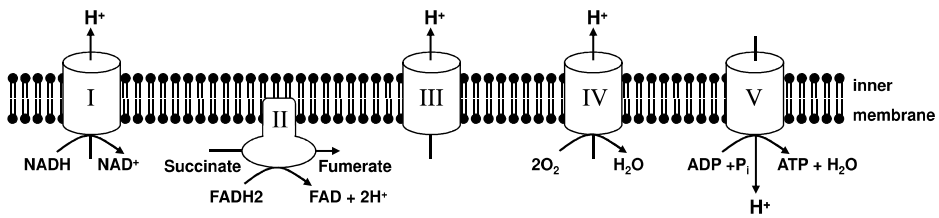
mitochondrial membrane. The back flow of protons through complex 5 drives the synthesis of ATP from adenosine diphosphate (ADP) (35-39). This process is called oxidative phosphorylation and is shown in figure 1. Normally, the respiratory chain is not active, even when NADH, FADH2 and oxygen are present. Only when ADP is generated by catabolic activity, the increased ADP level activates the respiratory chain. Thus, cells can only oxidize glucose, fatty acids and amino acids when ADP is generated by metabolic activity. Activation of the respiratory chain is coupled to conversion of ADP into ATP. In addition, mitochondria can exist in an uncoupled state. In this situation, protons flow back into the mitochondrial matrix, independent of ATP synthesis, with generation of heat as result. By that, uncoupled mitochondria can oxidize large amounts of fuel. Uncoupling proteins and chemical agents like dinitrophenol and high concentrations of fatty acids induce mitochondrial uncoupling.

The complexes from the respiratory chain consist of ~90 subunits encoded by both the nuclear and mitochondrial genome. Since the mitochondrial genome encodes for only 13 of these subunits the majority of subunits are encoded by the nuclear genome (37).

Insulin secretion and mitochondria

In the pancreatic beta-cell, mitochondria play a key role in insulin secretion. Human beta-cells express high Km Glut2 transporters, which are present at the cell membrane and transport glucose into the cytosol. Glucose is phosphorylated into glucose-6-phosphate by a beta cell specific hexokinase, called glucokinase. This is also a high Km enzyme (40-42). Subsequently, glucose-6-phosphate is further

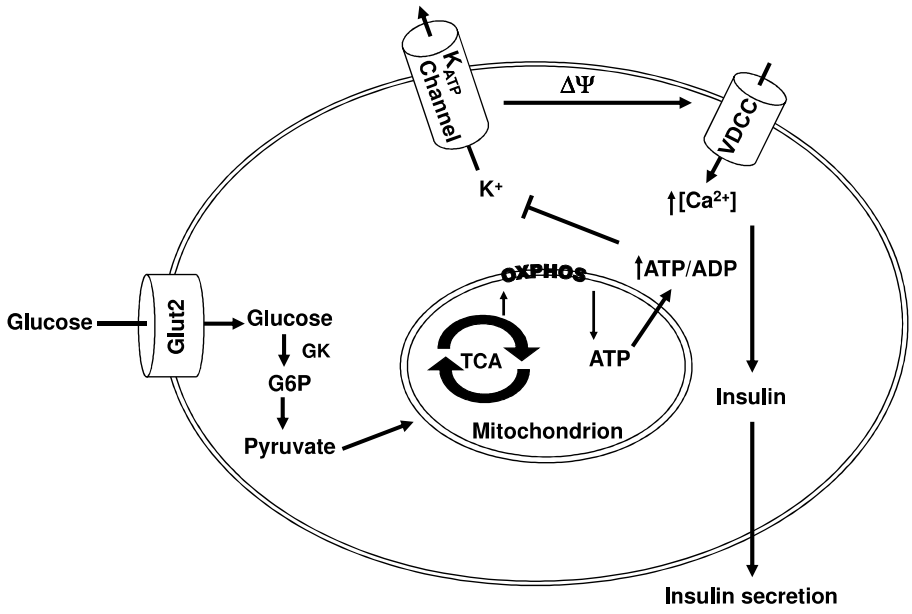
Figure 1. Oxidative phosphorylation by the respiratory chain



Complexes I – V are shown, embedded in the inner mitochondrial membrane. These complexes generate a proton flow directed outwards the mitochondrion, resulting in ATP synthesis by complex V.

metabolized resulting in the synthesis of ATP via processes described in the previous section. The presence of two high Km enzymes at the entrance of this glycolytic pathway makes that the flux through this pathway is very sensitive to variations in glucose concentrations around the physiological concentration of 5mmol/L (40;41). Therefore, variations in the ATP/ADP ratio in the cytoplasm depends on variations in the glycemic state. An increase in plasma glucose levels will increase the ATP/ADP ratio. An increased ATP/ADP ratio inhibits the ATP sensitive potassium channel, resulting in depolarization of the cell membrane. This activates the voltage-dependent calcium channel (VDCC) leading to an increased Ca^{2+} concentration in the cytoplasm, which is the main trigger for insulin secretion by fusion of insulin granules with the cell membrane (38;42). This process is shown in figure 2. Mitochondrial dysfunction could lead to an impaired glucose induced insulin secretion and subsequently type 2 diabetes.

Figure 2. Glucose stimulated insulin secretion



Glucose is transported into the beta-cell and processed into pyrovate. This enters the mitochondrion and is further processed into ATP, which is the main trigger for insulin secretion

Insulin resistance and mitochondria

Mitochondria are also involved in the oxidation of fatty acids. This process is called beta-oxidation and degrades fatty acids into acetyl-CoA, which is subsequently oxidized through the TCA cycle. In physically active muscle, in which large amounts of ADP are generated, fatty acids are the main fuel for ATP-synthesis. Fatty acids normally induce insulin resistance in muscle cells when insulin-stimulated glucose uptake is considered. This physiological adaptation process ensures that during fasting, when carbohydrate is scarce and fatty acids are generated by lipolysis in adipocytes, the physically active muscle uses fatty acids for energy supply, so that sufficient glucose remains available to provide the brain with energy since the brain uses predominantly glucose as fuel.
An inherited defect in mitochondria is often associated with triglyceride deposits in muscle, suggesting impaired removal of fatty acids. A similar situation is observed in HIV-patients treated with Highly Active Anti Retroviral Therapy containing nucleoside analogues, in which mitochondrial DNA content is decrease (43;44). This excess of fatty acids contributes to insulin resistance of muscle and liver, a situation also seen in type 2 diabetes. By this mechanism a mitochondrial dysfunction may contribute to the development of insulin resistance and type 2 diabetes (45-47). Furthermore, fatty acids are toxic to pancreatic beta-cells leading to a decline in insulin secretory capacity.

Aim of Part 1

Evidence is accumulating that mitochondrial dysfunction is a risk factor for the onset of type 2 diabetes. It has been shown that mitochondrial encoded enzymes of the oxidative phosphorylation are down regulated in type 2 diabetes patient muscle and these muscles have an impaired bioenergetic capacity (48;49). As described above, proper mitochondrial function is involved in both insulin secretion and insulin sensitivity. Alterations in these processes are the hallmarks of type 2 diabetes. Relatively rare mutations in the mitochondrial DNA are shown to be associated with MIDD in part through a decreased insulin secretion (50;51). Furthermore, it has been shown that insulin resistant offspring of type 2 diabetes patients have an impaired mitochondrial activity. However, common SNPs in the

mitochondrial genome are not found to be associated with type 2 diabetes (52;53). Since the majority of mitochondrial proteins are encoded by the nuclear genome and translocated to the mitochondria, we hypothesized that defects in nuclear encoded mitochondrial proteins may be associated with type 2 diabetes. The aim of this part is:

Analyzing the association of nuclear encoded mitochondrial targeted genes with type 2 diabetes susceptibility.

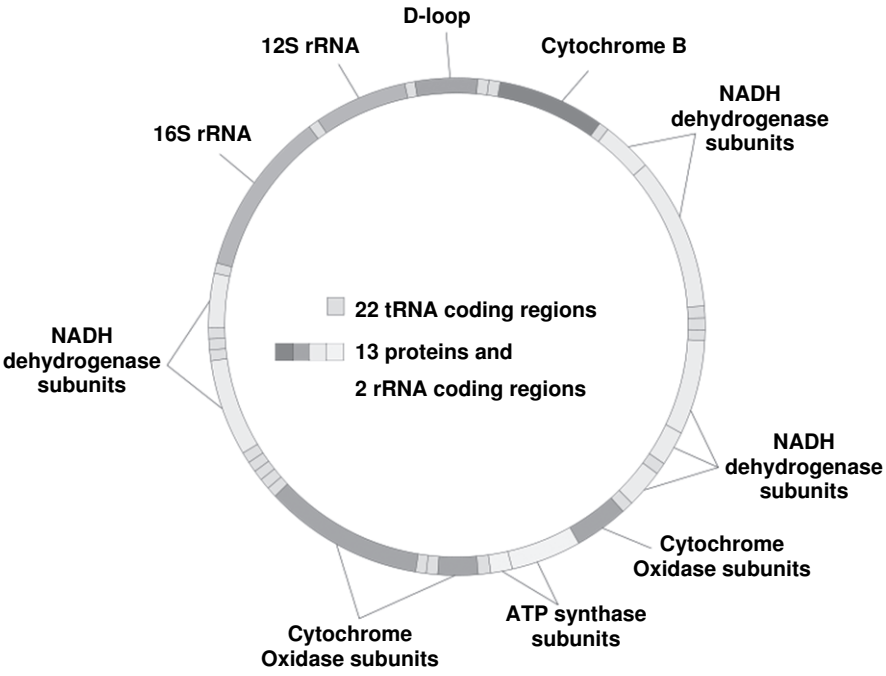
In chapter 2 I describe our search for an association of various SNPs in the *LARS2* gene with type 2 diabetes. The protein encoded by this gene is involved in mitochondrial protein synthesis. It encodes the charging enzyme for the mitochondrial leucyl-tRNA(UUR). A mutation in the mitochondrial leucyl-tRNA(UUR) gene can result in MIDD, but also mitochondrial myopathy, mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS) and chronic progressive external ophthalmoplegia (CPEO) (50;54). A particular polymorphism in the *LARS2* gene was previously found to be associated with type 2 diabetes (55). I investigated potential associations between several SNPs in *LARS2* with type 2 diabetes in various cohorts from the Netherlands, UK, Denmark Sweden and Finland.
In chapter 3 we have analyzed a selection of 13 candidate genes, all encoding mitochondrial proteins, for association with type 2 diabetes in the Netherlands and putative associations are replicated in cohorts from the Netherlands and Denmark. These candidate genes were selected from four clusters regarded essential for correct mitochondrial protein synthesis and biogenesis: aminoacyl tRNA synthetases, translation initiation factors, tRNA modifying enzymes and mitochondrial DNA transcription and replication.

Part 2: mitochondrial DNA content and type 2 diabetes

Mitochondrial DNA

As described in part 1 of this introduction, mitochondria have their own genome. This circular genome is approximately 16.6 kb in length and encodes for 13 genes of the oxidative phosphorylation, 2 rRNAs and 20 tRNAs (figure 3). Mitochondrial DNA (mtDNA) is predominantly maternally inherited. The quantity of mtDNA, the so-called mtDNA content, varies between different cell types. Cells contain approximately 1000 – 10,000 mtDNA copies (37;56). Mitochondrial activity in human fibroblasts declines upon aging, probably by accumulating somatic mutations in their DNA (57;58).

Figure 3. Map of mitochondrial DNA



Mitochondria have their own DNA of ~16.6 kb, encoding for all tRNAs, 2 rRNAs and 13 subunits for the oxidative phosphorylation.

Replication of mitochondrial DNA

Replication of mtDNA is not dependent on cell division, but there is continuous mtDNA turn-over. Mitochondrial DNA is synthesized by DNA polymerase gamma (POLγ), a RNA-dependent DNA polymerase. This enzyme consists of 2 subunits, POLγA and POLγB. The proteins replicative mitochondrial helicase (TWINKLE) and mitochondrial Single-Stranded DNA-Binding protein (mtSSB) are responsible for unwinding and stabilizing of the mtDNA respectively (56;59-62). Mice expressing a proof reading defective POLγA, are characterized by accumulation of point mutations and deletions in the mtDNA, resulting in decreased life span and aging phenotypes like weight loss, hear loss and osteoporosis (63).

Transcription of mitochondrial DNA

Transcription of mtDNA is directed from two promotor sites, the Heavy Strand Promotor and Light Strand Promotor. Transcription is performed by the Mitochondrial RNA Polymerase (POLRMT). POLRMT forms a complex with the Mitochondrial Transcription Factor A (TFAM) and one of the two other transcription factors; Mitochondrial Transcription Factor B1 (TFB1M) or Mitochondrial Transcription factor B2 (TFB2M), which play important roles in DNA binding, unwinding, and prevention of RNA/DNA hybrid formation. After transcription, the mRNA is processed into proteins by the mitochondrial protein synthesis machinery consisting of nuclear encoded proteins and two mtDNA encoded rRNAs (56;64-66).

Aim of Part 2

As described in part1, mitochondrial function is altered in type 2 diabetes patients. Whether this is a cause or consequence is largely unknown. It has been shown that patients treated with highly active antiretroviral therapy have 30-50% decreased mtDNA content and are at increased risk for developing diabetes and the metabolic syndrome. Furthermore, a reduction of peripheral fat and development of central obesity is observed (44;67). This indicates that a reduction in mtDNA content is rather a cause than consequence of type 2 diabetes development. It has also been shown that mitochondrial content declines during aging in pancreatic islets and decreased mitochondrial content in beta-cells is

associated with decreased insulin secretion (68;69). Furthermore, a small study showed evidence that low mtDNA content in blood precedes the development of type 2 diabetes, further suggesting that mtDNA content may contribute to the development of type 2 diabetes (70). In a twin study it has been observed that mtDNA content is higher correlated in monozygotic twin pairs, compared to dizygotic twin pairs in blood, indicating that regulation of mtDNA content has a genetic component (71). Another study showed that this heritability might be linked with a genomic region on chromosome 10q (72). Taken together variation in the mtDNA content is a plausible candidate to modulate the risk for type 2 diabetes. The aim of this part is:

Analysis of the heritability of mitochondrial DNA content in different tissues in relation to the risk for type 2 diabetes.

In *chapter 4* the heritability of mtDNA content is examined, using monozygotic and dizygotic twins, derived from the Dutch Twin Register. Mitochondrial DNA content is analyzed in samples obtained by buccal swabs. These cells represent a more homogenous cell sample compared to whole blood which was used in other studies. Furthermore, the association between mtDNA content and the onset of type 2 diabetes is examined in a case control study in the Netherlands and in two prospective studies from the Netherlands and Sweden.

Part3: Genes regulating fasting plasma glucose concentrations

Plasma glucose levels are tightly regulated. Despite fluctuations in food intake and physical exercise, the variation in plasma glucose is limited. Deregulation of the glucose homeostasis may lead to hyperglycemia, which is the major hallmark of type 2 diabetes. Variation of FPG levels within healthy limits (FPG < 7 mmol/L) is clinical important as it has been found that FPG levels in the higher region of the healthy range result in an elevated risk for heart disease and type 2 diabetes later in life (8;73-76). Furthermore, FPG levels in pregnant women are an important predictor of the offspring birth weight, which is associated with the development of type 2 diabetes later in life of the offspring (77;78). FPG levels are approximately 50% genetically determined (79). Therefore, a genetic predisposition for increased FPG levels may also represent an elevated risk for type 2 diabetes. A main component of the glucose sensing system, controlling FPG, consist of the pancreatic Glut2-Glucokinase system and its downstream pathway (80).

The aim of this part of my thesis is:

To analyze the effect of known FPG genes on FPG levels and subsequent risk for type 2 diabetes.

The results of this part are described in *chapter 5*. Using a population based study in the Netherlands we examined the effects of known FPG genes on several clinical variables like FPG and HbA_{1C}. Next we used a case-control study from the same region in the Netherlands to investigate the combined effect of these established FPG genes on type 2 diabetes risk.

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Chapter 2

Genetic association analysis of *LARS2* with type 2 diabetes

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Abstract

Aims/hypothesis

LARS2 has been previously identified as a potential type 2 diabetes susceptibility gene through the low frequency (LF) H324Q (rs71645922) variant (MAF 3.0%). However, this association did not achieve genome-wide levels of significance. The aim of this study was to establish the true contribution of this variant and common variants in *LARS2* (MAF >5%) to type 2 diabetes risk.

Methods

We combined genome-wide association study (GWAS) data (n=10128) from the DIAGRAM consortium, with independent data derived from a tagging SNP approach in Dutch individuals (n=999), and took forward two SNPs of interest for replication in up to 11163 Dutch subjects. In addition, because inspection of the GWAS data identified a cluster of LF variants with evidence of type 2 diabetes association, we attempted replication of rs9825041 (a proxy for this group) and the previously identified H324Q variant in up to 35715 subjects of European descent.

Results

No association of the common SNPs in *LARS2* with type 2 diabetes was found. Our replication studies for the 2 LF variants, rs9825041 and H324Q failed to confirm an association with type 2 diabetes in Dutch, Scandinavian and UK samples (OR 1.03 (0.95-1.12), p=0.45, n=31962 and OR 0.99 (0.90-1.08), p=0.78, n=35715 respectively).

Conclusion

In this study, the largest study examining the association of sequence variants in *LARS2* with type 2 diabetes susceptibility we find no evidence to support previous data indicating a role in type 2 diabetes susceptibility.

Introduction

Changes in mitochondrial function are observed in patients with type 2 diabetes and their first degree relatives. Previous studies have indicated that genes involved in oxidative phosphorylation are down regulated in the muscle cells of type 2 diabetes patients (1). Furthermore, the muscle mitochondria from patients with type 2 diabetes have an impaired bioenergetic capacity (2). Mitochondria also play an important role in insulin secretion and sensitivity (3;4). Previously, our group has shown that a mutation in the mitochondrial DNA encoded tRNA-Leu(UUR) gene is associated with maternally inherited diabetes and deafness (5). In addition, an H324Q (rs71645922) variant in the nuclear encoded mitochondrial *LARS2* gene has shown an association with type 2 diabetes in work previously carried out by our group (6). The *LARS2* gene encodes for the mitochondrial leucyl tRNA synthetase (EC 6.1.1.4), which catalyzes the aminoacylation of both mitochondrial leucyl tRNAs with leucine and is therefore essential for mitochondrial protein synthesis. By analyzing the coding region for the *LARS2* gene we found an H324Q (rs71645922) variant, and demonstrated an association with type 2 diabetes susceptibility in a meta-analysis of four independent cohorts from the Netherlands and Denmark (OR = 1.40 (95% CI 1.12-1.76, P = 0.004, n = 7836) (6).

In recent years the advent of genome-wide association studies (GWAS) and the accumulation of large data sets capable of detecting associations to levels of genome wide significance appropriate for such studies ($p<5*10^{-8}$) has identified close to 20 loci impacting on type 2 diabetes susceptibility. However, low frequency variants such as H324Q are generally poorly captured by such studies. We set out therefore to re-evaluate the possible contribution of this LF variant to type 2 diabetes susceptibility in appropriately sized samples. We also used a combination of publicly available (DIAGRAM consortium) and newly derived tagging SNP data to undertake the most comprehensive assessment of the *LARS2* locus yet performed.

Materials and methods

Study samples

The first part of our study was aimed at the identification of common alleles associated with increased type 2 diabetes susceptibility using DIAGRAM consortium data and a tagging SNP approach. For this we genotyped several European samples.

The first sample we included was from the Hoorn Study (here designated as NL1) (7). From this Dutch population based study from the city of Hoorn, in North-Western Netherlands, we selected 519 normal glucose tolerant (NGT) subjects and 480 type 2 diabetes subjects. Glucose tolerance was assessed using a fasting oral glucose tolerance test (OGTT), according to 1999 World Health Organization (WHO) criteria (8). This sample was used for the analysis of common variation in *LARS2* with a tagging SNP approach. Variants in *LARS2* identified from the DIAGRAM meta-analysis and the tagging SNP approach were then taken forward for replication in three Dutch samples, designated NL2, 3 and 4 respectively.

The second sample from the Netherlands (designated NL2) included 1517 controls and 821 cases (9;10). The1517 controls were randomly selected from the New Hoorn Study (NHS), which is an ongoing, population based study from the city of Hoorn, which does not overlap with the original Hoorn Study (NL1). We included 147 cases from the NHS and the remainder of the cases (n=674) were recruited from the diabetes clinics of the Leiden University Medical Centre (LUMC, Leiden) and from the Vrije Universiteit medical centre (VUmc, Amsterdam). All subjects in this replication sample were Dutch Caucasians and all NGT subjects underwent an OGTT according to WHO criteria(8).

The third replication sample was ascertained from the Breda study (NL3) (11;12). This is a case control study from the city of Breda, in Southern Netherlands. The 920 controls were from the Dutch blood bank and self reported a non-diabetic state. The 501 cases had type 2 diabetes based on WHO criteria (8).

For the fourth replication sample we selected 5183 NGT subjects and 1222 type 2 diabetes subjects from the population based ERGO study from the city of Rotterdam in the South-western region of the Netherlands (NL4) (13).

In total 8139 controls and 3024 type 2 diabetes cases were included in our replication study in the Netherlands.

The second part of this study was focused on the follow up of 2 low frequency variants in *LARS2* and for this we carried out replication in samples from the Netherlands (NL1-4) as well as samples from the UK (UK1,2), Denmark (DK1), Finland (FI1,2) and Sweden (SE1).

We included one replication sample from Denmark (designated DK1) (14). This sample consists of 514 NGT controls which are randomly selected from public registers at the Steno Diabetes Center and the Research Centre for Prevention and Health, Copenhagen, Denmark. The 706 cases were recruited from the Steno Diabetes Center. NGT subjects underwent an OGTT according to WHO criteria (8).

Two UK samples were included. The first (UK1) was the UKT2DGC (United Kingdom Type 2 Diabetes Genetics Consortium) case-control sample comprising 4124 type 2 diabetes cases and 5126 controls ascertained in Tayside, Scotland. Details of the ascertainment scheme and recruitment criteria for this sample have been described elsewhere (15;16): the enlarged sample used here represents continuing recruitment to this resource under precisely the same criteria. The second sample (UK2) consists of 1853 type 2 diabetes cases ascertained as part of the BDA Warren 2 collection (Exeter, London, Oxford, Norwich and Newcastle) and 10220 control samples. The latter represent the full British 1958 Birth Cohort (n=7133) and The United Kingdom Blood Services Collection of Common Controls (UKBS) (n= 3087), a subset of which featured in the WTCCC genome wide association scan (both samples were collected throughout the UK) (15;16).

Finally, we included samples from Finland and Sweden. One was a case-control sample from the Botnia region of Finland, here designated as FI1. This sample consisted of 353 controls and 402 cases. The second sample originated from Sweden (Skara and Malmö), here designated as the Swedish case-control study (SE1) and consisted of 468 controls and 480 cases (17;18). Furthermore, we included a set of trios originating from the Botnia region of Finland (FI2). This sample consisted of 211 probands (multiple diabetic sibs) and 370 parents. All study samples are summarized in table 1.

In total 25191 controls and 10800 type 2 diabetes cases were included for the follow up of the LF variants.

All studies were approved by the appropriate medical ethical committees and were in accordance with the principles of the Declaration of Helsinki. All participants provided written, informed consent for this study.

Common SNP selection

Common SNPs (MAF >5%) in the *LARS2* locus were selected for follow-up based on the data from the DIAGRAM meta-analysis (gene boundaries chr3: 45373001…45698001) (22). SNPs with a P<0.05 were genotyped in the Dutch replication samples (NL1-4). Furthermore, tagging SNPs in *LARS2* were selected for genotyping in the NL1 sample using the HapMap database and Tagger software (19;20) (selection criteria and SNPs shown in supplementary table S1).

Table 1. Description of study samples.

Study	Subjects (%male)		Mean Age (years)(SD)		Mean BMI (Kg/m^2)(SD)	
	Controls	cases	controls	cases	controls	cases
NL1	519 (55)	480 (52)	65 (8)	67 (8)	26.4 (4.5)	28.8 (4.6)
NL2	1517 (44)	821 (50)	53 (7)	61 (11)	25.5 (3.6)	29.0 (4.6)
NL3	920 (61)	501 (46)	48 (13)	71 (10)	n.a.	27.8 (4.1)
NL4	5183 (41)	1222 (39)	69 (9)	73 (9)	26.0 (3.9)	27.4 (4.0)
DK1	514 (46)	706 (48)	57 (10)	59 (10)	25.9 (3.8)	29.3 (5.1)
UK1	5126 (51)	4124 (55)	60 (13)	66 (6)	26.9 (11.4)	31.2 (13.8)
UK2	10220 (50)	1853 (61)	42 (7)	57 (9)	27.2 (6.4)[a]	31.8 (6.7)
FI1	353 (53)	402 (55)	60 (10)	61 (10)	26.1 (3.6)	28.7 (4.5)
FI2	370 (50)[b]	211 (47)[c]	n.a	40 (9)	28.5 (5.5)	n.a.
SE1	468 (52)	480 (53)	66 (12)	67 (11)	27.5 (4.1)	27.9 (4.1)

n.a. not available

a. Based on the British 1958 Birth Cohort (7133) and Panel 2 of the United Kingdom Blood Services Collection of Common Controls (n=1643)

b. parents

c. probands

Genotyping and quality control

SNPs selected for follow-up in our replication samples were genotyped using Taqman SNP genotyping assays (Applied Biosystems, Foster City, USA). Tagging SNPs were genotyped in the NL1 sample using the Sequenom platform (Sequenom, San Diego, USA). Assays showing overlapping clusters, success rates below 95% or not obeying Hardy Weinberg Equilibrium (HWE) ($p<0.05$) were excluded from analysis. Duplicate samples (~5%) showed complete concordance.

Statistical analysis

Differences in genotype distribution and allele frequencies were analyzed using a chi-squared test. ORs were calculated using an additive model, which was the best fit for the data. Homogeneity of ORs between the different samples was calculated with a Tarone's test after which a common OR was calculated with a Mantel-Haenszel test using a fixed effects model. Results from OGTT (only normal glucose tolerant subjects) were analyzed with univariate analysis of variance, using additive, recessive and dominant models and correction for age, BMI and gender as possible confounders. Association in the Botnia trios was assessed by the transmission disequilibrium test (TDT). All general statistics were calculated using SPSS 16.0 (SPSS Inc, Chicago, USA). For statistics involving the geographical distribution of the H324Q (rs71645922) variant in the UK population (described below) we used StatXact v 6.0 (Cytel software corps, Cambridge, MA, USA). Power calculations were performed using Quanto (21). From the DIAGRAM consortium meta-analysis of common variants we selected for replication all common SNPs with a $p<0.05$. At this alpha the DIAGRAM consortium meta-analysis had at least 80% power to detect a variant with OR ≥1.20 (MAF >0.05) (22). Combined with our Dutch replication sample we had at least 80% power to replicate the association of a variant with an OR ≥1.09 at the observed MAFs of 0.19 (rs952621) and 0.24 (rs17637703) respectively (alpha = 0.05) or OR ≥1.12 at alpha = 10^{-4}). Power of the tagging SNP approach in NL1 was limited (80% power to detect a variant with an OR≥1.6 (alpha=0.05, MAF=0.05) or OR≥1.45 at the observed lowest MAF of 0.10) therefore we replicated in NL2-4 only our strongest signal from the NL1 sample (rs17637703, $p = 0.07$).

Whilst extensive GWAS have indicated that the effect sizes of common variants influencing type 2 diabetes risk are modest, the potential remains for low-frequency variants to have effects on type 2 diabetes risk that are more substantial which was corroborated by our previous observation regarding the H324Q variant (6). Power calculations at the start of the project demonstrated that we had at least 99% power to detect an effect size similar to our initial finding concerning H324Q (rs71645922) (OR 1.4) and at least 80% power to detect an OR of 1.13 (α=0.05) (6). From the DIAGRAM meta-analysis we used an alpha of 0.05 to select other LF SNPs for replication. At this alpha the power in DIAGRAM was 80% to detect association for variants with ORs ranging from 1.24 (MAF = 0.03) to 1.45 (MAF = 0.01 and alpha = 0.05). For replication of the two low frequency variants (observed MAFs ~0.03(H324Q, rs71645922) and ~0.05 (rs9825041) respectively) we had in our complete replication sample at least 80% power to detect an OR ≥1.13 (25191 controls and 10800 type 2 diabetes cases and alpha = 0.05).

Results

Common LARS2 variants in available DIAGRAM *GWAS data*

We analyzed the data from the DIAGRAM GWAS meta-analysis (22) for the *LARS2* gene (100% coverage (MAF > 5%), according to HapMap phase 2, April 2007, CEU population) and observed 1 common SNP (rs952621, directly typed) showing weak evidence of association with type 2 diabetes (OR = 1.11 (1.02 – 1.20), p = 0.01 for the T allele). This SNP was also captured in our complementary tagging SNP approach (NL1) and we found an OR of 1.13 (0.89 – 1.43), p = 0.33 for the same allele. However, additional genotyping in the Dutch samples (NL2,4) and meta-analysis of all data resulted in a common OR of 1.05 (0.99 – 1.11), p = 0.13 (n=19870). As there was no convincing evidence of association in our samples, this SNP was not analysed further.

No other common SNP in *LARS2* showed evidence for association with type 2 diabetes in the GWAS data. The same was true of the tagging SNP analysis conducted in the NL1 sample (supplementary table s1). In the latter analysis, rs17637703 showed weak evidence of association (OR = 1.22 (95% C.I. 0.99 – 1.50), p = 0.07) but this was not confirmed in the Dutch replication samples (the

common OR was 0.98 (0.91 – 1.06), p = 0.62 (n = 10087), in line with the DIAGRAM result for this SNP (OR 1.02 (0.94 – 1.10)).

Low frequency variants in LARS2

In addition to the common variants, the DIAGRAM meta-analysis also captured fourteen LF SNPs (0.01<MAF<0.05) within the *LARS2* gene, ten of which are in high LD with each other (r^2 > 0.95 according to HapMap, supplementary figure S1) and showed some evidence for association with type 2 diabetes (ORs 1.17 – 1.21; p 0.02 – 0.05). We selected rs9825041 (OR = 1.20 (1.03 – 1.39), p = 0.02) as a proxy for the group for genotyping in the replication samples but no association with type 2 diabetes was observed (table 2). Homogeneity of ORs was tested with a Tarone's test (p = 0.67) and we calculated a common OR across all studies of 1.03 (0.95 – 1.12), p = 0.45, (8959 cases, 23003 controls).

Follow up of the H324Q (rs71645922) variant in LARS2

Finally, we examined the association of the H324Q (rs71645922) variant with type 2 diabetes in our replication samples from the Netherlands (NL2-4), UK (UK1, 2), Sweden (SE1) and Finland (FI1). This variant was not captured by the GWAS and was not captured by any of the SNPs mentioned above (r^2 < 0.17). Subjects in the Dutch replication samples that were included in our original study of this variant were excluded from analysis (n = 914 from the NL4 study). The replication samples did not confirm our previously observed association. A meta-analysis of all available studies including our previous data from the Netherlands (NL1) and Denmark (DK1, 2) (6) resulted in an overall OR of 0.99 (0.90 – 1.08), p = 0.78, n = 35715 subjects (10399 type 2 diabetes subjects, table 2). In addition, we did not observe a significant excess of transmission of the risk allele in the Botnia trios (FI2, transmitted / untransmitted = 18 / 14, OR 1.29 (0.64 – 2.59), p = 0.48).
To investigate possible heterogeneity between the studies we performed several analyses. For age stratification we created, based on the age distribution in the Dutch samples the following age strata; ≤60 years, 61-70 years and >70 years. A decreased frequency of the risk allele was observed in type 2 diabetes subjects with increasing age in most but not all samples (data not shown, available on

request), but this did not reach statistical significance. Furthermore we looked at age at diagnosis of type 2 diabetes and allele frequencies in those with early onset diabetes (≤45 years) and those with an age at diagnosis above 45 years. Although the allele frequency was slightly higher in those with early onset diabetes this was not statistically significant nor was the age at diagnosis in carriers and non-carriers (all $p > 0.05$, data not shown). Stratification for gender and BMI (where data available) did not affect the outcome in the Dutch studies (NL1-NL4, data not shown) and was therefore not further investigated.

H324Q (rs71645922) shows marked variation in MAF between the various European-descent samples examined (control MAF ranges from 1.9% to 4.8%). In the two large UK control samples, for example, there was a highly significant ($p = 5 \cdot 10^{-7}$, using an exact implementation of the Cochran Armitage trend test) difference in allele frequencies between UK1 (recruited exclusively in Scotland) and UK2 (recruited throughout the UK) that made us consider the possibility that this variant was showing variation in allele frequency along the south-north cline previously described in the WTCCC study and others (16;23-26).To test this, we made use of information on the region of ascertainment available for the UK 1958 Birth Cohort and UK Blood Service and analysed genotype frequencies based on subdivisions of the UK into 4 major regions, namely (1) Scotland, (2) Northern England(Yorkshire and the Humber, North East, North West), (3) UK Midlands (East Midlands, West Midlands, Wales, East of England) and (4) South of England (South East, Greater London, and South West). We found some evidence (supplementary figure S2) for a North - South gradient across the UK. (MAF 4.66 %, 3.41%, 3.31%, and 3.28% respectively) with $p = 0.038$ calculated using the Jonckheere-Terpstra Test (StatXact v 6.0; Cytel software corporation, Cambridge, MA, USA). No such MAF gradient was observed in other European samples (supplementary figure S3)

Table 2. Genotyping results for rs9825041 and H324Q (rs71645922).

Study	rs9825041				H324Q (rs71645922)			
	Controls	Cases	OR 95% CI	P_{Add}	Controls	Cases	OR 95% CI	P_{Add}
NL1	5.5	7.3	1.35 (0.92 – 1.98)	0.15	1.9	4.3	2.26 (1.10 – 4.66)[a]	0.04
NL2	6.0	6.6	1.11 (0.85 – 1.44)	0.46	3.4	2.7	0.78 (0.49 – 1.25)	0.18
NL3	5.7	6.8	1.21 (0.88 – 1.66)	0.25	3.3	3.0	0.90 (0.57 – 1.41)	0.74
NL4	6.1	5.5	0.89 (0.74 – 1.08)	0.27	3.1	3.5	1.11 (0.84 – 1.47)[a]	0.47
DK1	4.2	4.7	1.12 (0.75 – 1.68)	0.62	2.2	2.8	1.24 (0.74 – 2.09)[a]	0.44
DK2	n.m.	n.m.	n.m.	n.m.	2.8	3.7	1.33 (0.97 – 1.82)[a]	0.08
UK1	4.9	4.9	1.00 (0.87 – 1.14)	1.00	4.8	4.4	0.91 (0.79 – 1.05)	0.19
UK2	4.8	5.1	1.05 (0.88 – 1.27)	0.57	3.6	3.4	0.94 (0.77 – 1.15)	0.59
FI1	n.m.	n.m.	n.m.	n.m.	4.2	4.9	1.18 (0.72 – 1.93)	0.51
SE1	n.m.	n.m.	n.m.	n.m.	4.5	4.2	0.94 (0.59 – 1.48)	0.79
M-A			1.03 (0.95 – 1.12)	0.48			0.99 (0.90 – 1.08)	0.82
DIAGRAM[b]			1.20 (1.03 – 1.39)	0.02			n.m.	n.m.

Minor allele frequencies are shown for cases and controls.
n.m.: not measured
M-A: meta-analysis
a. Data taken from reference (6). Results from the NL4 study are partially from this previous research (n = 914).
b. Meta analysis of the DIAGRAM consortium GWAS, n = 10128 (4549 cases and 5579 controls) (22).

Discussion

We found no evidence of common SNPs in *LARS2* being associated with type 2 diabetes in our samples. We therefore conclude that it is unlikely that common SNPs in *LARS2* are associated with type 2 diabetes susceptibility.

Several low frequency SNPs, which are all in high LD with each other ($r^2 > 0.95$) showed nominal evidence of association with type 2 diabetes in the DIAGRAM meta-analysis. However, we have been unable to confirm this association in our large replication samples from the Netherlands, Denmark and the UK (n = 31962) and therefore conclude that the nominal p-values observed in the GWAS are most likely consistent with statistical noise.

The previously observed association of the H324Q (rs71645922) variant in *LARS2* with type 2 diabetes was not confirmed in our replication samples (table 2). Power

calculations at the start of the project demonstrated that we had at least 99% power to detect an effect size similar to our initial finding concerning H324Q (rs71645922) (OR 1.4) and at least 80% power to detect an OR of 1.13. Before excluding the previous association as false, we considered the possibility of heterogeneity but found no evidence that age, age at diagnosis, BMI and gender were responsible. Another possibility, raised by the evidence for variation in H324Q MAF across the UK is that the previous association in Dutch and Danish subjects reflected the effects of hidden population structure. It seems, however, unlikely that population stratification effects were responsible for the original reports of H324Q (rs71645922) associations as the cases and controls in that study were recruited from the same relatively narrow geographic regions within the Netherlands and Denmark (6). Also in our additional Dutch replication cohorts we could not detect a MAF gradient across the country. However, there are differences in MAF between different countries (supplementary Figure S2, S3). Migration patterns in the UK appear to reflect an increase in the MAF of H324Q (rs71645922) and therefore this may be a potential migration marker, however this needs to be demonstrated in other populations. Since stratification for BMI and gender did not affect our result, we can exclude that these variables confounded our observation. The reason for the discrepancy between our first and current study is likely to reflect chance. Three other low frequency non-synonymous SNPs are present in the *LARS2* locus; K727N (rs36054230), E831D (rs9827689) and E868K (rs34965084). However, according to dbSNP and our own sequencing efforts (6) these SNPs are only identified in the African population and not polymorphic in the European population. Therefore, these additional non-synonymous variants were not analysed in this study. As our study does not include a thorough resequencing of the complete *LARS2* locus we cannot fully exclude that other yet unknown LF variants are present and associated with type 2 diabetes. Results from the 1000 genomes project should facilitate a thorough investigation of LF SNPs in *LARS2* in the future. In conclusion, our findings do not support the hypothesis that common variants in *LARS2* are major type 2 diabetes susceptibility factors. We have also conducted one of the largest (up to 35715 subjects) replication studies for two low frequency variants for type 2 diabetes and also these data do not support a significant role as

type 2 diabetes susceptibility variants. We therefore conclude that currently known genetic variation in *LARS2* does not play an important role in type 2 diabetes susceptibility.

Acknowledgements

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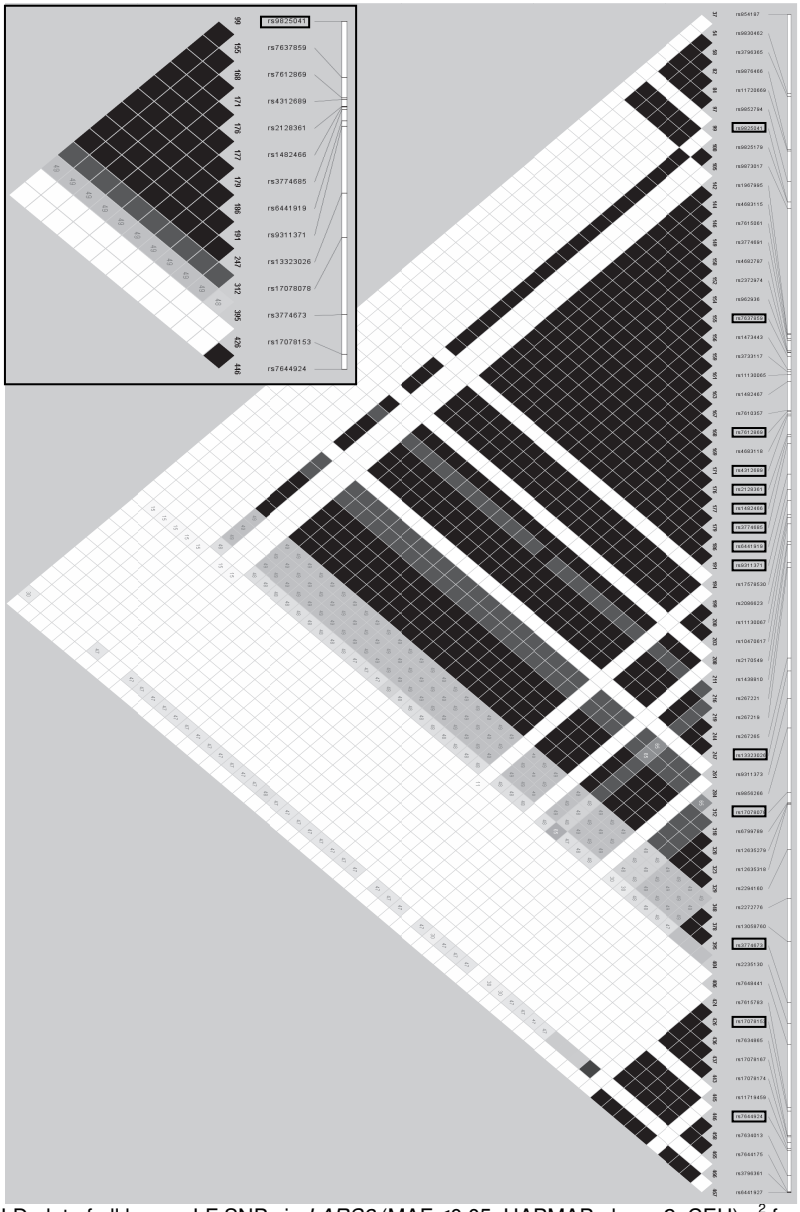
Supplementary figure S1. LD plot of all known LF SNPs in the LARS2 locus.

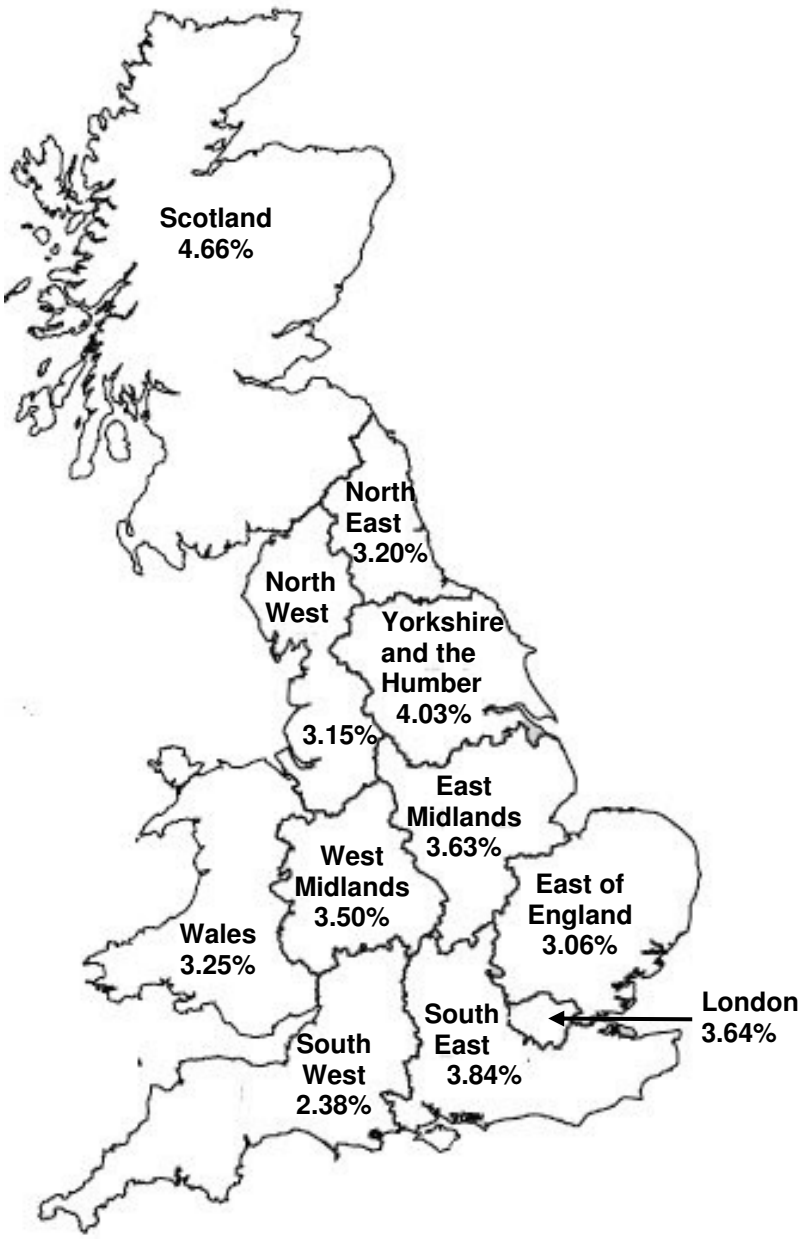


LD plot of all known LF SNPs in *LARS2* (MAF ≤0.05; HAPMAP phase 2, CEU). r^2 for each of the SNP pairs is given (black r^2=1, shades of gray 0< r^2<1 and white r^2=0).

The 14 LF SNPs directly genotyped in DIAGRAM are boxed. In the insert only the 14 LF SNPs included in the DIAGRAM scan are shown.

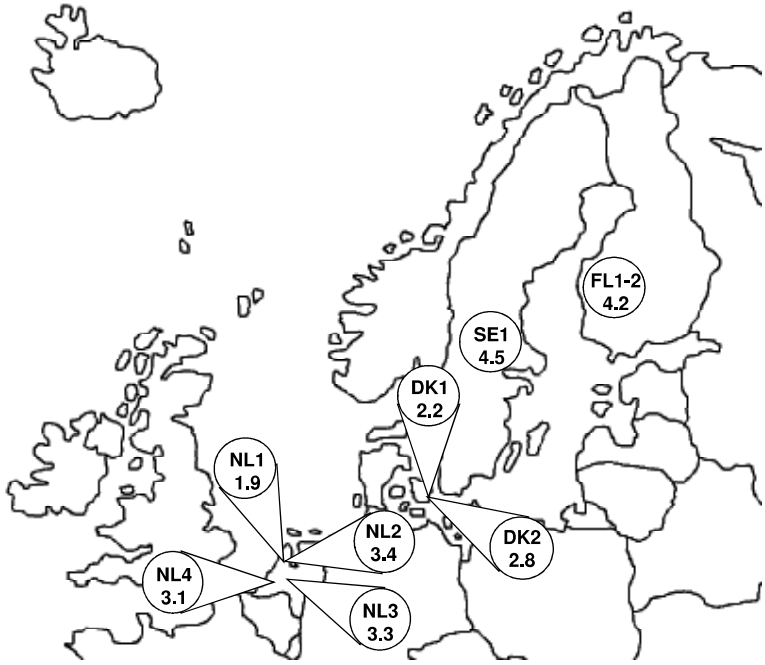
rs9825041 was used as a proxy for the block and is shown in the box.

Supplementary figure S2. Geographic stratification in the UK for the H324Q (rs71645922) variant.



The numbers represent minor allele frequencies of the H324Q (rs71645922) variant in each of the geographic regions.

Supplementary figure S3. Geographic stratification in other European samples.



The numbers is the circles represent minor allele frequencies of H324Q (rs71645922) in each of the study samples.

Supplementary table S1. Overview of tagging SNPs, including results from the NL1 study.

rs number	Position	Allele 1	Allele 2	MAF controls	MAF cases	OR 95% C.I.	p-value
rs854191	45409713	T	C	0.19	0.16	0.86 0.68 – 1.08	0.22
rs17637580	45411477	G	A	0.33	0.35	1.08 0.89 – 1.30	0.45
rs17077759	45411945	A	C	0.23	0.20	0.86 0.69 – 1.07	0.17
rs17637703	45415725	A	G	0.22	0.25	1.22 0.99 – 1.50	0.07
rs864391	45422021	G	A	0.23	0.20	0.88 0.71 – 1.09	0.23
rs6802884	45423962	T	C	0.19	0.17	0.91 0.72 – 1.14	0.42
rs17576051	45424984	A	G	0.11	0.10	0.94 0.71 – 1.25	0.72
rs3774696	45425473	A	C	0.24	0.27	1.16 0.95 – 1.42	0.17
rs9878643	45426336	G	A	n.a.	n.a.	n.a.	n.a.
rs6764923	45429693	A	G	0.40	0.37	0.88 0.69 – 1.13	0.32
rs17576289	45433737	A	G	0.14	0.12	0.87 0.67 – 1.12	0.29
rs4682783	45445335	A	G	n.a.	n.a.	n.a.	n.a.
rs4683113	45450337	A	G	0.42	0.42	1.00 0.80 – 1.25	1.00
rs9825692	45451366	G	A	0.28	0.31	1.17 0.97 – 1.42	0.12
rs952621	45451698	C	T	0.18	0.20	1.13 0.89 – 1.43	0.33
rs11926964	45464453	A	T	0.18	0.16	0.89 0.70 – 1.13	0.34
rs1877960	45471148	T	C	0.29	0.28	0.95 0.73 – 1.24	0.74
rs1949304	45475954	T	C	0.28	0.28	0.97 0.74 – 1.26	0.84
rs3774694	45481450	C	T	0.31	0.32	1.02 0.84 – 1.23	0.89
rs12487705	45492763	A	G	0.36	0.38	1.12 0.93 – 1.34	0.26
rs2128358	45524032	A	G	0.13	0.13	1.04 0.80 – 1.34	0.79
rs11706785	45546084	A	G	0.43	0.42	0.95 0.75 – 1.19	0.64
rs2118749	45547261	C	T	0.28	0.30	1.09 0.84 – 1.42	0.54
rs9825041	45502488	G	A	0.06	0.07	1.35 0.92 – 1.98	0.15

The *LARS2* locus was also investigated using a tagging SNP approach in the NL1 sample.
Common SNPs (MAF > 0.05), in *LARS2* were identified using HAPMAP (gene boundaries set at 45,405,079 - 45,565,332)[19].
Tagging SNPs were selected using the Tagger option in Haploview (NCBI build 36, phase II, April 2007, population CEU, LOD threshold 3.0 and $r^2 > 0.8$)[20].
Allele 1 is the major and allele 2 is the minor allele.
n.a.: not available.

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Chapter 3

Genetic association analysis of 13 nuclear encoded mitochondrial candidate genes with type 2 diabetes: the DAMAGE study

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Abstract

Aims/hypothesis

Mitochondria play an important role in many processes, like glucose metabolism, fatty acid oxidation and ATP synthesis. In this study we aimed to identify association of common polymorphisms in nuclear encoded genes involved in mitochondrial protein synthesis and biogenesis with type 2 diabetes using a two stage design.

Methods

In the first stage we analyzed 62 tagging SNPs in the Hoorn study (n = 999 subjects), covering all common variation in 13 biological candidate genes. These 13 candidate genes were selected from four clusters regarded essential for correct mitochondrial protein synthesis and biogenesis: aminoacyl tRNA synthetases, translation initiation factors, tRNA modifying enzymes and mitochondrial DNA transcription and replication. SNPs showing evidence for association with type 2 diabetes were measured in second stage genotyping (n = 10164 subjects).

Results

After a meta-analysis only one SNP in *SIRT4* (rs2522138) remained significant (p = 0.01). Extending the second stage with samples from the Danish Steno Study (n = 1220 subjects) resulted in a common OR of 0.92 (0.85 – 1.00), p = 0.06. Moreover, in a large meta-analysis of three genome wide association studies this SNP was also not associated with type 2 diabetes (p = 0.72).

Conclusion

In conclusion, we did not find evidence for association of common variants in 13 nuclear encoded mitochondrial proteins with type 2 diabetes.

Introduction

Mutations in genes involved in mitochondrial protein synthesis have been found to associate with a diabetic state. The 3243A>G mutation in the mitochondrial tRNA(Leu, UUR) gene, which impairs mitochondrial protein synthesis, is strongly diabetogenic as are some additional mutations in mtDNA encoded tRNA genes (1;2). Recently, we found that an H324Q variant in the nuclear encoded mitochondrial leucyl tRNA synthetase (*LARS2*) results in an increased type 2 diabetes susceptibility (3). It seems that a change in the activity of mitochondrial protein synthesis makes the organism more vulnerable to develop glucose intolerance.

A change in mitochondrial protein synthesis is expected to result in an unbalance in the stoichiometry of the proteins composing the respiratory chain. Some proteins of the respiratory chain are synthesized through the mitochondrial protein synthesis machinery, whereas the others are synthesized in the cytosol and imported into the mitochondrion. Impaired activity of the mitochondrial protein synthesis machinery is therefore expected to result in an unbalance between the nuclear DNA- and mitochondrial DNA-encoded proteins of the respiratory chain. A similar situation of an unbalance may arise when the rate of mitochondrial biogenesis is altered.

In type 2 diabetes a reduced activity of the respiratory chain is seen, which is also present in first degree relatives (4;5). The biochemical basis of this phenomenon may arise through an unbalanced expression of the various proteins. Based on these considerations, we hypothesized that polymorphisms in genes involved in mitochondrial protein synthesis and mitochondrial biogenesis may alter the balanced expression of proteins of the respiratory chain. As a result these polymorphisms are expected to modify the risk for type 2 diabetes. It is estimated that the complete mitochondrial proteome consists of approximately 1500 proteins, which are mostly encoded by the nuclear genome (6). Common variations in genes encoded by the mitochondrial genome are not associated with the onset of type 2 diabetes (7;8). Based on a literature study, we selected 13 candidate genes divided in several clusters. These candidates are all nuclear genes encoding for mitochondrial proteins.

Because of previous association of *LARS2* with type 2 diabetes the first cluster consisted of the mitochondrial aminoacyl-tRNA synthetases *DARS2*, *TARS2* and *IARS2*. *DARS2* and *TARS2* are located on a widely replicated type 2 diabetes linkage locus on chromosome 1q (9). Furthermore, variants in the $tRNA^{Ile}$ gene were shown to be associated with metabolic disorders (10).

The second cluster consisted of the two mitochondrial translation initiation factors, *MTIF2* and *MTIF3*. These two genes are involved in initiation of the mitochondrial protein synthesis and dysfunction could lead to altered expression of mitochondrial proteins and subsequently to oxidative stress and late onset disease (11-13). We have selected them above other important translation factors, like *TUFM*, *TSFM* and *GFM1*, because mutations in the latter have already been associated with severe early onset disease (14).

The third cluster consisted of *MTO1*, *TRMU* and *GTPBP3,* which all encode tRNA modifying enzymes. Variation in these genes could lead to dysfunctional enzymes and subsequently result in impaired tRNA modification and mitochondrial disease (15-17).

In our fourth cluster we included *TFAM* and *PPRC1*, which are involved in transcription and replication of the mitochondrial genome. Previously, it has been shown that a low mitochondrial copy number is associated with type 2 diabetes, although this is not confirmed in all studies (18;19). *TFAM* is one of the main regulators of mitochondrial transcription and replication and thus of the mitochondrial copy number (20;21). *PPRC1* is a *PPARGC1A* related gene and involved in mitochondrial biogenesis (22).

In addition, we selected 3 genes, which did not fit in a specific cluster but may affect mitochondrial biogenesis and function. *SIRT3* and *SIRT4*, which are Sir2 homologues, are involved in mitochondrial function and insulin secretion (23;24). Moreover, *SIRT3* has been associated with longevity (25). *GPAM* is the final candidate gene, which is involved in triacylglycerol and phospholipid synthesis. *GPAM* knock out mice show hyperinsulinemia and reduced glucose tolerance (26;27). Therefore, we hypothesized that defects in *GPAM* could lead to insulin resistance and subsequently type 2 diabetes.

Dysfunction of these 13 candidate genes potentially affects mitochondrial function and may therefore increase type 2 diabetes susceptibility. Therefore, the aim of the present study was to analyze the association of common variants in these gene loci with type 2 diabetes, using a tagging SNP approach.

Materials and methods

SNP selection

Tagging SNPs were selected using the HapMap database (www.HAPMAP.org) and Tagger (28;29). The threshold for the minor allele frequency (MAF) was set at 0.05. Using Tagger 62 SNPs were selected, which cover all common variation in the selected genes according to HapMap data (phase 2, April 2007 (Build 36), population CEU, $r^2 > 0.8$). Aggressive tagging with 2- and 3-marker tests was used. This resulted in 4 multimarker tests. The remaining tests were all single marker tests. Gene boundaries include 5' and 3' UTR regions. Details about the SNP selection for the individual genes, including gene boundaries, are summarized in table 1.

Genotyping and quality control

A two stage design was used. For first stage genotyping the Sequenom platform (Sequenom, San Diego, USA) was used. First, the allelic discrimination plots were visually observed for good clustering. Assays with bad clustering were not further analyzed. SNPs with a success rate below 95% or not obeying Hardy Weinberg Equilibrium (HWE) ($p < 0.01$) were excluded from analysis. SNPs which did not fit in the Sequenom assay or failed quality control (QC) were genotyped with Taqman SNP genotyping assays (Applied Biosystems, Foster City, USA) and evaluated with the same QC guidelines. Duplicate samples (~5%) showed identical genotypes. SNPs showing evidence for association with type 2 diabetes ($p < 0.05$) were selected for second stage genotyping. Taqman SNP genotyping assays were used for this.

Table 1. Gene boundaries and SNPs in the selected candidate genes.

Gene	Chr	Gene boundaries for SNP selection	SNPs (successful)	% covered (MAF > 0.05)
TARS2	1	148726544 - 148746371	4 (4)	100
DARS2	1	172060581 - 172094305	2 (1)	28
IARS2	1	218334078 - 218387999	6 (4)	81
MTIF2	2	55317262 - 55349888	7 (7)	100
MTO1	6	74228209 - 74267896	5 (5)	100
TFAM	10	59815181 - 59825901	5 (5)	100
PPRC1	10	103882777 - 103900078	3 (3)	100
GPAM	10	113899611 - 113933507	6 (6)	100
SIRT3	11	205031 – 226361	10 (10)	100
SIRT4	12	119224546 - 119235427	3 (3)	100
MTIF3	13	26907783 - 26922711	3 (3)	100
GTPBP3	19	17309379 - 17314530	4 (4)	100
TRMU	22	45109962 - 45131900	4 (3)	81

Gene boundaries and coverage according to HapMap data. Gene boundaries according to genome build 36.

The widest gene boundaries were used when more isoforms are known.

Study cohorts

The first stage genotyping was performed in the Hoorn study (30). This population based study consists of 519 normal glucose tolerant (NGT) subjects (aged 65 ± 8 years, 55% male) and 480 type 2 diabetes subjects (aged 67 ± 8 years, 52% male). Glucose tolerance was tested using a fasting oral glucose tolerance test (OGTT), according to World Health Organization (WHO) criteria (31).

For the second stage genotyping three cohorts from the Netherlands were pooled. The first cohort was the New Hoorn Study (NHS), which is an ongoing second non overlapping population based study in Hoorn (32). From this study we randomly selected 1517 NGT and 147 type 2 diabetes subjects. All subjects underwent an OGTT according to WHO criteria (31). In order to increase power we included 674 subjects with type 2 diabetes from the diabetes clinics of the Leiden University Medical Center (LUMC, Leiden) and from the Vrije Universiteit medical center (VUmc, Amsterdam). All subjects were Dutch Caucasians. In total the NHS sample includes 1517 controls (aged 53 ± 7 years, 44% male) and 821 cases (aged 61 ± 11 years, 50% male).

The second cohort was the Breda study (33;34). This case/control study consists of 920 healthy controls (according to self report) from the Dutch blood bank (aged 48 ± 13 years, 61% male). The 501 cases (aged 71 ± 10 years, 46% male) are type 2 diabetes patients according to WHO criteria (31).

The third cohort was the ERGO study from Rotterdam (35). This population based study consists of 5183 NGT subjects (aged 69 ± 9 years, 41 % male) and 1222 (73 ± 9 years, 39 % male) type 2 diabetes patients.

In total for the second stage genotyping we used 7620 controls and 2544 type 2 diabetes cases. The characteristics of both stages are summarized in table 2 (characteristics of the independent cohorts are summarized in supplementary table s1). For additional replication of our strongest signal we extended the second stage with samples from the Danish Steno study (36). This case/control study consisted of 514 NGT controls (aged 57 ± 10, 46% male) randomly selected from public registers at the Steno Diabetes Center and the Research Center for Prevention and Health. The 706 cases (aged 59 ± 10 years, 48% male) were recruited from the Steno Diabetes Center.

Table 2. Descriptive statistics of the first and second stage.

	First stage		Second stage	
	controls	cases	controls	cases
Subjects	519	480	7620	2544
(% male)	(55)	(52)	(44)	(45)
Age - years	65	67	63	68
(std. dev.)	(8)	(8)	(12)	(12)
BMI - kg / m^2	26	29	26	27
(std. dev.)	(5)	(5)	(4)	(5)
FPG - mmol / L	5.4	7.7	5.5	8.3
(std. dev.)	(0.4)	(2.2)	(0.5)[1]	(2.5)[1]

1) available for 2438 controls and 334 cases

First stage consists of subjects from the Hoorn study (30). Second stage is a pooled sample of subjects original from the NHS, Breda studies and ERGO (33-35).

FPG = Fasting Plasma Glucose

Statistical analysis

Differences in genotype distribution and allele frequency were analyzed using a chi-squared test. An additive model was the best fit for most of the SNPs. Rs1049432 (*TFAM*) and rs4917960 (*PPRC1*) might better fit a recessive model and were therefore also analyzed with this model. A common allelic OR covering both stages was calculated in a meta-analysis, using a Mantel-Haenszel test. Homogeneity of ORs between the different cohorts was tested using a Tarone's test. Results from OGTT (only normal glucose tolerant subjects) were analyzed with univariate analysis of variance, using additive, dominant and recessive models and correction for age, BMI and gender as possible confounders. Power calculations were performed using Quanto (37). Power to detect modest effects in the first stage was limited (80% power to detect OR 1.45, MAF = 0.1). Therefore we did not correct for multiple testing but rather performed extensive replication of all signals from the first stage with $p < 0.05$. All statistics were calculated using SPSS 14.0 (SPSS Inc, Chicago, USA).

Results

Of the 62 SNPs tested in the first stage genotyping 58 SNPs passed QC guidelines. Of these SNPs we selected the top seven SNPs for second stage genotyping ($p < 0.05$). Data from genome wide association studies (GWAS), which came available after completion of our study resulted in the inclusion of two additional SNPs, rs4397793 in *TFAM* and rs2792751 in *GPAM*. Compared to our data both showed a similar effect in at least one of the publically available databases of GWAS (38;39). In total 9 SNPs were selected for genotyping in the second stage (table 3).

We analyzed the second stage results for rs1049432 in *TFAM* and rs4917960 in *PPRC1* first with a recessive model because that was the best fit for the first stage data ($p_{rec} = 0.002$ and $p_{rec} = 0.07$ resp). However, 2nd stage results showed no support for this model, therefore the additive model was used for further analyses. There was no evidence of heterogeneity of ORs between the independent cohorts, therefore we pooled all second stage data. A meta-analysis of both stages was performed in order to calculate a common OR for the 9 selected SNPs (table 3).

Only the G allele of rs2522138 in *SIRT4* remained significant (p = 0.01), but this was mainly caused by stage 1 results. In order to confirm the observed association we expanded the second stage with the Danish Steno study resulting in a common odds ratio of 0.92 (0.85 – 1.00), p = 0.06.

The C allele of rs4917960 in *PPRC1* was borderline significant after the meta-analysis (OR = 1.06 (1.00 – 1.13) p=0.06). This association was nominal and mainly caused by stage 1 data. The ORs in all independent cohorts are shown in supplementary table 2. Correction for age, gender and BMI (when available) did not influence the results.

Finally, we analyzed OGTT data for association of the selected genes with differences in clinical variables like glucose and insulin. Furthermore, association with BMI was assessed when the data were available. No differences were observed using different models (data not shown).

Table 3. Results of first and second phase genotyping.

Rs-number	Gene	MAF [1]		1st Stage		2nd Stage		Meta-analysis	
		controls	cases	OR 95% CI	P_{Add}	OR 95% CI	P_{Add}	OR 95% CI	P_{Add}
rs1937 [2]	*TFAM*	0.14	0.09	0.66 0.50-0.88	0.005	1.00 0.86-1.17	0.97	0.91 0.79-1.04	0.17
rs4397793	*TFAM*	0.41	0.37	0.86 0.72-1.03	0.10	1.04 0.97-1.11	0.25	1.02 0.95-1.08	0.62
rs1049432	*TFAM*	0.19	0.14	0.77 0.61-0.97	0.03	1.00 0.91-1.09	0.95	0.97 0.89-1.05	0.39
rs4917960	*PPRC1*	0.38	0.42	1.12 1.00-1.43	0.05	1.05 0.98-1.12	0.21	1.06 1.00-1.13	0.06
rs17129583	*GPAM*	0.12	0.16	1.32 1.02-1.70	0.04	0.92 0.84-1.02	0.12	0.97 0.88-1.06	0.50
rs2792751	*GPAM*	0.26	0.24	0.87 0.71-1.06	0.18	1.01 0.94-1.09	0.81	0.99 0.93-1.06	0.82
rs535716	*SIRT3*	0.24	0.19	0.78 0.63-0.96	0.02	1.05 0.97-1.13	0.25	1.01 0.94-1.09	0.78
rs2522138 [3]	*SIRT4*	0.18	0.13	0.69 0.54-0.88	0.003	0.93 0.85-1.02	0.13	0.92 0.85-1.00	0.06
rs17085633	*MTIF3*	0.44	0.50	1.25 1.05-1.50	0.01	1.02 0.97-1.09	0.55	1.05 0.98-1.11	0.15

1) MAF in first stage genotyping (Hoorn study).
2) Second stage genotyping only in NHS and Breda study (n = 3759).
3) Meta-analysis includes Steno study.

After completion of our study, a meta-analysis of three type 2 diabetes GWAS appeared (40). The coverage of common variation in the 13 selected genes in the GWAS was between 80 and 100% except *GTPBP3*, which was covered for 50%. We compared our results with the GWAS data, using the OR-based analysis (table 4). The G allele of rs2522138 in *SIRT4* was not associated with type 2 diabetes susceptibility in the GWAS data (OR = 1.02 (0.93 - 1.11), p = 0.72). The C allele of rs4917960 in *PPRC1* showed an OR of 1.04 (0.97 – 1.12), p = 0.23) in the GWAS, which is in the same direction as observed in our study (OR = 1.06 (1.00 – 1.13), p = 0.06). Two SNPs located in *IARS2* (the C alleles of rs17007135 and rs2289191) showed evidence of nominal association in the GWAS meta-analysis ($p = 6 * 10^{-4}$ and p = 0.003, respectively.). Unfortunately, the assays for these two SNPs failed in our study. None of the other SNPs in our 13 genes showed evidence of association in the type 2 diabetes GWAS meta-analysis.

Table 4. Comparison DIAGRAM meta-analysis and our replication SNPs

Rs number	Gene	Meta-analysis GWAS		Meta-analysis of our study	
		OR 95% CI	p-value	OR 95% CI	p-value
rs1937	*TFAM*	1.02 0.91 - 1.14	0.76	0.91 0.79 – 1.04	0.17
rs4397793	*TFAM*	1.03 0.96 - 1.09	0.43	1.02 0.95 – 1.08	0.62
rs1049432	*TFAM*	1.02 0.94 - 1.12	0.69	0.97 0.89 – 1.05	0.39
rs4917960	*PPRC1*	1.04 0.97 - 1.12	0.23	1.06 1.00 – 1.13	0.06
rs17129583	*GPAM*	1.00 0.92 – 1.09	0.99	0.97 0.88 – 1.06	0.50
rs2792751	*GPAM*	1.04 0.98 - 1.11	0.22	0.99 0.93 - 1.06	0.82
rs535716	*SIRT3*	1.03 0.96 - 1.11	0.42	1.01 0.94 – 1.09	0.78
rs2522138	*SIRT4*	1.02 0.93 - 1.11	0.72	0.92 0.85 – 1.00	0.06
rs17085633	*MTIF3*	1.00 0.94 - 1.06	0.89	1.05 0.98 – 1.11	0.15

T2D: type 2 diabetes

Discussion

We analyzed 58 tagging SNPs in 13 genes involved in mitochondrial function for association with type 2 diabetes susceptibility using a two stage design. After meta-analysis of the data of both stages only rs2522138 in *SIRT4* remained borderline significant. However, after extension of the second stage with samples from the Steno study, this association did not remain significant.

For several gene variants measured in our study, there is substantial heterogeneity of ORs between the first and second stage, but not within each stage (Table 3 and supplementary table s2). Heterogeneity between the independent cohorts might be caused by the differences in the age, gender or selection criteria used and might obscure true association (supplementary table s1). However, we were unable to identify the reasons for this heterogeneity in our study. Differences in LD between the measured and causal SNP are also an unlikely cause, because the study subjects in our cohorts are from similar ancestry (41). In the context of testing multiple SNPs, our nominal significant results in *SIRT4* and *PPRC1* should be interpreted as consistent with statistical noise.

One of the limitations of our study is that it is statistically underpowered to detect SNPs with a very small impact or low allele frequency. Although power in the first stage alone was low, we had at least a power of 80% at an observed minor allele frequency ≥ 0.13 to detect ORs comparable to those recently reported in GWAS (1.12 – 1.37) when first and second stage are combined (40). Our negative results for these 13 genes are confirmed by the DIAGRAM GWAS meta-analysis, which has a much higher power to detect also more modest effects (38-40;42). Taken together, we can exclude that common variants in the selected genes have a major impact on type 2 diabetes susceptibility. Rare variants are not covered by our approach so we cannot exclude a role for such variants. Deep sequencing and analysis of novel rare variants in large well phenotyped cohorts should provide more insight into these important issues.

Other nuclear encoded mitochondrial genes are also not among the top hits of reported type 2 diabetes GWAS (38-40;42;43). Nevertheless, previous studies suggested that they may be involved in type 2 diabetes susceptibility (3;44-47). Further combined and detailed analysis of the available GWAS datasets and

analysis of copy number and so-called low frequency intermediate-penetrance variants are necessary to fully explore the role of nuclear encoded mitochondrial genes in the pathogenesis of type 2 diabetes.

In conclusion, we were unable to detect association of common SNPs in 13 nuclear encoded mitochondrial candidate genes with type 2 diabetes or related continuous traits.

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Supplementary table S1. Descriptive statistics of all individual cohorts.

	Hoorn Study		NHS		Breda Study		ERGO Study		Steno Study	
	controls	cases	controls	cases	controls	cases	controls	cases	controls	cases
Subjects	519	480	1517	821	920	501	5183	1222	514	706
(% male)	(55)	(52)	(44)	(50)	(61)	(46)	(41)	(39)	(46)	(48)
Age	65	67	53	61	48	71	69	73	57	59
(std. dev.)	(8)	(8)	(7)	(11)	(13)	(10)	(9)	(9)	(10)	(10)
BMI	26	29	26	29	n.a.	28	26	27	n.a.	n.a.
(std. dev.)	(5)	(5)	(4) [1]	(5)[1]		(4)	(4)	(4)		
FPG	5.4	7.7	5.3	7.6	n.a.	n.a.	5.7	8.7	n.a.	n.a.
(std. dev.)	(0.4)	(2.2)	(0.4)[2]	(2.1)[2]			(0.6)[3]	(2.6)[3]		

1) Only available for NHS subjects selected from the ongoing study, not from diabetic subjects from the LUMC and VUmc diabetes clinics (n=1664).
2) 1517 controls and 147 cases
3) 920 controls 187 cases
n.a. = not available
FPG = Fasting Plasma Glucose

Supplementary table S2. Results of replicated SNPs for every independent cohort

Rs number	1st stage				2nd stage				Steno Study	
	Hoorn Study		NHS		Breda Study		ERGO Study			
	OR 95% CI	P_{Add}	OR 95% CI	P_{Add}	OR 95% CI	P_{Add}	OR 95% CI	P_{Add}	OR 95% CI	P_{Add}
rs1937	0.66 0.50-0.88	0.005	1.08 0.88-1.33	0.46	0.89 0.70-1.13	0.37	n.a.	n.a.	n.a.	n.a.
rs4397793	0.86 0.72-1.03	0.10	1.10 0.95-1.28	0.20	1.00 0.85-1.17	1.00	1.06 0.97-1.16	0.22	n.a.	n.a.
rs1049432	0.77 0.61-0.97	0.03	1.11 0.91-1.35	0.29	0.93 0.74-1.17	0.56	0.99 0.88-1.12	0.90	n.a.	n.a.
rs4917960	1.12 1.00-1.43	0.05	1.09 0.94-1.26	0.28	1.00 0.86-1.18	0.97	1.03 0.94-1.13	0.51	n.a.	n.a.
rs17129583	1.32 1.02-1.70	0.04	0.75 0.60-0.93	0.01	1.09 0.88-1.36	0.46	0.90 0.79-1.04	0.15	n.a.	n.a.
rs2792751	0.87 0.71-1.06	0.18	1.08 0.92-1.27	0.35	0.94 0.79-1.12	0.50	1.01 0.91-1.11	0.92	n.a.	n.a.
rs535716	0.78 0.63-0.96	0.02	1.05 0.88-1.25	0.59	0.97 0.81-1.16	0.78	1.06 0.96-1.18	0.27	n.a.	n.a.
rs2522138	0.69 0.54-0.88	0.003	0.98 0.82-1.18	0.89	0.84 0.67-1.07	0.15	0.98 0.86-1.10	0.71	1.13 0.91-1.42	0.27
rs17085633	1.25 1.05-1.50	0.01	1.10 0.95-1.28	0.21	0.97 0.83-1.14	0.72	0.99 0.91-1.09	0.91	n.a.	n.a.

n.a. = not available

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Chapter 4

The association of mitochondrial content with prevalent and incident type 2 diabetes

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Abstract

Aims/hypothesis

It has been shown that mitochondrial DNA (mtDNA) content is associated with type 2 diabetes (T2D) and related traits. However, empirical data, often based on small samples, did not confirm this observation in all studies. Therefore, the role of mtDNA content in T2D remains elusive. In this study we assessed the heritability of mtDNA content in buccal cells and analyzed the association of mtDNA content in blood with prevalent and incident T2D.

Methods

Mitochondrial DNA content from cells from buccal and blood samples were assessed using a real time PCR based assay. Heritability of mtDNA content was estimated in 391 twins from The Netherlands Twin Register. The association with prevalent T2D was tested in a case / control study from the Netherlands (n=329). Incident T2D was analyzed using prospective samples from Finland (n=444) and the Netherlands (n=238).

Results

A heritability of mtDNA content of 35% (19%-48%) was estimated in the twin families. We did not observe evidence of an association between mtDNA content and prevalent or incident T2D and related traits. Furthermore, we observed a decline in mtDNA content with increasing age, which was male specific (p=0.001).

Conclusion

In this study we show that mtDNA content has a heritability of 35% in Dutch Twins. There is no association between mtDNA content in blood and prevalent or incident T2D and related traits in our study samples.

Introduction

Mitochondria contribute to multiple cellular processes such as ATP synthesis, beta-oxidation of fatty acids and apoptosis. They have their own circular genome, of approximately 16kb which exists in multiple copies per mitochondrion. Mitochondrial DNA (mtDNA) encodes for 2 rRNA's, all mitochondrial tRNA's and 13 subunits of the respiratory chain. These 13 subunits are synthesized by the mitochondrial protein synthesis machinery inside the mitochondrial matrix. Since the complete pool of mitochondrial proteins consists of approximately 1500 proteins, only a fraction of them is encoded and synthesized inside the mitochondrion. Quantities of mtDNA, the so-called mtDNA content, vary between individuals and tissues within an individual. In muscle dynamic adaptation is observed in relation to physical exercise (1;2). Mutations in the human *POLG* gene and knock down of Tfam in mouse models, two of the most important regulators of mtDNA content, result in mtDNA depletion, showing that nuclear factors are involved in the regulation of mtDNA content (3;4). Furthermore, it has previously been shown in humans that the mtDNA content in blood cells is partially heritable (5;6). In this study we readdress the heritability of mtDNA content using buccal swabs from mono- and dizygotic twins and their siblings.

Evidence is accumulating that mtDNA content is associated with type 2 diabetes. However, there is debate about whether mitochondrial dysfunction is primary or secondary to type 2 diabetes. HIV infected patients treated with Highly Active Anti Retroviral Therapy (HAART) containing nucleoside analogues, show a 30-50% decreased mtDNA content, redistribution of body fat, insulin resistance and an increased risk for development of type 2 diabetes, favouring the hypothesis that mitochondrial dysfunction is a cause rather than a consequence of development of type 2 diabetes (7-9).

It has been shown that low mtDNA content precedes type 2 diabetes (10). However, in a sample of 141 non-diabetic, adult offspring who had one parent with T2D, no difference in mtDNA content in blood was observed compared to controls (11). Other studies showed evidence that mtDNA content is associated with the pattern of triglyceride storage, insulin secretion, insulin sensitivity and glucose

metabolism (12-15). Furthermore, mtDNA content is decreased in Goto-Kakizaki rat, which is a genetic model for type 2 diabetes (16).
Since the association of mtDNA content with type 2 diabetes is not confirmed in all studies the role of mtDNA content in the onset of type 2 diabetes remains not fully understood. The aim of the current study was to elucidate this important issue. We assessed the heritability of mtDNA content in participants from the Netherlands Twin Register while taking into consideration several life style variables. Next, we assessed the association of mtDNA content with prevalent and incident cases of type 2 diabetes in a Dutch case-control study and in selected samples from two prospective studies form Finland and The Netherlands.

Materials and methods

Sample selection

Heritability

For estimation of the heritability participants were recruited from the Netherlands Twin Register (17;18). The sample consisted of 391 twins and 12 of their siblings from 212 families as part of a study on depression and anxiety (19) and smoking behavior (20). DNA was extracted from buccal cells. Assessment of zygosity was based on DNA polymorphisms. There were 183 complete twin pairs (38 monozygotic male (MZM), 23 dizygotic male (DZM), 60 monozygotic female (MZF), 45 dizygotic female (DZF) and 17 DZ pairs of opposite sex) and 25 incomplete pairs (5 MZM, 3 DZM, 12 MZF and 5 DZF). The sibling group consisted of 5 males and 7 females.
Information on BMI, smoking, and exercise was obtained from longitudinal surveys and from data collected at the time of DNA sampling.

Case / control study of prevalent type 2 diabetes

For the case control study we selected 175 normal glucose tolerant (NGT) Caucasian participants from the Dutch New Hoorn Study (NHS). 154 prevalent type 2 diabetes patients from NHS and the Diabetes Care System West-Friesland

(DCS) were matched for age (21-23). NHS is a population based study from the city of Hoorn, the Netherlands, which aims to examine potential determinants for glucose intolerance and related disorders. Glucose tolerance was assessed by oral glucose tolerance test (OGTT) following WHO '99 criteria (24). DCS aims to improve diabetes care by coordinating the diabetes care involving all caregivers and providing education of patients in order to improve patient empowerment. Patients are referred to the DCS by their physicians and are from the same geographical region as the NHS. DNA was extracted from whole blood.

Prospective study of incident type 2 diabetes

In order to prospectively analyze the association between mtDNA content and incident cases of type 2 diabetes we selected samples from 2 prospective studies. The first was the Botnia study, here designated prospective 1, which aims at the identification of genes increasing susceptibility to type 2 diabetes (25). This family study includes 2770 participants, which were healthy at baseline, originating from the Botnia region in Finland. Glucose tolerance at baseline and after follow up was tested using OGTT following WHO '99 criteria (24). DNA was extracted from whole blood, drawn at baseline. DNA from 133 participants who converted to type 2 diabetes (converters) was available. 311 non-converters were matched based on age, and BMI at baseline and gender. We allowed the inclusion of impaired glucose tolerant (IGT) and impaired fasting glucose (IFG) participants since these covered a large proportion of the converter group. The converter group included 55 NGT, 37 IGT, 20 IFG and 24 IGT+IFG participants at baseline. The non-converter group included 245 NGT, 45 IGT, 26 IFG and 15 IGT+IFG participants at baseline. Family-history (first degree relatives) of type 2 diabetes was present in 83.8% of the converter and 67.2% of the non-converter group.

The second prospective study was the Dutch Rotterdam study (n = 7983), here designated prospective 2, which is a population based study aiming to investigate determinants of disease occurrence and progression in the elderly (26). DNA was extracted from buffy coat, obtained from blood drawn at baseline. Diabetic state at baseline was assessed using a random glucose tolerance test, which was shown to have a good correlation with OGTT (27). Glucose tolerance after follow up was

assessed using OGTT following WHO '99 criteria (24). From this study 113 converters were available. We matched 125 non-converters based on age and BMI at baseline and gender. Entry of IGT participants at base line was allowed (n = 20 in the converter group and n = 1 in the non-converter group).
All studies were approved by the appropriate medical ethical committees and were in accordance with the principles of the Declaration of Helsinki. Study samples are summarized in table 1.

Table 1. Characteristics of study samples

	Twins / Heritability	Dutch case/control		Prospective 1		Prospective 2	
		Cases	Controls	Converters	Non-converters	Converters	Non-converters
N	403	154	175	133	311	113	125
(%male)	(38)	(49)	(49)	(50)	(45)	(60)	(50)
Age	38.7	55	52	53	53	66	65
(stdev)	(11.7)	(4)	(6)	(12)	(9)	(6)	(5)
BMI	24.0	31.1	25.8	28.8	26.8	28.5	28.4
(stdev)	(3.7)	(5.8)	(3.5)	(4.3)	(2.9)	(3.5)	(2.7)

Mean with SD are shown.
n.a.: not available

Measurement of mitochondrial DNA content

Mitochondrial DNA content was assessed using a modification of the quantitative real time PCR based method we described previously (28). In short, the relative amount of mtDNA was quantified by comparison with a nuclear target, which was the beta-globin gene (*HBB*). A fragment of mtDNA was amplified between nucleotide position 3780 and 3842, which is located in *MT-ND1* on the heavy strand transcript (primer sequences available on request). Quantitative real time PCR was performed using the Applied Biosystems 7900HT (Applied Biosystems, Applied Biosystems, Foster City, USA). Absolute QPCR SYBR Green ROX mix was used (Thermo Fisher Scientific Inc., Waltham, MA, USA). For quantification reference curves were used, which were serial dilutions of a standard DNA. A ratio between mtDNA and *HBB* was calculated (mtDNA / *HBB*), which is here used as the mtDNA content. Each sample was measured at least in triplicate and mean values were calculated. Specificity of amplification was tested by dissociation curves and water controls. Approximately 50% of samples were repeated and showed a high concordance in all study samples with initial results ($r^2 \geq 0.8$).

Mitochondrial DNA content is assessed in several tissues (buccal cells, whole blood and buffy coats). It is known that DNA extraction from these different samples can result in variance in mtDNA content. Therefore, differences in mtDNA content between the independent study samples is observed (29;30).

Statistical analysis

Heritability study

Twin studies make use of the genetic relatedness of twins and their family members. MZ twins are genetically identical while DZ twins share on average 50% of their segregating genes, like other siblings (31). Twin studies compare the correlation of a phenotype, such as mtDNA content, within MZ twin pairs with the resemblance within DZ twin and sibling pairs. If the correlation in MZ twins is about twice as large as the correlation in DZ twins, familial resemblance is explained by additive genetic effects (A). When the DZ correlation is more than half the MZ correlation, there is evidence for environmental (C) effects shared by twins and siblings from the same family. Differences within MZ twin pairs are due to unique environmental influences (E), which also include measurement error . The observed variance thus can be decomposed into variance components A, C and E (32).

Statistical analyses were performed using structural equation modeling as implemented in the software package Mx (33). The raw data full information maximum likelihood approach in Mx was used to fit different models to the data, for example ACE and AE models. Testing of sub models was done by means of likelihood-ratio tests, by subtracting the negative log likelihood (-2LL) for the more restricted model (e.g. AE) from the -2LL for the more general model (e.g. ACE). This yields a statistic that is distributed as χ^2 with degrees of freedom (df) equal to the difference in the number of parameters in the two models. We first fitted a saturated model to the data in which means, variances, and correlations were estimated as well fixed effects of age, sex, BMI and physical activity on mtDNA content.

Case / control study of prevalent type 2 diabetes

The association of mtDNA content with prevalent type 2 diabetes in the case control study was analyzed with logistic regression with adjustment for age, gender and BMI. Mitochondrial DNA content in each cohort was adjusted for age, gender and BMI with general linear models. Continuous traits where analyzed with linear regression with adjustment for age, BMI and gender (were appropriate) only in NGT participants. SPSS (SPSS Inc, Chicago, USA) was used for these statistical analyses.

Prospective study of incident type 2 diabetes

Since the prospective 1 sample (Botnia study) is a family based study, we adjusted for family relation. In order to do so, we made use of general estimating equations with logistic regression, which takes into account family relatedness when calculating the standard errors. Conversion to type 2 diabetes was adjusted for age, BMI and glucose tolerance at baseline and gender and family history of diabetes. Estimated average mtDNA content was calculated with generalized estimated equations with linear regression, with similar adjustments as mentioned above.

The prospective 2 sample (Rotterdam study) is a sample of the population of Rotterdam, The Netherlands. We used standard logistic regression with adjustment for age, BMI and glucose tolerance at baseline and gender. Estimated average mtDNA content and continuous traits were analyzed with a general linear model, using adjustment for age, BMI and glucose tolerance at baseline and gender.

Power calculation

Prior to the study, power calculations were performed, using Lenth's power and sample-size calculator (http://www.stat.uiowa.edu/~rlenth/Power/index.html website accessed on May 20 2008). We assumed a population standard deviation of 30%. For the association with prevalent or incident type 2 diabetes a sample size of 160 cases and controls is needed for 80% power to detect a difference of at least 10% (alpha = 0.05).

Results

Heritability

Regression of gender, age, exercise and BMI on mtDNA content indicated that mean mtDNA content was lower in subjects who do not exercise, in women, who are older and who have a higher BMI. These four covariates thus explain a significant part (4%) of the variance in mtDNA content. Genetic modeling with these covariates yielded twin correlations of 0.34 for MZ and 0.19 for DZ/sib pairs. The corrected mean mtDNA was 1.28 with an SD of 0.02. Comparing an ACE model (-2LL = 1996.76) with an AE model (-2LL = 1996.77) indicated that C (shared environmental effects) could be dropped without loss of fit. Finally, fitting a model (E Model) that specified no familial (genetic) variance showed a significantly worse fit (-2LL = 2014.24, χ^2 of 17.49 with 1 df when compared to AE model; $p = 2 \cdot 10^{-5}$), showing that genetic factors do have a significant effect on mitochondrial content. Heritability under the best model (AE) was estimated at 35% (CI 19%-48%).

Prevalent type 2 diabetes and mitochondrial DNA content

Mitochondrial DNA content was assessed in a case control study ascertained from the Dutch NHS and DCS, originating from the same geographic location. Estimated mean mtDNA content in prevalent cases of type 2 diabetes and controls was 0.30 (0.28 – 0.31) and 0.29 (0.28 – 0.30) respectively, p = 0.51 (table 2). Also after

Table 2. Association of mitochondrial DNA content with (future) type 2 diabetes

Study	mtDNA content		p-value
	Case	Control	
Case / Control[a]	0.30 (0.28 – 0.31)	0.29 (0.28 – 0.30)	0.51
Prospective 1[b]	0.38 (0.36 – 0.40)	0.40 (0.39 – 0.42)	0.08
Prospective 2[c]	0.55 (0.50 – 0.59)	0.51 (0.46 – 0.56)	0.18

Estimated mitochondrial DNA content is shown as the ratio between nDNA and mtDNA with 95% CI and p-values.
a. Adjusted for age, BMI and gender using general linear model.
b. Adjusted for age, BMI, gender, family history of type 2 diabetes, pedigree clustering and glucose tolerance at baseline using generalized estimated equations.
c. Adjusted for age, BMI, gender and glucose tolerance at baseline using general linear model.

logistic regression with adjustment for possible confounders (BMI, age and gender) we could not detect an effect on type 2 diabetes susceptibility (p = 0.56). Furthermore, we could not find any evidence for a correlation between mtDNA content and other relevant variables (supplementary table S1 and S2).

Incident type 2 diabetes and mitochondrial DNA content

Mitochondrial DNA content was first assessed in the prospective 1 sample. Estimated mean mtDNA content in converters and non-converters was 0.38 (0.36 – 0.40) and 0.40 (0.39 – 0.42) respectively, p = 0.08 (table 2). Using logistic regression with adjustment for possible confounders (age, gender, BMI, glucose tolerance at baseline and family clustering) we also observed a weak regression towards decreased mtDNA content with increased type 2 diabetes risk, but this did not reach statistical significance (p = 0.10). No associations between mtDNA content and OGTT derived variables were observed.

Next, mtDNA content was assessed in the prospective 2 sample. Estimated mean mtDNA content in converters and non-converters was 0.55 (0.50 – 0.59) and 0.51 (0.46 – 0.56) respectively, p = 0.18 (table 2). Logistic regression with adjustment for possible confounders gave a similar result (p = 0.17).

All studies showed a trend towards decreasing mtDNA content with increasing age (supplementary table S1). However, for most studies, this did not reach statistical significance. Therefore, we pooled all data in order to increase power and repeated the analysis. Mitochondrial DNA content of each independent study was normalized using the average mtDNA content to make the data comparable. To avoid heterogeneity, we excluded the twin data for this analysis, since this was the only study for which DNA was extracted from buccal swabs. Using generalized estimating equations with correction for family relatedness and study sample, we observed a statistical significant decrease in mtDNA content with increasing age (B = -0.003 (-0.005 to -0.0007), p = 0.009). Correction for BMI did not influence the data. However, the association seems to be gender specific. No effect of age on mtDNA content was observed in females (p = 0.53), while a strong association was observed in males (B = -0.005 / year (-0.008 to -0.002), p = 0.001). Furthermore,

BMI showed a trend towards association with mtDNA content (B = -0.81 (-1.65 to 0.03), p = 0.06) in the pooled data set. After correction for age and gender, the association is borderline significant (B = -0.92 (-1.78 to -0.07), p = 0.04). Exercise data for the pooled sample was only available from the case / control and prospective 1 sample and did not influence this association (data not shown). We also observed different mtDNA content between males and females. Females showed a ~5% higher mtDNA content (p = 0.007). Correction for age and BMI revealed an estimated mtDNA content in males vs. females of 0.96 (0.93 to 0.98) and 1.01 (0.98 to 1.04) respectively (p = 0.007, supplementary table S2). The pooled sample was not corrected for exercise, since assessment of exercise was performed with different questionnaires.

Discussion

Heritability

In this study we showed that mtDNA content has a heritability of 35% (CI: 19% – 48%) in buccal cells. This partially confirms results from other groups who analyzed mtDNA content in blood and reported heritability between 33% and 65% (5;6). However, the reported heritability of 65% is higher than our finding. This might be caused by a different ethnicity of participants or different types of tissue used.
In our study mtDNA content in buccal cells is not affected by smoking behaviour, so this could not bias our results. However, two other studies did show modest effects of smoking on mtDNA content in buccal cells and whole blood (6;34).

Prevalent type 2 diabetes and mitochondrial DNA content

Inconsistency in literature is observed concerning the association of mtDNA content with type 2 diabetes (10;11). In our study we could not detect a relation between mtDNA content in blood and prevalent type 2 diabetes in a case / control setup. Post-hoc power calculations showed that we had >95% power to detect an effect comparable to the results of Lee H.K. et al (mitochondrial content 35% lower in cases of type 2 diabetes) (10) and 80% power to detect a difference of at least 10%. Therefore, we conclude that mtDNA content in blood is not associated with type 2 diabetes in our sample selection.

Incident type 2 diabetes and mitochondrial DNA content

In order to analyze the relation between mtDNA content and incident cases of type 2 diabetes we analyzed two prospective studies, but we could not detect a consistent association with incident type 2 diabetes. A previous study had suggested a 25% decrease in converters (10). We had >95% power to detect a similar effect in both prospective 1 and prospective 2 (Botnia and Rotterdam) studies. This indicates that mtDNA content in blood is not a good predictor of future type 2 diabetes. While the prospective 1 study shows a trend towards decreased risk for type 2 diabetes with increased mtDNA content, the prospective 2 study shows an opposite effect. This might be caused by the differences between the studies. For instance, participants from the prospective 1 sample are younger, than those of the prospective 2 sample. Furthermore, there are more IGT and IFG subjects in the non-converter group of the prospective 1 sample, compared to the prospective 2 sample. However, analyzing only NGT subjects does not change the outcome.

We conclude that mtDNA content in blood is not associated with incident type 2 diabetes, which argues against previous observations where low mtDNA content in blood was associated with type 2 diabetes, triglyceride storage, glucose homeostasis, insulin sensitivity and insulin secretion (10;12-15). A major difference between our and previous studies is the difference in ethnicity. All positive findings arose in Asian participants from Korea, while no associations were observed in Caucasians (10-14). Our data indicate that mtDNA content in blood is not useful as predictor of type 2 diabetes in Caucasians. We cannot rule out any effects of mtDNA content in other tissues like muscle, pancreas and adipose tissue on type 2 diabetes and related traits like insulin resistance and secretion. Muscle seems a highly relevant tissue to examine mitochondrial function since associations with mitochondrial activity and insulin resistance are observed (35;36) and it has already been reported that mtDNA content decreases with aging in muscle, but also in pancreas, liver and blood (5;37-39). It seems that the decline in mtDNA content in muscle is faster than in blood. This may be related to the high level of oxidative metabolism in these tissues. Furthermore, it is speculated that the faster proliferative rate of hematopoietic stem cells provides them with the opportunity to

remove damaged mtDNA and perhaps a better maintenance of mtDNA copynumber (37;38).
In our pooled dataset we observed a negative correlation between mtDNA content and age, which was male specific. To our knowledge, we are the first to show that the decline in mtDNA content might be male specific. The observed gender effect on mtDNA content, also observed by Xing J et al (6), is probably caused by this gender specific correlation between mtDNA content and ageing. One might speculate that overall mitochondrial fitness is better retained in females, which might explain the observed difference in life span between males and females. However, this hypothesis needs further investigation. The association between mtDNA content and BMI was very weak and was not observed by others (10-15). Therefore, it is likely that this is not a true association.

In summary, we have confirmed the heritability of mtDNA content, but could not find evidence for an association of mtDNA content in blood with prevalent or incident type 2 diabetes. The observed decline in mtDNA content in males might suggest enhanced mitochondrial fitness in elderly women. However, this needs further study in more suitable cohorts and experimental settings.

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Supplementary table S1. Regression of mitochondrial DNA content with clinical variables

	Twins / Heritability study (n = 403)	Case / Control study (n = 329)	Prospective 1 study (n = 444)	Prospective 2 study (n = 238)
Age	-0.001 (-0.003 to 0.001) 0.35	-0.002 (-0.004 to 0.0001) 0.06	-0.0008 (-0.002 to 0.0001) 0.09	-0.002 (-0.006 to 0.002) 0.29
BMI	-3.05 (-5.17 to -0.92) 0.005	0.55 (-2.18 to 3.27) 0.69	-0.78 (-3.31 to 1.76) 0.55	-2.26 (-4.57 to 0.04) 0.05
WHR	-0.03 (-0.08 to 0.02) 0.27	0.02 (-0.08 to 0.13) 0.68	0.01 (-0.04 to 0.05) 0.77	0.01 (-0.05 to 0.06) 0.85
0hr gluc	0.49 (0.08 to 0.91) 0.02	0.27 (-0.28 to 0.81) 0.34	0.19 (-0.17 to 0.55) 0.29	n.a.
2hr gluc	n.a.	1.04 (-0.94 to 3.02) 0.30	0.72 (-0.45 to 1.90) 0.23	n.a.

Regression coefficients are shown with 95% CI and p-values.
WHR: Waist Hip Ratio
n.a.: not available
For the case / control study, only NGT participants are included (n = 175)
Regression coefficients, 95% CI and p-values are shown.

supplementary table S2. Gender effect on mitochondrial DNA content in healthy participants

Study	Male		Female
Twins / Heritability	1.01 (0.96 to 1.06)		1.01 (0.97 to 1.06)
p-value		0.98	
Case / Control	0.98 (0.94 to 1.03)		0.98 (0.92 to 1.01)
p-value		0.95	
Prospective 1	0.97 (0.93 to 1.01)		1.02 (0.98 to 1.07)
p-value		0.07	
Prospective 2	0.92 (0.87 to 0.97)		1.04 (0.98 to 1.09)
p-value		0.002	
Pooled	**0.96 (0.93 to 0.98)**		**1.01 (0.98 to 1.04)**
p-value		**0.007**	

Relative mitochondrial DNA content, normalized using the average mtDNA content of every independent study sample are shown with 95% CI and p-values. Netherlands Twin Register is excluded from the pooled sample, since DNA extractions are from buccal cells.
Estimated mean mitochondrial DNA content and 95% CI are calculated using generalized estimating equations with adjustment for age and BMI.

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Chapter 5

Combined effects of single-nucleotide polymorphisms in *GCK*, *GCKR*, *G6PC2* and *MTNR1B* on fasting plasma glucose and type 2 diabetes risk

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Abstract

Aims/hypothesis

Variation in fasting plasma glucose (FPG) within the normal range is a known risk factor for the development of type 2 diabetes. Several reports have shown that genetic variation in the genes for glucokinase (*GCK*), glucokinase regulatory protein (*GCKR*), islet-specific glucose 6 phosphatase catalytic subunit-related protein (*G6PC2*) and melatonin receptor type 1B (*MTNR1B*) is associated with FPG. In this study we examined whether these loci also contribute to type 2 diabetes susceptibility.

Methods

A random selection from the Dutch New Hoorn Study was used for replication of the association with FGP (2361 non-diabetic participants). For the genetic association study we extended the study sample with 2628 participants with type 2 diabetes. Risk allele counting was used to calculate a four-gene risk allele score for each individual.

Results

Variants of the *GCK*, *G6PC2* and *MTNR1B* genes but not *GCKR* were associated with FPG (all, $p \leq 0.001$; *GCKR*, $p = 0.23$). Combining these four genes in a risk allele score resulted in an increase of 0.05 mmol/l (0.04–0.07) per additional risk allele ($p = 2 \times 10^{-13}$). Furthermore, participants with less than three or more than five risk alleles showed significantly different type 2 diabetes susceptibility compared with the most common group with four risk alleles (OR 0.77 [0.65-0.93], $p = 0.005$ and OR 2.05 [1.50-2.80], $p = 4 \times 10^{-6}$ respectively). The age at diagnosis was also significantly associated with the number of risk alleles ($p = 0.009$).

Conclusion

A combined risk allele score for single-nucleotide polymorphisms in four known FPG loci is significantly associated with FPG and HbA_{1c} in a Dutch population-based sample of non-diabetic participants. Carriers of low or high numbers of risk alleles show significantly different risks for type 2 diabetes compared with the reference group.

Introduction

Variation in fasting plasma glucose (FPG) levels within the normal range are associated with an increased risk of developing type 2 diabetes and coronary heart disease (1;2). Furthermore, it is known that FPG is partially genetically determined (3). Several loci influencing FPG levels have been identified. These loci encode glucokinase (*GCK*), glucokinase regulatory protein (*GCKR*) and islet-specific glucose 6 phosphatase catalytic subunit-related protein (*G6PC2*) (4-10). Recently, the gene encoding melatonin receptor type 1B (*MTNR1B*) was identified as a fourth locus influencing FPG (11-13). In this study we investigated the combined effect of these loci on FPG levels in the Netherlands and analysed their single and combined effects on the risk of type 2 diabetes.

Methods

Study samples

Study sample for continuous trait analysis

For this part of the study we used participants from the ongoing New Hoorn Study, a population-based cohort study in the Netherlands, which examines potential determinants of glucose intolerance and related disorders (14;15). From this study, 2361 non-diabetic white participants (46% male, aged 53±7 years) were selected from the original random sample of the population register of the town of Hoorn, the Netherlands. Glucose tolerance status was assessed with OGTT using the 1999 WHO criteria (16).

Case–control sample for genetic association with type 2 diabetes

As a control sample we used all participants with normal glucose tolerance from the above-mentioned sample (*n*=2041). Subjects with impaired glucose tolerance (IGT) and/or impaired fasting glucose (IFG) (*n*=320) were excluded from the control group because they have an increased risk of type 2 diabetes.

For the case sample we used all known (*n*=90) and newly identified (*n*=90) cases from the New Hoorn Study. To improve power we added cases from Diabetes Care System West Friesland (DCS, *n*=1906) (17). The DCS aims to improve diabetes

care by coordinating diabetes care, involving all caregivers and providing education for patients in order to improve patient empowerment. Patients are referred to the DCS by their physicians and are from the same geographical region as those taking part in the New Hoorn Study. We also included 542 type 2 diabetes patients from the diabetes clinics at Leiden University Medical Centre (Leiden, the Netherlands) and VU University Medical Centre (Amsterdam, the Netherlands), who were referred to the clinic by their physicians. In total we selected 2628 participants with type 2 diabetes (55% males, aged 64±11 years) for the case–control study. All participants in our study were of white ethnicity. The study was approved by the appropriate medical ethics committees and was in accordance with the principles of the Declaration of Helsinki.

Genotyping and quality control

Based on previous publications, we selected the single-nucleotide polymorphisms (SNPs) rs1799884 in *GCK* (4), rs1260326 (P446L) in *GCKR* (7), rs560887 in *G6PC2* (9) and rs10830963 in *MTNR1B* (11-13) for genotyping with Taqman SNP genotyping assay (Applied Biosystems, Foster City, CA, USA). All genotype frequencies were similar between the case subgroups. For quality control the allelic discrimination plots were visually observed for good clustering. Plates with bad clustering or a success rate below 95% were repeated. Next, we assessed Hardy–Weinberg equilibrium ($p>0.05$) and genotyped approximately 5% duplicate samples, which all showed identical genotypes.

Statistical analysis

Differences in FPG and other clinical variables (HbA_{1c}, 2 h glucose, triacylglycerol, LDL, HDL, total cholesterol, BMI and waist–hip ratio) were analysed in non-diabetic participants using linear regression, adjusted for BMI, age and sex as possible confounders. All analyses were performed using an additive model, because previous studies had shown that this model was the best fit. In order to combine the effects of all SNPs, risk alleles were counted and used as a sum score (18). A risk allele was defined as an allele that results in an increased FPG as described in the literature. Differences in genotype distribution, allele frequency and risk allele

scores between participants with normal glucose tolerance and those with type 2 diabetes were compared using standard contingency tables with Fisher's exact test, and allelic ORs were calculated with logistic regression adjusted for age, sex and BMI. Subjects with either IGT or IFG were excluded from this analysis. Using Bonferroni correction for multiple hypothesis testing, $p<0.001$ was considered statistically significant for association of FPG loci with clinical variables (36 tests). For the case–control study, $p<0.01$ was considered significant (four tests). All statistics were calculated using SPSS 16.0 (SPSS, Chicago, IL, USA).

Power calculations

Power calculations were performed using Quanto (19). We had an estimated power of 80% to detect a minimal per allele effect in clinical variables between 0.056 and 0.069 mmol/l, depending on allele frequency (α=0.001). For the association study with type 2 diabetes we had an estimated power of 80% to detect a minimal OR between 1.13 and 1.16 for single gene effects (α=0.01). For all power calculations we assumed an additive model.

Table 1 Association of SNPs with FPG (n=2361) and type 2 diabetes (n=4669)

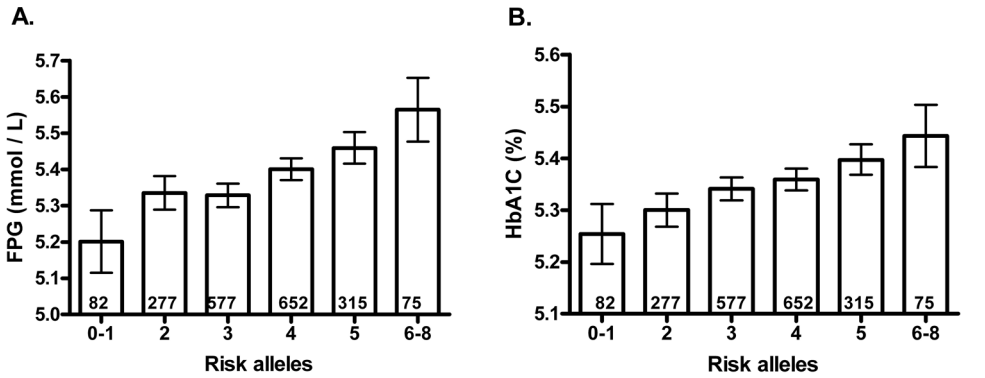
Locus	FPG, mmol/l (genotype count)			Effect/allele mmol/l	P_{add}	T2D OR 95% CI	P_{Add}
	AA	AB	BB				
GCK	5.39±0.01 (1523)	5.45±0.02 (620)	5.47±0.05 (65)	0.06 0.03 to 0.09	0.001	1.12 1.00–1.25	0.06
GCKR	5.35±0.03 (267)	5.39±0.01 (956)	5.38±0.01 (924)	0.01 −0.02 to 0.03	0.23	0.94 0.86–1.02	0.13
G6PC2	5.32±0.03 (218)	5.36±0.01 (930)	5.43±0.01 (1077)	0.06 0.04 to 0.09	5×10^{-6}	0.96 0.87–1.05	0.32
MTNR1B	5.37±0.01 (1269)	5.44±0.01 (891)	5.52±0.04 (135)	0.08 0.05 to 0.11	7×10^{-8}	1.12 1.02–1.23	0.02

Estimated FPG levels (mean±SD) per genotype are adjusted for age, sex and BMI
Effect per allele on FPG levels, 95% CI and *p* values, adjusted for age, sex and BMI, were generated by linear regression
The B genotype carries the risk allele
Odds ratios are for associations of independent SNPs with type 2 diabetes and were calculated based on allele frequency in 2041 controls and 2628 type 2 diabetes participants
T2D, type 2 diabetes

Results

All SNPs passed quality control guidelines. Associations between SNPs and clinical variables were analysed in the non-diabetic participants only. Results of association with FPG levels were comparable to those reported in the literature (all $p \leq 0.001$), except for *GCKR*, for which we could not detect a significant effect on FPG levels ($p=0.23$; results shown in Table 1). However, *GCKR* showed nominal evidence for decreased 2 h glucose, but did not reach a formally significant *p* value ($p=0.008$; see Electronic supplementary material [ESM] Table 1). Furthermore, *GCK* and *G6PC2* showed increased HbA_{1c} levels ($p=5\times10^{-8}$ and 3×10^{-5}; ESM Table 1). In line with our FPG results, *GCKR* was not associated with HbA_{1c} levels ($p=0.50$). However, we did confirm the previously reported association of the T allele of rs1260326 (*GCKR*) with increased triacylglycerol levels ($p=9\times10^{-7}$; ESM Table 1) (5). Other clinical variables were not associated with any of the analysed variants (ESM Table 1). We analysed the combined effect of all SNPs by calculating the risk allele score for each individual. We observed a combined effect of the risk alleles on FPG levels. The increase in FPG level per additional risk allele was 0.05 mmol/l (0.04–0.07 mmol/l), $p=2\times10^{-13}$ (Fig. 1a). A similar result was

Fig. 1 Combined effect of GCK, GCKR, G6PC2 and MTNR1B on FPG and HbA_{1c} in non-diabetic participants from the New Hoorn Study.



A Fasting plasma glucose. Numbers within the bars are numbers of participants per allele group. The per allele effect was 0.05 (0.04–0.07) mmol/l ($p=2\times10^{-13}$).
Error bars represent 95% CI.
B HbA_{1c}. Numbers within the bars represent the number of participants per allele group. The per allele effect was 0.03% (0.02–0.04) ($p=5\times10^{-10}$).
Error bars represent 95% CI

observed for HbA_{1c}: 0.03% (0.02–0.04) increase per additional risk allele, $p=5\times10^{-10}$ (Fig. 1b). We also analysed whether the rate of the age-related increase in FPG was affected by the number of risk alleles. However, we did not observe any differences in these rates between the risk allele scores in our cross-sectional data set (ESM Fig. 1). Separate analysis of only the participants with normal glucose tolerance (n=2041) did not alter any of the results (data not shown)

Next, we analysed the association of these single variants and the risk allele score with type 2 diabetes susceptibility. Only rs10830963 (*MTNR1B*) and rs1799884 (*GCK*) showed weak evidence for association with type 2 diabetes (p=0.02 and p=0.06 respectively; Table 1 and ESM Table 2). Risk allele scores were calculated for the participants with normal glucose tolerance and those with type 2 diabetes and all risk allele groups were compared with the reference group having four risk alleles, since this was the most common group (31%). The lower risk allele groups showed a protective effect on type 2 diabetes, while the risk allele groups with more than four risk alleles showed an increased risk of type 2 diabetes (Table 2). Those with fewer than three risk alleles had a significantly reduced risk of type 2 diabetes (OR 0.77 [0.65-0.93], p=0.005) whereas those with more than five had a significantly increased risk of type 2 diabetes compared with the reference group (OR 2.05 [1.50–2.80], $p=4\times10^{-6}$). Adjustment for age, sex and BMI did not alter the results.

We also noted a significant correlation with the age at diagnosis of type 2 diabetes in our study sample. We observed a per allele effect of −0.46 (−0.80 to −0.11) years in age at diagnosis per additional risk allele (p=0.009) (Table 2). At the extremes of the distribution, i.e. 0 or 1 versus 6–8 risk alleles, there was a difference of almost 4.5 years in age at diagnosis between the two groups (p=0.002) (Table 2).

Table 2 Association of risk allele scores with type 2 diabetes

Risk alleles	Count (frequency)		Age at diagnosis[a] (years, SD)	OR for T2D (95% CI)	*p* value T2D
	Controls (*n*=2041)	Cases (*n*=2628)			
0 or 1	76 (4.2)	115 (4.9)	57.5±1.1	0.75 (0.55–1.02)	0.07
2	243 (13.5)	352 (15.0)	57.3±0.6	0.78 (0.64–0.95)	0.02
3	522 (29.0)	667 (28.4)	56.1±0.5	0.89 (0.76–1.04)	0.14
4	605 (33.6)	685 (29.1)	55.6±0.4	1.00	ref
5	288 (16.0)	381 (16.2)	56.1±0.6	1.17 (0.97–1.41)	0.11
6-8	65 (3.6)	151 (6.4)	52.9±0.9	2.05 (1.50–2.80)	4×10^{-6}

[a] Age at diagnosis was available for 2132 participants with type 2 diabetes
$Beta_{age\ at\ diagnosis}$=–0.46 (–0.80 to –0.11) years, *p*=0.009 adjusted for sex
OR for type 2 diabetes for <3 versus 4 risk alleles was 0.77 (0.65–0.93), *p*=0.005
OR for type 2 diabetes for >4 versus 4 risk alleles was 1.33 (1.12–1.58), *p*=0.001
T2D, type 2 diabetes

Discussion

Several studies have shown that SNPs in *GCK*, *GCKR*, *G6PC2* and *MTNR1B* are associated with FPG levels (4-7;9;11-13;20). In this study we replicated these findings in a Dutch population, with the exception of the association of *GCKR* with FPG. However, our results for *GCKR* are in the same direction as those of most other studies and it should be noted that some other recent publications reported considerable variability in effect size between different samples (8) or failed to replicate this observation (11). *GCK* and *G6PC2* were associated with HbA_{1c} in our study, which confirms previous observations (13;21).

We observed a significant combined effect of all variants on FPG levels. This confirms a recent observation in a French study (13). The association of FPG levels with the risk allele count was also reflected in increased HbA_{1c} levels, arguing against previous findings in which it was suggested that FPG and HbA_{1c} have independent underlying risk loci (22;23). Our cross-sectional data suggest that these loci cause a physiological disturbance of glucose homeostasis by raising the set point of insulin secretion, leading to an elevation of FPG depending on the number of risk alleles present, which is not further affected by ageing. However, longitudinal studies and a wider age span would be needed to confirm this observation.

To our knowledge, this is the first report showing that the analysed loci have a combined effect on type 2 diabetes susceptibility, although the contribution of each individual variant to the risk of type 2 diabetes is very low or undetectable (Table

1). Our data show that carriers of fewer than three risk alleles are at decreased risk of type 2 diabetes whereas those with more than five risk alleles have increased susceptibility to type 2 diabetes compared with the most common risk allele group of four risk alleles. We also noted a significantly different age at diagnosis between the different groups, indicating that the number of risk alleles also influences the age at which the disease becomes manifest. This might also have implications for the development of complications. If replicated, our results imply that these loci not only influence FPG levels, probably through an altered set point for glucose at which an insulin response is elicited, but also jointly increase the risk of type 2 diabetes and the age at diagnosis.

In conclusion, we replicated the combined effect of *GCK*, *GCKR*, *G6PC2* and *MTNR1B* risk alleles with FPG. Furthermore, we showed that the risk allele score is also associated with HbA_{1c} and that carriers of a low or high number of risk alleles have significantly different susceptibilities to the development of type 2 diabetes and age at diagnosis of the disease.

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Supplementary table s1. Association of GCK, GCKR, G6PC2 and MTNR1B with clinical variables in

Variant	FPG (mmol / L)	2hrG (mmol / L)	HbA_{1C} (%)	TG (mmol / L)
***GCK* (rs1799884)**				
GG	5.39 (0.01)	5.37 (0.04)	5.34 (0.01)	1.39 (0.02)
GA	5.45 (0.02)	5.49 (0.06)	5.40 (0.01)	1.39 (0.03)
AA	5.47 (0.05)	5.68 (0.18)	5.44 (0.03)	1.31 (0.09)
p-value	0.001	0.06	$5 \cdot 10^{-8}$	0.86[a]
***GCKR* (rs1260326)**				
TT	5.35 (0.03)	5.40 (0.08)	5.33 (0.02)	1.56 (0.05)
TC	5.39 (0.01)	5.36 (0.04)	5.35 (0.01)	1.39 (0.02)
CC	5.38 (0.01)	5.19 (0.04)	5.35 (0.01)	1.30 (0.02)
p-value	0.23	0.008	0.50	$9 \cdot 10^{-7}$[a]
***G6PC2* (rs560887)**				
AA	5.32 (0.03)	5.42 (0.09)	5.30 (0.02)	1.33 (0.05)
AG	5.36 (0.01)	5.34 (0.05)	5.34 (0.01)	1.37 (0.03)
GG	5.43 (0.01)	5.33 (0.04)	5.37 (0.01)	1.39 (0.02)
p-value	$5 \cdot 10^{-6}$	0.67	$3 \cdot 10^{-5}$	0.30[a]
***MTNR1B* (rs10830963)**				
CC	5.37 (0.01)	5.40 (0.04)	5.35 (0.01)	1.38 (0.02)
CG	5.44 (0.01)	5.38 (0.05)	5.37 (0.01)	1.38 (0.03)
GG	5.52 (0.04)	5.50 (0.12)	5.40 (0.02)	1.45 (0.07)
p-value	$7 \cdot 10^{-8}$	0.62	0.01	0.26[a]

Association of Fasting Plasma Glucose (FPG), 2 hours glucose (2hrG), HbA_{1C}, triglycerides (TG), LDL, HDL,
Data represent estimated means, adjusted for age, gender and BMI and standard deviations are given.
a: Non transformed values are shown. P-values are calculated with 10Log transformed triglyceride values.

non diabetic subjects from the NHS (n = 2361).

LDL (mmol / L)	HDL (mmol / L)	TC (mmol / L)	BMI (kg / m^2)	WHR
3.31 (0.02)	1.53 (0.01)	5.45 (0.03)	25.97 (0.10)	0.89 (0.001)
3.36 (0.04)	1.51 (0.02)	5.50 (0.04)	25.91 (0.15)	0.89 (0.002)
3.29 (0.11)	1.48 (0.05)	5.36 (0.12)	25.89 (0.47)	0.89 (0.006)
0.39	0.46	0.40	0.94	0.79
3.33 (0.05)	1.52 (0.02)	5.54(0.06)	25.85 (0.23)	0.89 (0.003)
3.32 (0.03)	1.52 (0.01)	5.46 (0.03)	25.78 (0.12)	0.89 (0.002)
3.35 (0.03)	1.54 (0.01)	5.47 (0.03)	25.94 (0.12)	0.89 (0.002)
0.76	0.37	0.53	0.66	0.63
3.23 (0.06)	1.57 (0.03)	5.39 (0.07)	25.87 (0.26)	0.89 (0.004)
3.31 (0.03)	1.51 (0.01)	5.44 (0.03)	25.93 (0.12)	0.89 (0.002)
3.36 (0.03)	1.52 (0.01)	5.50 (0.03)	25.87 (0.12)	0.89 (0.002)
0.12	0.18	0.18	0.93	0.99
3.34 (0.03)	1.52 (0.01)	5.48 (0.03)	25.88 (0.11)	0.89 (0.001)
3.28 (0.03)	1.54 (0.01)	5.43 (0.03)	25.93 (0.13)	0.89 (0.002)
3.34 (0.08)	1.48 (0.03)	5.47 (0.08)	26.63 (0.33)	0.89 (0.004)
0.29	0.27	0.54	0.09	0.82

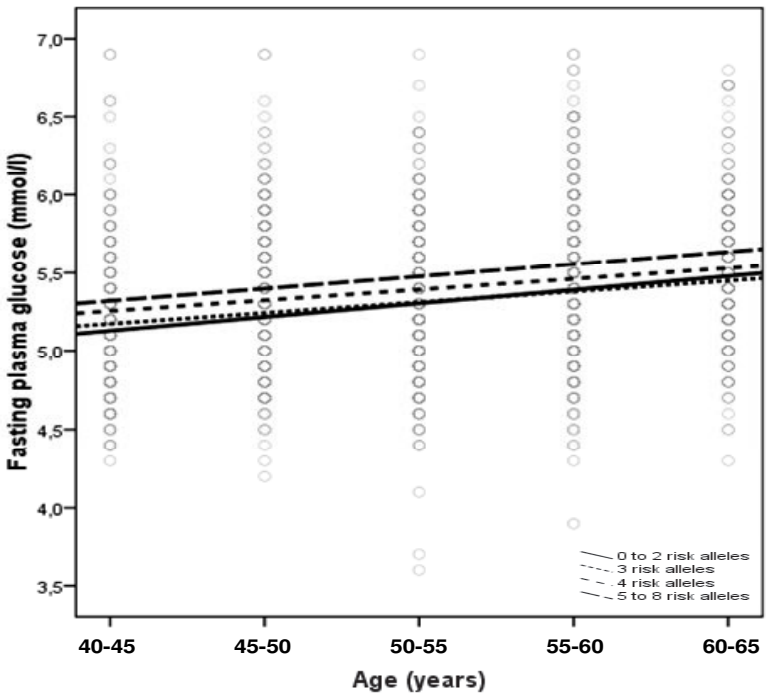
total cholesterol (TC), BMI and waist-hip ratio (WHR) with SNPs in *GCK*, *GCKR*, *G6PC2* and *MTNR1B*.

Supplementary table s2. Genotyping results of the independent SNPs in 2041 controls and 2628 cases.

SNP	Gene	risk allele	Allele frequency risk allele (AA, AB, BB)		OR (95% CI)	P-value
			controls (n)	cases (n)		
rs1799884	*GCK*	A	16.8 (641) 1326, 531, 55	18.4 (917) 1668, 743, 87	1.12 1.00 – 1.25	0.06
rs1260326	*GCKR*	C	65.8 (2562) 235, 864, 848	64.2 (3232) 313, 1174, 1029	0.94 0.86 – 1.02	0.13
rs560887	*G6PC2*	G	69.6 (2750) 192, 816, 967	68.6 (3502) 263, 1074, 1214	0.96 0.87 – 1.05	0.32
rs10830963	*MTNR1B*	G	25.0 (994) 1111, 764, 115	27.1 (1377) 1343, 1011, 183	1.12 1.02 – 1.23	0.02

Allele frequencies (counts) and genotype counts (AA, AB, BB) are shown. B represents the risk allele.

Supplementary Figure 1: Age related increase in FPG in non-diabetic subjects.



For ease of interpretation we have divided the non-diabetic subjects into four groups depending on the number of risk alleles. Group 1, 0 to 2 risk alleles (n=359); group 2, 3 risk alleles (n=580); group 3, 4 risk alleles (n=652) and group 4, >5 risk alleles (n=393). Unadjusted trend lines for each group are shown. Beta's with (95% CI) are calculated with linear regression adjusted for gender and BMI. Group 1: $\beta = 0.013$ (0.007-0.019); Group 2; $\beta = 0.011$ (0.006-0.016); Group 3; $\beta = 0.011$ (0.007-0.016); Group 4; $\beta = 0.013$ (0.007-0.019); all $P<1.0*10^{-4}$)

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Chapter 6

Summary and discussion

This thesis deals with a genetic study on the involvement of mitochondria in setting the risk for type 2 diabetes and related parameters. It comprises three themes:

(1) Nuclear encoded mitochondrial proteins (chapters 2 and 3);

(2) Mitochondrial DNA content and type 2 diabetes (chapter 4);

(3) Genes regulating fasting plasma glucose concentrations (chapter 5).

Part 1: Nuclear encoded mitochondrial proteins (chapters 2 and 3)

In Chapter 2 I describe the association study of the mitochondrial leucyl-tRNA synthetase gene (*LARS2*) with type 2 diabetes. The *LARS2* gene is a candidate gene for type 2 diabetes involved in the same biochemical pathway as the mitochondrial tRNA-Leu(UUR) gene. Mutations in the latter gene are low frequency high penetrance mutations for the maternally inherited diabetes and deafness syndrome.

Previously an H324Q variant in this gene was found to be associated with type 2 diabetes in four independent populations from the Netherlands and Denmark (1). This variant is potentially able to affect proper function of the mitochondrial leucyl tRNA synthetase as we obtained biochemical evidence that the H324Q variant may lead to increased acylation of mitochondrial tRNA-Leu with Isoleucine (E. Reiling et al., unpublished). As an extension of our previous finding we analyzed common tagging SNPs (MAF > 0.05) and low frequency SNPs (MAF 0.01 - 0.05) in *LARS2* in the Dutch Hoorn study. Potential signals, including the previously identified H324Q variant, were followed up in up to 35715 subjects from the Netherlands, Denmark, Sweden, Finland and the UK. Access to these samples was by collaboration with J.M. Dekker, G. Nijpels, A.G. Uitterlinden, M.H. Hofker, O. Pedersen, T. Hansen, L. Groop and M.I. McCarthy. After follow up no putative associations remained significant, including the H324Q variant. These data exclude the *LARS2* gene as a major type 2 diabetes susceptibility gene. In order to elucidate the failure of replication of the previously found association of H324Q with type 2 diabetes we analyzed our data for heterogeneity by stratification for several variables like age and geographic location of study samples. Given the potential role of mitochondria in ageing the variant may affect life expectancy in some

subgroups of the cohorts. In addition, the frequency of some genetic variants have been found to exhibit a geographic north-south trend. A decrease in MAF at increasing age was observed for type 2 diabetes subjects in most but not all study samples. This could indicate an increased mortality in type 2 diabetes subjects carrying the H324Q risk allele, but this did not reach formal levels of statistical significance. Furthermore, a MAF gradient from south to north was observed in the UK. However, this was not observed in our initial study (1) and additional samples from The Netherlands, Denmark, Sweden and Finland, making this an unlikely explanation for the initial false positive finding. Most likely, the first finding was a false positive, caused by chance. These findings highlight the potential pitfalls one can encounter when analyzing low frequency variants.

In chapter 3 I describe a candidate gene study on the association of variants in nuclear-encoded mitochondrial protein genes with type 2diabetes. In total 13 candidate genes were selected. The rationale for selecting these genes is accumulating evidence that mitochondria play a causal role in the onset of type 2 diabetes. For instance a 3243A>G mutation in the mitochondrial tRNA-Leu(UUR) gene is associated with maternally inherited diabetes and deafness, a specific subtype of diabetes. Carrier frequency of this mutation is 0.3-2%, depending on ethnicity. In addition, a number of other rare point mutations in mitochondrial DNA (mtDNA) are high penetrance diabetogenic mutations (2). However, common SNPs in mtDNA did not show evidence for association with type 2 diabetes (3;4). This makes it unlikely that additional, polymorphisms in mtDNA exist which contribute to type 2 diabetes. However, mitochondrial dysfunction has been shown in muscle from type 2 diabetes patients and their non-diabetic, insulin resistant, first degree relatives (5-7). Since the majority of mitochondrial proteins are encoded by the nucleus, we focussed on nuclear encoded mitochondrial genes that are thought to be key players in mitochondrial maintenance and function. We genotyped tagging SNPs covering all common variation in these candidate genes (CEU population, MAF > 0.05) in our first stage sample (the Dutch Hoorn Study, n = 999). Potential signals ($p < 0.05$) were followed up in the second stage comprising of Dutch samples from the New Hoorn Study, Breda Study and ERGO study (n = 10164).

Only one SNP (rs2522138 in *SIRT4*) remained significant, but after extending the second stage with a sample from Denmark (n = 1220) the signal was no longer significant. Therefore, I conclude that common variation in the selected candidate genes is not associated with type 2 diabetes. Although the first stage was underpowered to detect modest associations (80% to detect an OR of 1.45, MAF = 0.1), results were in line with results from Genome Wide Association Studies (GWAS), making it unlikely that common variation in our candidate genes is associated with type 2 diabetes (8-12). This study underlines the importance of extensive replication of novel association signals.

Since we selected only 13 candidate genes and the total pool of mitochondrial protein is expected to be approximately 1500 (13), we cannot conclude from our data that common SNPs in nuclear encoded mitochondrial genes do not contribute to the development of type 2 diabetes. However, nuclear encoded mitochondrial proteins are also not among the top hits of GWAS (8-12). Since the DIAGRAM meta-analysis used LD information from the HapMap database and covered ~2,000,000 SNPs, a large proportion of common DNA sequence variation is covered by this analysis. Also this study did not show evidence for association of nuclear encoded mitochondrial proteins with type 2 diabetes (12). Therefore, it seems likely that common genetic defects in nuclear encoded mitochondrial proteins do not have a major contribution to type 2 diabetes susceptibility.

However, while GWAS, like the DIAGRAM meta-analysis, are well powered to identify association of common SNPs with type 2 diabetes (MAF > 0.05), they are underpowered to detect associations of low frequency and rare variations (MAF < 0.05) with type 2 diabetes considering modest effect sizes. Moreover, genotype data from the HapMap database about the Caucasian population is based on 90 subjects (CEU population) (14). Therefore, it is likely that low frequency variation was partially missed during sequencing of these 90 subjects and subsequently these are not analysed for association with type 2 diabetes using LD.

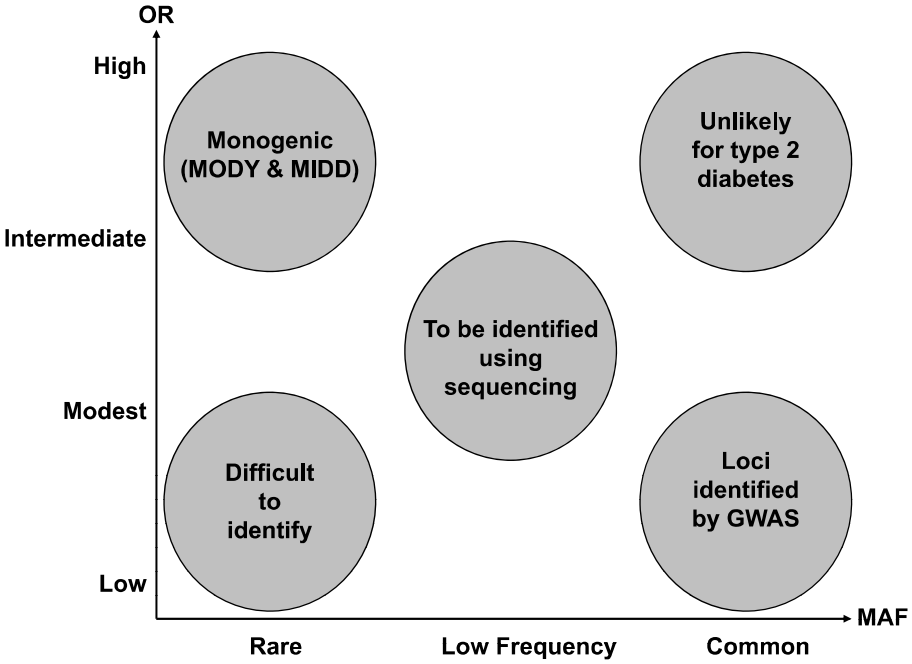
Taken together, it is not likely that common SNPs in nuclear encoded mitochondrial proteins predispose to type 2 diabetes susceptibility. However, this conclusion cannot be drawn for low frequency variation. Since the HapMap database does not provide sufficient genotype data about that kind of genetic variation, resequencing

of large samples (>100 subjects) should be applied in order to identify low frequency variation prior to large scale genotyping. Currently, the 1000 genomes consortium (www.1000genomes.org) is aiming to achieve a nearly complete catalogue of common human genetic variation with a MAF above 0.01. Three sets of 400-500 individuals from broad geographic regions are being resequenced and the data will be published on a free accessible database. This project will potentially generate helpful information, which can further improve our understanding of the involvement of nuclear encoded mitochondrial proteins, including the influence of low frequency variation (MAF = 0.01 – 0.05). However, our study concerning the *LARS2* gene (chapter 2 of this thesis), illustrates the pitfalls of studying low frequency variation. Population heterogeneity caused by for instance age or geographic stratification can bias the observations resulting in false positive (or negative) findings. Therefore, caution should be taken when analyzing low frequency variation. Very large, homogeneous study samples (n > 30000) will be needed, not only to obtain sufficient power to identify diabetogenic SNPs with modest impact, but also to confirm novel findings, since these are easily biased by above mentioned heterogeneity. When analyzing their data, researchers should be aware of these pitfalls. Sequencing will also provide more insight in another form of genetic variation; the so-called copy number variations (CNV). CNVs are deletions or insertions of stretches of DNA sequences. It is likely that these CNVs affect gene function and therefore disease susceptibility. Until now, little is known about the existence and role of CNVs. Sequencing projects will reveal more detailed information about this issue (15). It has been shown that the smaller and common CNVs are inherited like SNPs (16). If their effect on T2DM is also comparable to that of common SNPs (OR < 1.5), they might have been undetected by family studies. However, large CNVs with a higher impact on type 2 diabetes susceptibility (OR > 1.5) would have been detected by family studies. On the other hand, low frequency CNVs with a modest impact on type 2 diabetes mellitus, might also been missed previously.

For analyzing rare variation (MAF < 0.01) a different strategy has to be applied, since case-control studies will be underpowered for this. Resequencing and genotyping should be performed in families with a high prevalence of type 2

diabetes to study rare variation, the so-called linkage study as explained in the introduction of this thesis. The very rare variations will potentially result in a technical problem because the MAF will almost reach the same value as the genotyping error, resulting in yet another difficulty in analyzing rare variation. The theoretical arrangement of rare, low frequency and common variation is schematically shown in figure 1. Common variants with high impact on type 2 diabetes are not expected, because they would have been detected in GWAS.

Figure 1. arrangements of rare, low frequency and common variation



Addapted from McCarthy MI et al, Nat Rev Genet. 2008 May

In conclusion, our study excludes the association of common variation in 14 selected candidate genes including the *LARS2* gene, with type 2 diabetes. All these genes encode for mitochondrial proteins involved in mitochondrial protein synthesis and biogenesis. Furthermore, it is not likely that common variation in other mitochondrial targeted genes is associated with type 2 diabetes, based on GWAS data. However, we cannot exclude the involvement of low frequency and rare variations in these genes even when they have relative high diabetogenic potential (OR > 1.4). This should be elucidated in future research.

Part 2: Mitochondrial DNA content and type 2 diabetes (chapter 4)

Proper mitochondrial function contributes to many cellular processes related to maintenance of glucose homeostasis, such as insulin resistance of muscle, glucose-induced, insulin secretion, apoptosis of pancreatic beta-cells, removal of fatty acids by beta-oxidation and setting the energy status of the brain. In chapters 2 and 3 of this thesis, I described our findings that common variants in key-mitochondrial proteins are unlikely to be linked to type 2 diabetes susceptibility. Another factor that determines mitochondrial activity within a cell is variation in the number of mitochondrial DNA (mtDNA) molecules per cell, expressed as number of mtDNA molecules per nuclear genome. For that reason we also analyzed mtDNA content in blood in relation to the risk of an individual for developing type 2 diabetes. This study is described in chapter 4.

In view of the situation that the risk for diabetes has a genetic component we first assessed whether mtDNA content is determined by heritability. We found a heritability of 35% (19 - 48) in buccal cells. This confirms findings of others, which showed comparable results in blood (17;18). We could not detect an association of mtDNA content in blood with prevalent type 2 diabetes, incident type 2 diabetes or related traits. This contradicts previously published studies, in which it was shown that a low mtDNA content precedes type 2 diabetes onset and is associated with insulin resistance, glucose metabolism, insulin secretion and patterns of triglyceride storage (19-22). However, our results are supported by another study, in which no differences were observed in mtDNA content between first degree relatives of type 2 diabetes patients and control participants without family history of type 2 diabetes (23). The original studies are often much smaller than our study. Therefore, it might be possible that those were false positives, caused by bias during sample selection. Another possibility is that differences in ethnicity causes the discrepancy between different studies, since associations of mtDNA content with type 2 diabetes and related traits are only observed in people of Asian descent. We did observe evidence for an inverse relation of mtDNA content with age, which is in line with previous observations in blood and muscle (17;24). However, our results indicate that this is specific for males, resulting in a lower mtDNA content in elderly males compared to elderly females.

One of the drawbacks of our and previous studies, is that blood was used for DNA extraction. Since this is a heterogeneous cell type, results can be biased by for instance the inflammation status of individuals leading to variations in leukocyte composition. Furthermore, different amounts of platelets in the blood samples (platelets do not contain a nucleus but many mitochondria) potentially result in an overestimation of the mtDNA content in blood.

We observed a heritability of only 35% indicating that environmental factors are supposed to play an important role in determining mtDNA content. This seems to be reflected by differences in mtDNA content in the independent study samples, used for this research. Although these differences may be partially caused by different DNA extraction techniques, it is also possible that biological differences between the samples have caused the variance in mtDNA content.

Previous studies showing an association between mtDNA content in blood and type 2 diabetes or related traits are much smaller than our study and are therefore likely to be biased by sample selection. We conclude that mtDNA content in blood does not associate with type 2 diabetes or related traits in white European individuals. However, from our data we cannot draw the same conclusion for other tissues like muscle and liver. Several groups showed a relation between mitochondrial function and type 2 diabetes in patient muscle (6;7). This suggests that blood may not be the correct tissue for these analyses, but muscle tissue should be analyzed instead. However, mtDNA content in muscle depends on physical exercise adding an additional complexity. Patients with a sedentary life style, who are at risk for diabetes as a result of this life style, are therefore also expected to have lower mtDNA content in their muscles. This illustrates the complex nature of conclusions based on association.

Others showed a decrease in mtDNA content in beta-cells upon ageing (25). This is of clinical importance since beta-cell function also declines during ageing and the decreasing mtDNA content is a plausible candidate mechanism. The beta-cell is therefore another important tissue to analyze, but difficult to obtain

Remarkably, when an acute decline in mtDNA content of 30-50% is induced in humans, as result of highly active antiretroviral therapy (HAART), one observes a rapid development of insulin resistance and a more gradual steatosis of the liver,

both major risk factors for type 2 diabetes (26;27). However, this induced decrease in mtDNA content did not result in acute development of type 2 diabetes, indicating that pancreatic beta-cells seem to have a relatively large spare capacity in mitochondrial function.

Taken together, the role of mtDNA content in the onset of type 2 diabetes awaits further investigation in different tissues. For predictive uses concerning type 2 diabetes, mtDNA content in blood is not a useful parameter.

As described in this discussion, we can not fully exclude that genetic variation in nuclear encoded mitochondrial proteins and variation in mtDNA content in blood is associated with type 2 diabetes. It is important that low frequency and rare genetic variation, CNVs and mtDNA content in different tissues are analyzed. However, one should also consider the possibility that mitochondrial dysfunction is not a common pathogenic mechanism for type 2 diabetes. Changes in mitochondrial parameters as described by others (6;7), might as well be caused by hyperglycemia, obesity or decreased exercise instead of being the pathogenic factor causing the disease. In fact, a study in diabetic Goto-Kakizaki rats provides evidence that mitochondrial oxidative capacity declines as a result of long-lasting metabolic dysfunction (24). Furthermore, Kelley *et al* showed that mitochondrial dysfunction is already present in obese subjects and further deteriorates when type 2 diabetes develops (6). Another possibility is that mitochondrial dysfunction does increase type 2 diabetes susceptibility, but that this dysfunction is not caused by a defect in the mitochondrion itself but by factors controlling mitochondrial activity. For instance, it is known that calcium fluxes in- and outwards the mitochondrion plays an important role in glucose stimulated insulin secretion (GSIS). It might be possible that mitochondrial dysfunction is a result of impaired calcium signaling caused by for instance dysfunctional endoplasmic reticulum or disturbed GLP-1 signaling, leading to decreased GSIS and subsequently type 2 diabetes (28). By this means, assessing causes of mitochondrial dysfunction directly, like decreased mtDNA content, would not result in the identification of increased type 2 diabetes susceptibility

Taken together, my personal interpretation of all the data is that genetic variation in mitochondrial components are not major contributors to the risk of an individual for diabetes.

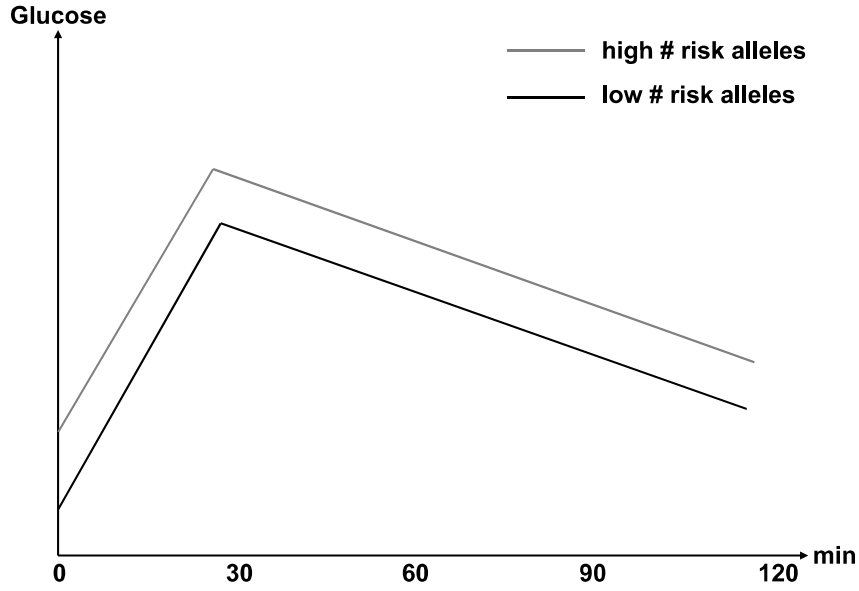
Part 3: Genes regulating fasting plasma glucose concentrations (chapter 5)

Previously, SNPs in four genes have been shown to be associated with fasting plasma glucose (FPG) levels: *GCK*, *GCKR*, *G6PC2* and *MTNR1B* (9;29-37). It is known that elevations in FPG levels within healthy range predicts a higher risk for type 2 diabetes and coronary heart disease later in life (38;39)

For that reason I studied the analysis of the combined risk alleles of these 4 genes, on type 2 diabetes susceptibility. This study is described in chapter 5. SNPs were genotyped (rs1799884 (*GCK*), rs1260326 (*GCKR*), rs560887 (*G6PC2*) and rs10830963 (*MTNR1B*)) in a Dutch sample. I observed that these SNPs have a combined effect on FPG levels, which is also reflected by increased HbA_{1C}. This finding confirms a previous observation in a French study (37). In addition, I also observed that the combined risk allele score of these genes is associated with a decreased type 2 diabetes susceptibility for those with a low number of risk alleles and with increased type 2 diabetes susceptibility for those with a high number of risk alleles, compared to individuals carrying the most common risk allele group (4 risk alleles, 31% of the study sample). Furthermore, a high number of risk alleles also associates with a lower age at diagnosis of type 2 diabetes. This indicates that the known FPG genes have in combination an effect on type 2 diabetes susceptibility and age at diagnosis of type 2 diabetes as well, although their single gene effects are minimal or absent. Our data also suggest that the rate of increase of FPG concentrations upon ageing does not differ between individuals with a high number of risk alleles and those with a low number of risk alleles. This indicates that carriers of multiple risk alleles have an increased fasting glucose set point when compared to carriers of less risk alleles. Thus, it seems that these genetic variants determine the initial setting of the beta-cell glucose sensor, like the MODY2 polymorphisms in the glucokinase gene. A prospective study is needed to

strengthen this finding. The OGTT curve should than be increased over the entire length, as indicated in figure 2. This should be investigated in future research. Published studies have combined the risk alleles of all confirmed type 2 diabetes genes, resulting in a large difference in odds ratio between the extremes of the risk allele groups. Remarkably, the predictive value of genetic risk factors is limited compared to other non-genetic type 2 diabetes risk factors like age and BMI (40-42). Therefore, the known genetic risk factors for type 2 diabetes do not improve the prediction of the disease when compared to anthropomorphic markers.
Including the four FPG genes to the list of type 2 diabetes risk genes and analyzing the predictive value of the combined group of genes might further improve our understanding of the predictive value for the development of type 2 diabetes using known risk loci. Currently, it seems unlikely that common genetic variation will be useful as diagnostic predictor of type 2 diabetes.
Until now, the best way to predict future type 2 diabetes risk, is by analyzing family history of type 2 diabetes, BMI and exercise level. It will be necessary to reveal all genetic risk factors for type 2 diabetes and related traits like FPG, BMI and insulin

Figure 2. Predicted OGTT curves of low and high risk allele carriers



secretion in order to achieve a meaningful genetic prediction. This will go beyond the scope of common SNPs, since also low frequency (MAF = 0.01 - 0.05) and rare variation (MAF < 0.01) have to be analyzed. In addition, other variation like CNVs needs to be assessed. Therefore, resequencing has to be performed in order to analyze these forms of genetic variation. Since technology shows a quick development this will be possible in the near future. The hypothesis is that these forms of genetic variation will have a large impact on type 2 diabetes susceptibility. If this is true, this approach might prove to be useful in disease prediction. If the odds ratio's of these genetic variations will be in the same range as those of common SNPs (OR between 1.1 and 1.3) the additive value will be little and studies will be easily underpowered to detect such associations. Future research will shed more light on these important issues.

Closing remarks

Until recently genetic research related to type 2 diabetes has struggled to prove its use in medical science. Only sparsely novel type 2 diabetes genes were identified. The studies described in this thesis again underline the difficulties one can encounter during the search for such diabetes risk genes, since we could not identify any novel type 2 diabetes gene by the candidate-gene approach. However, with the coming of GWAS, new hope emerged for genetic research since it identified several new loci in relatively short time. Unfortunately, until now no functional polymorphism has been identified and no major type 2 diabetes loci with large impact on disease risk have been depicted. Since the known type 2 diabetes loci do not have a significant predictive value for type 2 diabetes, the clinical relevance of type 2 diabetes genetic research is under fire. The hope was that by identification of high risk individuals for diabetes, progression of the disease could be specifically prevented in those individuals by early therapeutic intervention. Until now, this is not possible. Only by further genetic research, like assessing effect sizes of low frequency variation, rare variation and CNVs as described above, the value of genetic research can be clarified. Therefore, further investments in genetic research will be needed.

Although it is difficult to link the genetic variations in the various diabetes risk genes with a particular pathogenic mechanism, the genes in which these variants occur are often related to the function of the beta cell rather than with insulin action. This suggests that the onset of decreased insulin secretion has a large genetic compound, while insulin resistance is induced predominantly by influences like diet, exercise and ageing. This has contributed to a change in view on the pathogenesis of the disease as previously it was considered that insulin resistance as a result of a genetic predisposition was the driving factor for the pathogenesis of type 2 diabetes. However, another possibility might be that the current design of studies is not suitable to detect insulin resistance genes. If low frequency and rare variants and CNVs do have an impact on insulin resistance, they will be identified in the coming years. Furthermore, GWAS have identified susceptibility genes, which are not likely to be selected by candidate gene approach since their function was unknown or not involved in a likely type 2 diabetes pathogenic pathway. This is one

of the important benefits of the hypothesis-free approach of GWAS and provides more insight in the pathogenic pathway of type 2 diabetes. Therefore, genetics of type 2 diabetes already showed its use in the past and future research will hopefully further improve our understanding of type 2 diabetes.

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Nederlandse samenvatting

(summary in Dutch)

Type 2 diabetes is een chronische ziekte, waarvan het voorkomen wereldwijd fors toeneemt. De ziekte wordt gekarakteriseerd door chronisch verhoogde glucose spiegels, wat op den duur kan resulteren in glucose geïnduceerde complicaties zoals schade aan bloedvaten en het zenuwstelsel. Daarnaast kan de ziekte leiden tot cardiovasculaire aandoeningen waardoor de levensverwachting van type 2 diabetes patiënten met ongeveer 10 jaren afneemt. De ziekte ontstaat onder andere door een verminderde reactie van de lever, spier- en vetweefsel op insuline. In combinatie met een verminderd vermogen van de pancreas om insuline uit te scheiden, resulteert dit in verhoogde glucose spiegels. Bij een glucose waarde boven de 7 mmol/L in nuchtere conditie, is er sprake van type 2 diabetes. Het is bekend dat factoren zoals overgewicht, weinig beweging en veroudering het risico op type 2 diabetes verhogen. Verder speelt ook genetische variatie een rol in de vatbaarheid voor deze ziekte. Het is echter nog niet geheel duidelijk welke genen hierbij een rol spelen en hoe groot de invloed van deze genen is op de vatbaarheid voor type 2 diabetes. In deze studie heb ik geprobeerd om meer helderheid te krijgen in de rol van genetische variatie bij het ontstaan van type 2 diabetes.

Het eerste gedeelte van deze studie is gewijd aan genen die betrokken zijn bij mitochondriële functie. Mitochondriën spelen een belangrijke rol bij het glucose metabolisme, insuline secretie en vetzuurverbranding en er is op grond hiervan gesuggereerd dat een slechte mitochondriële functie kan leiden tot type 2 diabetes. In totaal heb ik 14 kandidaat-genen geselecteerd, die allemaal een belangrijke rol spelen in het onderhouden van een goede mitochondriële functie. Alle geselecteerde genen worden gecodeerd door het nucleaire DNA en de eiwitten worden vervolgens geïmporteerd in het mitochondrion. Met behulp van de internetdatabase HapMap (www.hapmap.org) heb ik een selectie gemaakt van zogenaamde ‘single nucleotide polymorphisms’ (SNP’s), waarmee alle veel voorkomende variatie (frequentie > 5%) geanalyseerd kan worden. Al deze SNP’s zijn gemeten in de Nederlandse Hoorn studie (n = 999), dit is de eerste fase van dit project. Vervolgens zijn de SNP’s die statistisch bewijs vertoonden voor een associatie met type 2 diabetes ($p < 0.05$), opnieuw gemeten in drie andere

Nederlandse studies (Nieuwe Hoorn Studie, ERGO studie en Breda studie). Dit is de tweede fase van deze studie (n = 10164). Het doel van deze tweede fase is het bevestigen van potentiële associaties om toevalsbevindingen uit te sluiten. Slechts één SNP toonde na deze tweede fase zwak bewijs voor een associatie met type 2 diabetes ($p = 0.01$). Echter, nadat we de tweede fase hadden uitgebreid met een studie uit Denemarken (n = 1220), bleef dit niet statistisch significant ($p = 0.06$). Dit betekent dat veelvoorkomende variatie in onze kandidaat genen niet een rol speelt bij het ontstaan van type 2 diabetes. Tijdens afronding van deze studie, verschenen er verschillende genome wijde associatie studies (GWAS). GWAS worden gebruikt om veel voorkomende variaties verspreid over het gehele genoom te associëren met een bepaald fenotype (in dit geval type 2 diabetes). Resultaten van deze GWAS bevestigden onze negatieve bevindingen en toonden verder aan dat variatie in andere mitochondriële genen waarschijnlijk ook geen belangrijke rol spelen bij het ontstaan van type 2 diabetes.

In het tweede gedeelte van dit onderzoek, hebben we onderzoek gedaan naar het mitochondriële DNA (mtDNA). Dit is een klein circulair genoom in het mitochondrion, wat in verschillende hoeveelheden in de cel voorkomt. Omdat in het verleden in kleine studies is aangetoond dat een lage hoeveelheid mtDNA geassocieerd is met een verhoogd risico op type 2 diabetes, hebben wij dit verder onderzocht. Allereerst hebben we de erfelijkheid van de hoeveelheden mtDNA onderzocht, gebruik makende van het Nederlandse Tweelingen Register. Dit leidde tot de conclusie dat de hoeveelheid mtDNA voor ongeveer 35% wordt bepaald door genetische factoren. Vervolgens hebben we onderzocht of de hoeveelheden mtDNA ook effect hebben op de vatbaarheid voor type 2 diabetes. Hiervoor hebben we een zogenaamde case / control studie gebruikt uit de regio van Hoorn, waarbij mensen met en zonder type 2 diabetes met elkaar vergeleken worden. Verder gebruikten we twee prospectieve studies, waarbij voor een groep gezonde mensen voor een bepaalde periode wordt bijgehouden of ze type 2 diabetes hebben ontwikkeld. Bij geen van deze studies hebben we aanwijzingen gevonden dat variaties in hoeveelheden mtDNA het risico op type 2 diabetes beïnvloeden.

Onze conclusie is daarom dat verlaagde hoeveelheden mtDNA niet tot verhoogde vatbaarheid voor type 2 diabetes leiden, wat eerder wel gesuggereerd was.

In het derde gedeelte van dit proefschrift beschrijf ik het onderzoek naar de relatie tussen verschillende genen en 'fasting plasma glucose' (FPG) concentratie. Tot op heden is er van vier genen reproduceerbaar aangetoond dat zij de FPG concentratie beïnvloeden. Dit zijn de genen *GCK*, *GCKR*, *G6PC2* en *MTNR1B*. In deze studie heb ik SNP's in de genoemde genen (1 SNP per gen, op basis van eerder gepubliceerde resultaten van andere groepen) gemeten in een groep mensen met en zonder type 2 diabetes uit de regio van Hoorn. Omdat er bij het analyseren van 1 variant sprake is van een spreiding van 0 tot en met 2 risico allelen, is er bij 4 varianten sprake van een spreiding van 0 tot en met 8 risico allelen. Door middel van het optellen van het aantal risico allelen van ieder individu, was het mogelijk om een risico allel score te berekeningen en het effect van deze score op FPG concentraties en type 2 diabetes vatbaarheid te analyseren. Het bleek dat individuen die een hoge score hebben, niet alleen verhoogde FPG concentraties hebben, maar ook een hogere vatbaarheid voor type 2 diabetes vergeleken met mensen met een gemiddelde score. Verder lieten deze resultaten ook zien dat type 2 diabetes patiënten met een hoge score, gemiddeld eerder de ziekte ontwikkelden, ten opzichte van patiënten met een gemiddelde score.

Samenvattend konden wij geen genetische defecten ontdekken in nucleair gecodeerde mitochondriële genen en verschillen in hoeveelheden mtDNA die de vatbaarheid op type 2 diabetes beïnvloeden. Deze data wijzen erop dat mitochondriële disfunctie wellicht minder belangrijk is bij het ontstaan van type 2 diabetes dan eerder verondersteld. Mogelijk is mitochondriële disfunctie een gevolg van type 2 diabetes en niet andersom. Echter, dit onderzoek beslaat alleen maar veel voorkomende genetische variatie en mtDNA in bloed. Het is belangrijk dat in de toekomst ook minder frequentie genetische variatie en hoeveelheden mtDNA in andere weefsels worden onderzocht. Verder is het mogelijk dat niet genetische factoren mitochondriële functie verstoren en op deze manier type 2

diabetes risico verhogen. Een hypothese is bijvoorbeeld dat factoren zoals overgewicht, veroudering of weinig lichamelijke activiteit een negatief effect hebben op mitochondriële functie en zodoende de vatbaarheid voor type 2 diabetes verhogen. Verder blijkt uit onze data dat genen die FPG concentraties beïnvloeden gezamenlijk ook de vatbaarheid van type 2 diabetes beïnvloeden.

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Curriculum Vitae

The author of this thesis was born on the third of May, 1983, in Dedemsvaart The Netherlands. He graduated for MAVO education in 1999 at the MAVO Weidebeek in Wezep, and for HAVO education in 2001, at the Agnieten College in Zwolle. After this he attended “Biologie en Medisch Laboratorium Onderzoek” at the Saxion Hogeschool IJselland in Deventer. He performed his third year internship at the department of auto-immune biochemistry at the Nijmegen Centre for Medical Life Sciences under supervision of Dr. A.J.W. Zendman. His graduation internship was performed at the department of Molecular Cell Biology at the LUMC in Leiden, under supervision of Dr. L.M. ’t Hart and graduated in 2005. After his graduation, he stayed at this department for the PhD-project described in this thesis under supervision of Dr. L.M. ’t Hart and Prof. Dr. J.A. Maassen. Since August 2009 he is working as postdoctoral researcher at the RIVM in Bilthoven, The Netherlands.