The genetics of type 2 diabetes

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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that the H324Q variant may lead to increased acylation of mitochondrial tRNA-Leu with Isoleucine (E. Reiling et al., unpublished).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.

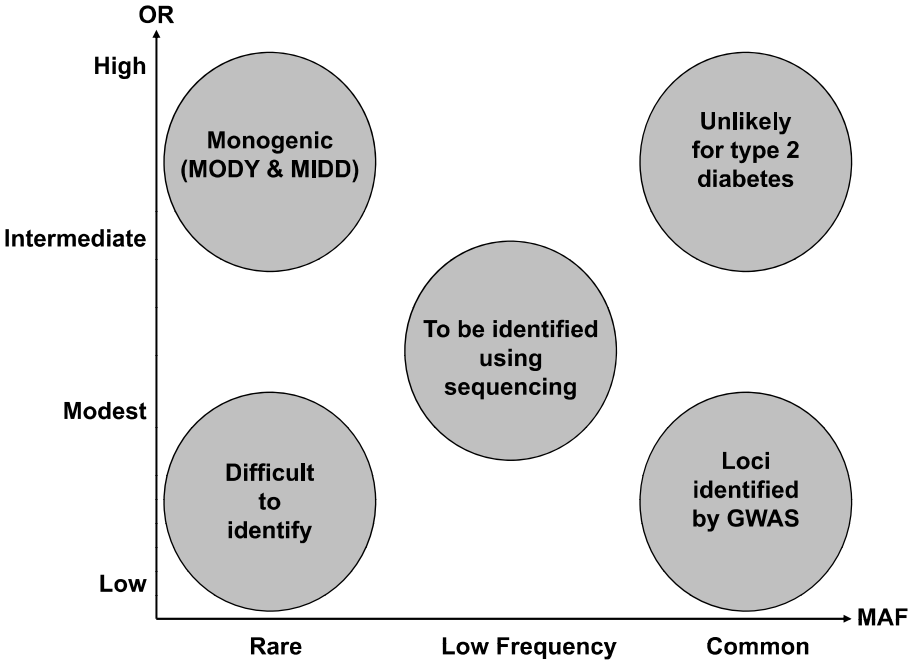
Taken together, it is not likely that common SNPs in nuclear encoded mitochondrial proteins predispose to type 2 diabetes susceptibility. However, this conclusion cannot be drawn for low frequency variation. Since the HapMap database does not provide sufficient genotype data about that kind of genetic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



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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)

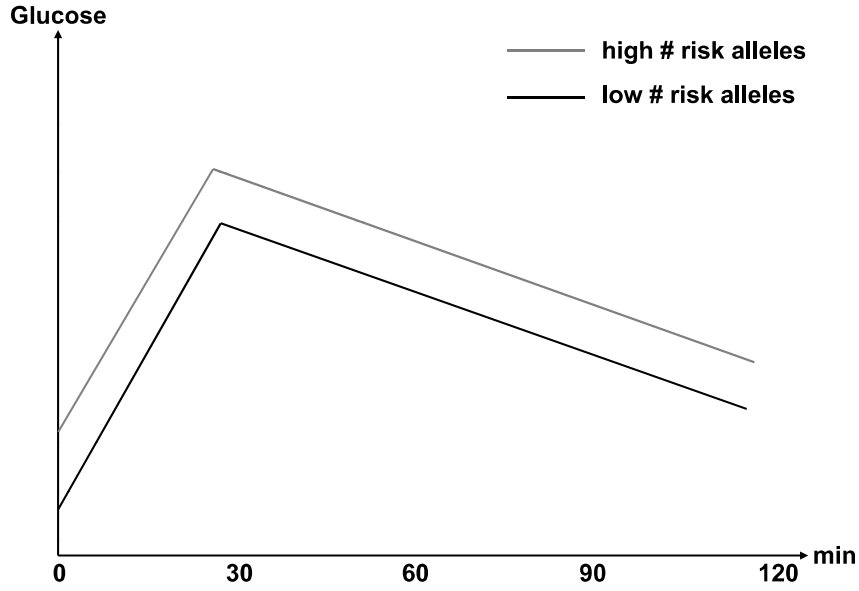
Previously, SNPs in four genes have been shown to be associated with fasting plasma glucose (FPG) levels: *GCK*, *GCKR*, *G6PC2* and *MTNR1B* (9;29-37). It is known that elevations in FPG levels within healthy range predicts a higher risk for type 2 diabetes and coronary heart disease later in life (38;39)

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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