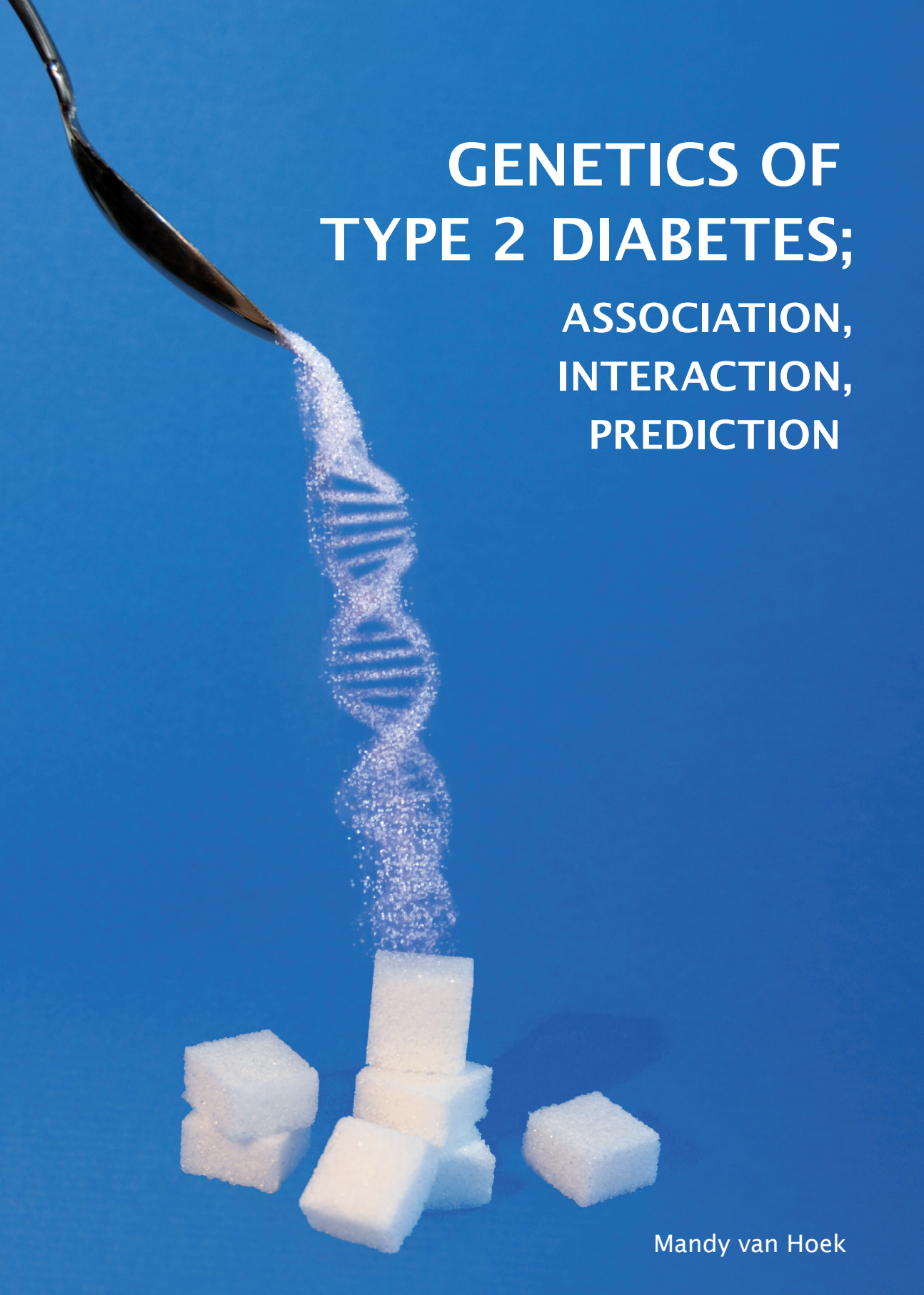

GENETICS OF TYPE 2 DIABETES;

ASSOCIATION, INTERACTION, PREDICTION

Mandy van Hoek

GENETICS OF TYPE 2 DIABETES; ASSOCIATION, INTERACTION, PREDICTION

Mandy van Hoek

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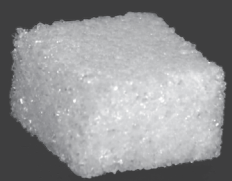
CONTENTS

PART I

INTRODUCTION TO THE THESIS

CHAPTER 1

GENERAL INTRODUCTION



1.1 TYPE 2 DIABETES

Type 2 Diabetes is a common chronic disease that results from an imbalance between the bodies insulin need and the production of insulin. It is a heterogeneous disorder in which the relative contributions of insulin resistance and defects in insulin secretion are highly variable between patients.[1,2]
Insulin is produced by beta cells located in the islets of Langerhans in the pancreas. It is a hormone with an extensive range of effects on metabolism. One of its main functions is to facilitate glucose uptake in various body tissues to allow glycogen storage. Besides a number of internal and external stimuli, the primary stimulus for insulin secretion are elevated glucose levels. When a relative lack of insulin remains untreated, this will eventually result in hyperglycaemia, i.e. diabetes.
Besides short term symptoms of hyperglycaemia, the main burden of type 2 diabetes are its long term complications; retinopathy, nephropathy, neuropathy and cardiovascular disease, which account for most of the morbidity and mortality of the disease.

Worldwide type 2 diabetes is a rapidly growing health care issue, with an estimated 171 million patients worldwide in 2000 and the expectancy that this number will be more than doubled by 2030.[3] Although many researchers in the past decennia have devoted their research to type 2 diabetes, much of the exact pathophysiology of the disease is still unknown.

1.2 RISK FACTORS

Type 2 diabetes is a typical multifactorial disease in which intricate interactions between environmental factors and genetics factors determine individuals' disease risks. Well known risk factors include age, obesity, lack of exercise and a positive family history for diabetes.[4] The latter indicates that type 2 diabetes has a strong genetic basis. This is supported by family- and twin studies.[5,6]

1.3 ETIOLOGICAL HYPOTHESES

Over the years, several hypotheses have arisen on genetic and environmental causes of type 2 diabetes. Two of these hypotheses have initiated a lot of research. These are the Barker hypothesis[7] and the 'thrifty genotype' hypothesis.[8]

The Barker hypothesis states that the fetal environment affects individuals' metabolic programming thereby influencing their risk of disease later in life. Individuals who suffer poor nutritional conditions *in utero* are hypothesized to be metabolically programmed to efficiently preserve and store nutrients. These adaptations are thought to have adverse effects later in life when the individual is exposed to an abundance of nutrition, as is the case in our modern western society. According to the hypothesis this may lead to increased risk of chronic conditions such as type 2 diabetes.
The 'thrifty gene' hypothesis encompasses a different view. It hypothesizes that our ancestors have been subjected to a process of natural selection. 'Thrifty genes' are genetic variants that allow efficient storage and use of nutrition in times of scarcity. This historic genetic advantage has now become a metabolic disadvantage in our modern age. This provides an explanation to why a detrimental disease such as type 2 diabetes has become common in our society.

1.4 GENETIC STUDIES IN TYPE 2 DIABETES

Unraveling the genetic basis of disease serves several purposes. First, it could reveal novel pathways involved in the disease pathophysiology. This would increase knowledge and could thereby lead us to new therapeutic targets. Second, it could bring us closer to personalized medicine, in which genetic profiles determine tailored preventive and therapeutic measures.
Type 2 diabetes is a polygenic disorder, which means that many genes are involved. Finding genetic variants involved in susceptibility to type 2 diabetes has been a major challenge. With increasing knowledge and improved technical possibilities, different approaches have become available.
Linkage studies have been successful in identifying genetic defects in monogenic disorders. However, in polygenic disorders such as type 2 diabetes success was limited. One important type 2 diabetes risk gene, *TCF7L2*, was found by means of fine-mapping of an established linkage signal.[9]
Candidate gene studies have been performed at a large scale. These studies rely on a prior hypothetical biological candidacy of a certain gene or pathway. These efforts have unfortunately revealed only few robustly replicated associations. The major findings found by this approach being the *PPARgamma, KCNJ11, WFS1* and *HNF1B* genes.[10-13]
Both linkage and candidate gene studies suffer from certain disadvantages for identifying genes in a polygenic disorder with expected small genetic effect sizes. Low prior odds of candidate genes and inadequate power are the main pitfalls of these approaches.

In recent years, genetic studies in common diseases have undergone a revolution in terms of knowledge of the genome (the HAPMAP project, the Human Genome project) and technical possibilities. This has eventually resulted in Genome Wide Association (GWA) studies. These studies provide the opportunity to test the association of thousands to millions of genetic markers without any prior hypothesis and to select the strongest signals based on ranking the results significance. With the availability of GWA studies, many new robustly associated type 2 diabetes genes were found.[14-20] These findings have offered new insights, pointing out the importance of beta-cell development and function in the pathophysiology of type 2 diabetes.

However, even with the current expanded list of robustly replicated genetic risk variants, the majority of the disease heritability remains unaccounted for. Amongst other explanations, gene-gene and gene-environment interactions may contribute to this missing heritability.

1.5 GENE-GENE AND GENE-ENVIRONMENT INTERACTIONS

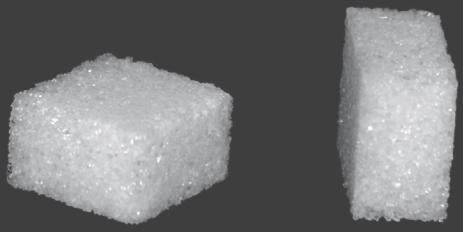
Most successful genetic studies have only been able to detect main effects of genetic variants. However, it is expected that part of the unexplained inherited risk could be attributable to gene-gene or gene-environment interactions, i.e. non-additive effects. So far, few empirical studies have been able to provide evidence for such effects. Lack of sufficient power, heterogeneity and lack of standardized measurement of environmental exposures in populations pose major difficulties for detecting and replication of such effects. Nonetheless, this could be a missing link needed for application of genetic information in clinical practice.

1.6 PRACTICAL APPLICATION OF TYPE 2 DIABETES GENETICS

One of the major promises of genetic knowledge is that it will lead to personalized medicine in which treatment and prevention could be tailored based on genetic risk. For monogenetic disorders this has been successful in the past. However, for complex diseases, such as type 2 diabetes, this still is a major challenge. As described above, during the past years there has been a great advance in the number of genes recognized to be involved. This has, however, not resulted in accurate prediction of disease risk. Nonetheless, many commercial companies offer predictive tests for complex disorders to the general public. Therefore, it is important to investigate the accuracy of prediction by genetic variants.

CHAPTER 2

AIMS AND SCOPE



2.0 AIMS AND SCOPE

The aim of present thesis is to investigate genetic causes of type 2 diabetes, their interactions with environmental factors, and their implications for treatment and prediction of the disease.

In part II (chapters 3-6), we investigate the associations and interactions of various genetic variants with environmental factors. In chapter three, this relates to *RBP4*, a retinol binding protein, and its interaction with dietary intake and plasma levels of vitamin A. In chapter 4, the effects of adiponectin polymorphisms on lipase activities and treatment effects of statins were investigated in patients with type 2 diabetes in the DALI study, a randomised placebo-controlled trial on the effects of statins in type 2 diabetes. In chapter 5, the intricate interactions between fetal environment and recently identified genetic risk factors were studied in a unique cohort exposed to fetal malnutrition, the Dutch famine study. In chapter 6, we investigate *APOC3*, a gene involved in lipid metabolism, and its association of genetic promoter variants with type 2 diabetes in lean versus obese subjects.

Part III (chapters 7 and 8) focuses on genetic prediction of type 2 diabetes. chapter 7 investigates the discriminative accuracy of a set of recently identified genetic risk variants and their value in addition to known clinical risk factors at a population-based level, in the Rotterdam Study. In chapter 8, the implications of updating commercial genetic tests with progressing genetic knowledge for individual risk prediction outcomes were studied.

Finally, in part IV, in chapter 9, I will discuss the main findings of this thesis, methodological issues related to our studies, implications of the findings for clinical practice and potential future research directions.

PART II

ASSOCIATION OF GENETIC VARIANTS WITH TYPE 2 DIABETES RISK AND INTERACTIONS WITH ENVIRONMENTAL FACTORS AND TREATMENT

CHAPTER 3

AN *RBP4* PROMOTER POLYMORPHISM INCREASES RISK OF TYPE 2 DIABETES

M. van Hoek,
A. Dehghan,
M.C. Zillikens,
A. Hofman,
J.C. Witteman,
E.J.G. Sijbrands.

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ABSTRACT

Background: Retinol Binding Protein 4 (RBP4), originally known for retinol transport, was recently identified as an adipokine affecting insulin resistance. The *RBP4 -803GA* promoter polymorphism influences binding of hepatic nuclear factor 1α and is associated with type 2 diabetes in case-control studies. We hypothesized that the *RBP4 -803GA* polymorphism increases type 2 diabetes risk at a population-based level. In addition, information on retinol intake and plasma vitamin A levels enabled us to explore the possible underlying mechanism.

Methods: In the Rotterdam study, a prospective, population-based, follow-up study, the *-803GA* polymorphism was genotyped. In Cox proportional hazards models, associations of the *-803GA* polymorphism and retinol intake with type 2 diabetes risk were examined. Moreover, the interaction of the polymorphism with retinol intake on type 2 diabetes risk was assessed. In a subgroup of participants the association of the polymorphism and vitamin A plasma levels was investigated.

Results: Homozygous carriers of the *-803A* allele had increased risk of type 2 diabetes (HR 1.83; 95%CI 1.26-2.66). Retinol intake was not associated with type 2 diabetes risk and showed no interaction with the *RBP4 -803GA* polymorphism. Furthermore, there was no significant association of the polymorphism with plasma vitamin A levels.

Conclusions: Our results provide evidence that homozygosity for the *RBP4 -803A* allele is associated with increased risk of type 2 diabetes in the Rotterdam population. This relationship was not clearly explained by retinol intake and vitamin A plasma levels. Therefore, we cannot differentiate between a retinol dependent or -independent mechanism of this *RBP4* variant.

INTRODUCTION

Insulin resistance and beta cell failure are major components of the pathogenesis of type 2 diabetes.[21] In the past years, it has become apparent that adipose tissue is an endocrine organ that secretes adipokines affecting insulin sensitivity.[22] Recently, retinol binding protein 4 (RBP4) was identified as a new adipokine that links glucose uptake in adipocytes to systemic insulin resistance.[23]

Originally, RBP4 was known as the only transport protein for retinol[24], but Yang et al. demonstrated a new function, by showing that adipose tissue-specific *Glut4* (also known as *SLC2a4*) knockout mice have increased serum levels of RBP4.[23] Downregulation of GLUT4 in adipose tissue is an important feature of insulin resistance.[25] RBP4 may be an important mechanistic link between downregulated GLUT4 in adipose tissue and systemic insulin resistance. This was confirmed in humans as well: RBP4 levels and the level of insulin resistance were correlated in people with obesity and impaired glucose tolerance, and patients with type 2 diabetes.[26] Moreover, RBP4 correlated with the level of insulin resistance in normoglycaemic men with a positive family history of type 2 diabetes, suggesting an underlying genetic predisposition.[26]

In a Mongolian case-control study, four single nucleotide polymorphisms in the *RBP4* gene were associated with increased risk of type 2 diabetes.[27] The *–803GA* polymorphism, located near an hepatic nuclear factor 1α (HNF1α)-binding motif, affects serum RBP4 levels and influences transcription efficiency and binding of HNF1α.[27] So far, the relationship between genetic variants in the *RBP4* gene and type 2 diabetes risk has not been studied prospectively.

The mechanisms by which RBP4 affects insulin sensitivity are largely unknown. Yang et al. showed that RBP4 impaired muscle insulin signaling and increased the expression of phosphoenolpyruvate carboxykinase (PEPCK) in mouse liver.[23] Whether these effects are based on retinol-dependent or -independent mechanisms is unclear.

The findings so far suggest that variation in the *RBP4* gene is involved in the pathogenesis of type 2 diabetes and that *RBP4* is a candidate gene for type 2 diabetes susceptibility. In a large prospective population-based study, we investigated the effect of the *RBP4 –803GA* polymorphism on type 2 diabetes risk. In addition, we assessed the association of retinol intake with risk of type 2 diabetes and its interaction with the *RBP4* polymorphism. Finally, we analysed the effect of the *RBP4* polymorphism on plasma vitamin A levels.

MATERIALS AND METHODS

STUDY POPULATION

Details of the Rotterdam Study have been described previously.[28] In short, the Rotterdam Study is an ongoing prospective, population-based, cohort study in 7983 inhabitants of a suburb in Rotterdam, designed to investigate determinants of chronic diseases in the elderly. Participants were aged 55 years or older. Baseline examinations were performed from 1990 until 1993. Follow-up examinations took place in 1993-1994, 1997-1999 and 2002-2004. Continuous surveillance on major disease outcomes was conducted between these exams. Information on vital status was derived from municipal health authorities. The medical ethics committee of the Erasmus Medical Center approved the study protocol and all participants gave their written informed consent.

DIABETES

In accordance with the guidelines of the World Health Organization[29] and the American Diabetes Association[30], diabetes was diagnosed at fasting plasma glucose levels ≥ 7.0mmol/l and/or a non-fasting plasma glucose levels ≥ 11.0mmol/l and/or treatment with anti-diabetic medication (oral medication or insulin) and/or a diagnosis of diabetes as registered by a general practitioner. At baseline, prevalent cases of diabetes were diagnosed by a non-fasting or post-load glucose level (after OGTT) ≥ 11.1mmol/l and/or treatment with anti-diabetic medication (oral medication or insulin) and the diagnosis diabetes as registered by a general practitioner.
In the current study, patients diagnosed with type 1 diabetes according to the general practitioners' records were excluded. For the present study, baseline data were collected between 1990 and 1993.

GENOTYPING

DNA material was available for genotyping 6,571 participants. The *-803GA* polymorphism (rs3758539) in the *RBP4* gene was genotyped by means of Taqman allelic discrimination assays. The assay was designed and optimised by Applied Biosystems, (Foster City, CA, USA; http://store.appliedbiosystems.com). We genotyped 90 blood bank samples to test for adequate cloud separation. In the Rotterdam study samples, 325 samples were genotyped *in duplo*, of which one gave an inconsistent result. To monitor contamination, 650 blanc samples were incorporated on the plates, of which all gave a blanc result. Reactions were performed on the Taqman Prism 7900HT platform. Genotyping was successful in 6,366 participants.

ASSESSMENT OF DIETARY INTAKE AND PLASMA LEVELS OF VITAMIN A

Dietary intake was assessed by means of an extensive, validated Semiquantitative Food-Frequency Questionnaire (SFFQ).[31] A trained dietician interviewed the participants. The food and drink intake from the SFFQ were converted to energy and nutrient intake according to the Dutch Food Composition Table.[32] In the current study, we used data on dietary intake of α-carotene(µg/day) and β-carotene(µg/day), β-cryptoxanthin(µg/day) and total energy (kcal/day). Alpha-carotene, β-carotene and β-cryptoxanthin were converted to retinol equivalents (RE; amount of RE = [µg retinol] + [µg β-carotene/6] + [µg α-carotene/12] + [µg β-cryptoxanthin/12] per day). Data on retinol intake were available for 5,642 participants.

Plasma levels of vitamin A were measured in a subgroup of 395 genotyped participants. At the second follow-up examination, blood samples were drawn after an overnight fast. Citrate plasma was immediately frozen and stored at -80°C. Total vitamin A plasma levels (retinol) were determined by a previously described method.[33] Briefly, reversed-phase HPLC with UV detection was performed using an RP C18 Column 100 x 4.6mm (Merck Lichrospher 100RP-18e; Merck, Darmstadt, Germany). Vitamin A was detected at 324nm. The inter-assay CV was 4.0%.

STATISTICAL METHODS

Analyses were performed with SPSS software version 12.0.1. Continuous variables are expressed as means ± SEM. Comparisons between groups were performed with ANOVA and a χ^2-testing for normally distributed continuous and categorical variables, respectively. Deviation from Hardy Weinberg equilibrium was assessed by means of χ^2 testing.

We tested the association of the *RBP -803GA* polymorphism, RE intake and their interaction with type 2 diabetes risk in Cox proportional hazards models. Participants with prevalent type 2 diabetes at baseline were excluded from the analyses, since they may contain selection biases such as survival bias. For the polymorphism the additive, dominant or recessive model was chosen based on the genotypic test and the best-estimated log-likelihood statistic in univariate Cox proportional hazards regression. For the interaction analysis an interaction term was created by entering the product of the *-803GA* polymorphism and retinol intake to a model with both independent variables. All models were adjusted for year of birth and sex. Additional models were adjusted for BMI.

RE intake was adjusted for energy intake by saving the standardized residuals of a linear regression model with RE intake as dependent variable and total energy intake as independent variable. These standardized residuals represent the remaining variation in RE intake after correcting for energy intake. These standardized residuals were entered as an independent variable in subsequent models.

RESULTS

BASELINE CHARACTERISTICS

In 6320 successfully genotyped persons, diabetes status was available. Of these, 658 had prevalent diabetes at baseline. Individuals whose DNA was not available or in whom genotyping did not succeed were 5.8 years older and contained 8.5% more women than the successfully genotyped group. This is explained by the fact that mostly elderly women in nursing homes did not provide DNA material for the study at baseline. Still, the genotyped and non-genotyped groups had similar distributions of BMI, waist circumference and presence of type 2 diabetes.

Baseline characteristics are shown in Table 1. Incident cases with type 2 diabetes were significantly younger, had higher BMI and waist circumference, lower HDL cholesterol and more often hypertension than individuals without type 2 diabetes. As expected, prevalent cases were significantly older at baseline compared to incident cases (73.5 ± 0.35 vs 68.1 ± 0.32, p<0.001) and people without diabetes (73.5 ± 0.35 vs 69.0 ± 0.13, p<0.001). They were excluded from further analyses.

The polymorphism was in Hardy-Weinberg equilibrium in the total population and inindividuals without type 2 diabetes. (χ^2 <1.02 df=2, p>0.33). In the total population, we found 27.8% heterozygotes for the *–803GA* polymorphism, while 2.8% were homozygous for the *A* allele. These percentages were 26.1 and 5.0% and 28.1% and 2.6% in individuals with and without incident type 2 diabetes, respectively; *-803AA* 5.0% vs 2.6% for incident cases vs individuals without type 2 diabetes, p=0.01.

Table 1. Baseline characteristics of all genotyped participants, participants without diabetes and incident cases of type 2 diabetes.

Characteristics	All participants	Individuals without type 2 diabetes	Incident cases with type 2 diabetes	P-value[a]
	n=6320	n=5080	n=582	
Age (years)	69.3 ± 0.11	69.0 ± 0.13	68.1 ± 0.32	0.02
Men (%)	40.6	40.5	44.0	0.10
Body Mass Index (kg/m^2)	26.3 ± 0.05	26.0 ± 0.05	28.0 ± 0.16	<0.001
Waist circumference (cm)	90.5 ± 0.15	89.6 ± 0.16	94.7 ± 0.46	<0.001
Systolic blood pressure (mmHg)	139.4 ± 0.3	138.0 ± 0.3	143.5 ± 0.9	<0.001
Diastolic blood pressure (mmHg)	73.8 ± 0.2	73.7 ± 0.2	75.5 ± 0.5	<0.001
Hypertension (%)	33.6	30.7	46.7	<0.001
Total cholesterol (mmol/l)	6.6 ± 0.02	6.6 ± 0.02	6.6 ± 0.05	0.86
HDL-cholesterol (mmol/l)	1.34 ± 0.005	1.37 ± 0.005	1.25 ± 0.01	<0.001
Current smoking (%)	22.2	22.5	25.5	
Former Smoking (%)	40.8	42.2	42.1	0.18

[a]p-value for comparison between incident cases and individuals without diabetes
Continuous data are expressed as means ± SEM.

The polymorphism was not associated with BMI, total cholesterol or HDL-cholesterol (data not shown).

COX PROPORTIONAL HAZARD RATIOS FOR TYPE 2 DIABETES OF THE *-803GA* POLYMORPHISM.

Based to the -log likelihood values and genotype frequencies in individuals with and without type 2 diabetes, the recessive model of inheritance was the best-fitting model. Homozygosity for the *A* allele was associated with a HR of 1.83 (95% CI 1.26-2.66, p=0.001) for type 2 diabetes compared with the reference group (*-803GG/GA*) adjusted for year of birth and sex. Figure 1 shows the effect of the *RBP4 -803GA* genotypes on type 2 diabetes-free survival in our population. The association remained similar after additional adjustment for BMI (*-803AA* HR 1.81; 95% CI 1.25-2.64, p=0.002; Table 2).

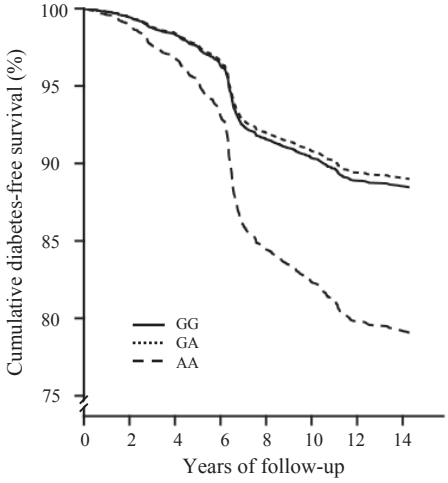

Figure 1. Diabetes-free survival according to *RBP4 -803GA* genotypes.

Table 2. HRs for type 2 diabetes according to *RBP4 -803GA* genotype

Genotype	HR1[a]	95% CI	p-value	HR2[b]	95% CI	p-value
-803GG/GA	1.0			1.0		
-803AA	1.83	1.26 -2.66	0.001	1.81	1.25 -2.64	0.002

[a]Adjusted for year of birth and sex
[b]Adjusted for year of birth, sex and BMI

RE INTAKE, VITAMIN A PLASMA LEVELS, THE *RBP4* POLYMORPHISM AND RISK OF TYPE 2 DIABETES

RE intake corrected for energy intake was not associated with risk of type 2 diabetes (HR=1.0; 95% CI 1.0-1.0, p=0.16) and did not interact with the *RBP4 -803GA* polymorphism on type 2 diabetes risk (HR=1.0; 95% CI 0.99-1.00, p for interaction=0.35). Vitamin A plasma level measurements were available in a subgroup of 395 genotyped participants. We did not observe significant differences between individuals with and without vitamin A plasma measurements with regards to BMI, waist circumference, sex and type 2 diabetes status. Individuals with measured vitamin A plasma levels were on average 4.3 years younger than those without vitamin A plasma measurements (65.4 ± 0.34 vs 69.7 ± 0.12, p<0.001), which is explained by the fact that they survived and participated until the second follow-up measurement. In this subgroup, the *RBP4* polymorphism was not significantly associated with vitamin A plasma levels. (Table 3)

Table 3. Plasma vitamin A levels according to *RBP4 -803GA* genotype

-803GA	n	Vitamin A (µmol/l)
GG	256	1.61 ± 0.02
GA	129	1.59 ± 0.03
AA	10	1.64 ± 0.09

DISCUSSION

RBP4 is a recently discovered adipokine that is thought to link adipocyte glucose metabolism to systemic insulin resistance.[23] In the present study, we demonstrated that homozygosity for a promoter variant in the *RBP4* gene was associated with increased risk of type 2 diabetes at a population-based level. Vitamin A intake was not associated with type 2 diabetes and did not influence the relationship of the polymorphic variant with type 2 diabetes risk. In a subgroup analysis, the *RBP4* polymorphism was not significantly associated with vitamin A plasma levels.

Munkhtula et al. were the first to describe the relationship between *RBP4* genetic variants and type 2 diabetes risk.[27] Genotype frequencies for the *-803GA* polymorphism in this Mongolian population were approximately equal to the genotype frequencies in our white population. Similarly to our results, the *-803A* allele was associated with increased risk of type 2 diabetes. However, a dominant mode of inheritance was found as opposed to our finding of a recessive pattern. The difference in race and differences in exposure to environmental factors between the populations may explain the discrepancy. In two separate studies, white carriers of an *RBP4* haplo-

type had a significantly increased risk of type 2 diabetes.[34,35] However, the individual *RBP4* polymorphisms were not related to risk of type 2 diabetes. In both studies, the haplotype associated with increased risk contained the -803G allele. This seems to contradict our findings, but the associated haplotype was not determined by the -803GA variant. The majority of the haplotypes found (four out of five) contained the -803G allele and were not associated with an increased risk, which makes it unlikely that the G allele contributed to the observed effect. The relatively small sample size of the study by Craig et al.[34] and the presence of considerably younger controls than cases in the study by Kovacs et al.[35] might have hidden the effect of the individual polymorphism. Hence, the findings in our large follow-up study do not necessarily contradict the two previous case-control studies: multiple *RBP4* variants may affect type 2 diabetes risk, including variants captured by the haplotypes described in the other references[34,35], or by the -803GA polymorphism itself.

Since RBP4 is the only known retinol transport protein, a retinol-dependent mechanism underlying its effect on type 2 diabetes risk seems a reasonable assumption. Within tissues, retinol is activated to retinoic acid isomers, which have a wide array of pleiotropic effects through interaction with retinoid acid X receptors (RXRs) and retinoic acid receptors[36], regulating transcription of over 300 target genes.[37] Consistent with a retinol-dependent mechanism Yang et al found that *RBP4* increased *PEPCK* expression in mice liver and cultured hepatocytes.[23] PEPCK is a gluconeogenic enzyme and retinoids regulate its transcription.[38] All retinoids in the body originate from the diet.[39,40] We did not find an association of retinol intake with type 2 diabetes risk. Conflicting results concerning this relationship have been published.[41-45] We did not observe interaction between retinol intake and the *RBP4* polymorphism on type 2 diabetes risk, nor did we find an association of the polymorphism with vitamin A plasma levels. However, since the effect on type 2 diabetes risk was confined to the *-803A* homozygotes and the analyses with vitamin A plasma levels contained only a small number of *-803A* homozygotes (n=10), these results should be interpreted with caution. Compared with the other genotypes the *-803A* homozygotes had a slightly higher mean plasma vitamin A level, although not significant. Therefore we cannot exclude the possibility that a retinol-dependent mechanism underlies the effect on type 2 diabetes risk. Moreover, the relationship between metabolites of retinol and insulin sensitivity may be complex, as some retinoic acids are thought to cause insulin resistance[46,47], whereas others seem protective through RXR-PPARγ activated pathways.[48] Based on our results we cannot exclude that RBP4 alters the amount of specific retinoic acid isomers locally or systemically. However, a number of retinol-independent mechanisms may operate: RBP4 may increase type 2 diabetes risk by binding of cell surface receptors[49,50], such as megalin and Stra6. Recently, it was shown that RBP4 can directly affect insulin signaling by blocking

the insulin-stimulated phosphorylation of IRS-1.[51] Furthermore, RBP4 may modulate transthyretin function. Transthyretin was recently shown to be involved in beta cell stimulus-secretion coupling.[52,53]

The relationship between genetic variation in the *RBP4* gene and type 2 diabetes had not previously been investigated prospectively at a population-based level. We identified all incident cases of type 2 diabetes in this large population-based study during a long period of follow-up. The availability of data on retinol intake in all individuals and vitamin A plasma levels in a subgroup allowed us to investigate potential associations and interactions between *RBP4* genetic variation and these parameters. To the best of our knowledge, this is the first study to examine this interaction.

In a previous study, the *-803GA* polymorphism was identified as a functional variant that affects HNF1α binding, *RBP4* transcription efficiency and RBP4 plasma levels. Unfortunately, RBP4 plasma levels in the Rotterdam Study are not available because of limitations in sample availability and lack of a readily available and reliable method to measure these levels in large populations and people with insulin resistance.[54] Kovacs et al. did not find an effect of the -803GA polymorphism on serum RBP4 levels in a case-control study.[35] Future studies are needed to obtain insight in the relationship between *RBP4* serum levels and the genetic variation in the RBP4 gene.

In conclusion, we have shown prospectively that a promoter polymorphism in the *RBP4* gene is associated with increased risk of type 2 diabetes in the Rotterdam population. Along with previous functional data our finding increases the confidence that the promoter polymorphism is a causal variant. Dietary intake of retinol did not influence type 2 diabetes risk or the association of the *RBP4* polymorphism with type 2 diabetes risk. Moreover, the *RBP4* polymorphism was not significantly associated with circulating vitamin A levels. However, we could not exclude a retinol-dependent mechanism underlying the association with type 2 diabetes.

CHAPTER 4

ADIPONECTIN POLYMORPHISMS AND PLASMA LEVELS INFLUENCE LIPASE ACTIVITIES AND THE HDL-CHOLESTEROL RESPONSE TO STATIN THERAPY IN PATIENTS WITH TYPE 2 DIABETES.

M. van Hoek,
A. van Tol,
L.C. van Vark-van der Zee,
H. Jansen,
J.J. Kastelein,
E.J.G. Sijbrands,
G.M. Dallinga-Thie.

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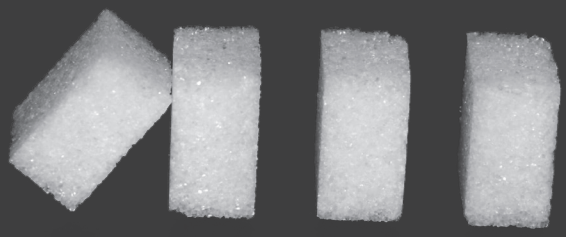

ABSTRACT

Background: In addition to its effects on insulin sensitivity, adiponectin is thought to play an important role in lipid homeostasis. However, the underlying mechanisms are unclear. We investigated the associations between adiponectin polymorphisms, adiponectin plasma levels, lipase activities and lipids. Furthermore, we assessed the effect lipid lowering therapy on these relationships.

Methods: In a placebo-controlled trial on the effects of atorvastatin in patients with type 2 diabetes, the associations between adiponectin polymorphisms *(-13391GA, 45TG)*, plasma adiponectin, and lipoprotein lipase (LPL) and hepatic lipase (HL) activities were studied at baseline and after 6 months of treatment with atorvastatin.

Results: The adiponectin *-11391A* allele associated with higher LPL activity (GGvsGA; 139 ± 37 vs 157 ± 44, p=0.03) and the *45TG* polymorphism was associated with HL activity (TTvsTG/GG; 418 ± 153 vs 356 ± 134, p=0.04). Baseline adiponectin associated positively with LPL activity (p=0.01) and HDL-cholesterol (p<0.001) and negatively with triglyceride levels (p=0.002). Atorvastatin treatment did not influence adiponectin levels. However, baseline adiponectin levels significantly interacted with atorvastatin treatment on HDL-cholesterol response (p=0.007). Patients in the highest adiponectin tertile had the largest increase of HDL-cholesterol (8.2-9.6%), while the increase in the lowest tertile was negligible (0.7-3.3%).

Conclusion: We conclude that adiponectin levels, which are partly explained by genetic variation in the adiponectin gene, associate with LPL activity. Statin therapy did not alter adiponectin levels, but baseline adiponectin levels determined the HDL-cholesterol raising effect of atorvastatin.

INTRODUCTION

Cardiovascular complications are a major burden of type 2 diabetes.[55,56] An unfavourable lipid profile characterized by raised triglyceride levels and low HDL-cholesterol contributes to increased risk of cardiovascular disease in patients with type 2 diabetes.[57]

High adiponectin levels have been associated with a decrease in cardiovascular disease risk in the general population[58] and in patients with type 2 diabetes.[59] This effect may be modulated through effects on lipid metabolism.[60] Adiponectin is associated with increased HDL-cholesterol and decreased triglyceride levels. These associations are independent of body mass index (BMI) and insulin sensitivity.[61,62]

A number of adiponectin polymorphisms have been linked to dyslipidemia[63] and cardiovascular disease risk.[64] It is still unclear whether there is a causal link between adiponectin and dyslipidemia, because the mechanisms mediating this relation are largely unknown.

Adiponectin levels have been associated with lipoprotein lipase(LPL)[65] and hepatic lipase(HL)[66-68] activities. LPL and HL are important enzymes in HDL-cholesterol and triglyceride metabolism.[69,70] The causality, i.e. the direction of the relations between adiponectin and lipase activities, is unclear.

Statins are indicated to prevent cardiovascular disease in patients with type 2 diabetes. Statins reduce LDL-cholesterol and serum triglycerides, but raise HDL-cholesterol levels only to a limited extent.[71] Studies on the effect of statin treatment on adiponectin plasma levels yielded conflicting results[72-75] and the only study in patients with type 2 diabetes was performed on top off rosiglitazone treatment.[72]

We hypothesized that adiponectin affects LPL and HL activity in patients with type 2 diabetes. To clarify the relation between these parameters, we investigated the associations of adiponectin gene polymorphisms with adiponectin plasma levels and lipase activities. Secondly, we assessed the effect of a statin treatment on adiponectin plasma levels. Thirdly, we investigated the interaction between plasma adiponectin and the response to statin treatment.

METHODS

SUBJECTS

The 'Diabetes Atorvastatin Lipid Intervention'(DALI) study is a randomized, double-blind, placebo-controlled trial on the effects of atorvastatin 10mg and 80mg daily in patients with type 2 diabetes. This study has previously been described in detail.[71] In brief, men and women aged 45-75 years, with a known duration of type 2 diabetes

of at least 1 year and a mild dyslipidemia (total cholesterol level between 4.0 and 8.0 mmol/l and fasting triglyceride level between 1.5 and 6.0mmol/l) were included. Type 2 diabetes was defined in accordance with the American Diabetes Association guidelines published in 2000.[76] Patients with an HbA_{1c} above 10% or a history of cardiovascular disease (myocardial infarction, percutaneous transluminal coronary angioplasty, coronary artery bypass grafting, proven manifest coronary artery disease, severe or unstable angina pectoris, clinically manifest heart failure or cardiac arrhythmia) were excluded.
When applicable, lipid-lowering therapy was withdrawn 6 weeks before start of the 2-week run-in phase. Finally, a total of 217 patients were randomized to placebo, atorvastatin 10mg or atorvastatin 80mg daily for a treatment period of 30 weeks.
The medical ethical committees of the participating institutions (University Medical Centres of Leiden, Rotterdam and Utrecht) approved the study protocol and all participants gave their written informed consent.

LABORATORY MEASUREMENTS

At baseline and after 30 weeks of treatment, blood was collected in EDTA tubes after an overnight fast of at least 12h. Plasma was centrifuged immediately (1,800g 15min 4°C) and was stored at -80°C until further analyses. Total cholesterol, triglycerides, HDL-Cholesterol and LDL cholesterol were measured as described previously.[71] CETP mass was measured using a sandwich ELISA.[77] Total adiponectin levels were measured with the Human Adiponectin/ACRP30 Quantikine ELISA kit (R&D systems inc) according to the manufacturer's instructions. Adiponectin levels are expressed as µg/ml. Adiponectin measurements succeeded in a total of 194 patients. They were included in all analyses in this paper.

POST HEPARIN LPL AND HL ACTIVITIES

LPL and HL activities were determined in fasting plasma as described previously.[78] In brief, 20 minutes after intravenous injection of heparin (50 IU/kg body weight), blood was obtained from the other arm in heparinized tubes. This was transported on wet ice to the laboratory. Plasma was immediately separated by centrifugation for 10 minutes, 3000 rpm and stored at -80°C till further analyses. All activity assays used gum acacia-stabilized [^{3}H]trioleylglycerol as a substrate. For determination of LPL activity, HL activity was inhibited by co-incubation with a goat antibody to human HL. HL activity was measured in the presence of 1M NaCl, to totally inhibit LPL activity. Fatty acids were extracted as calcium salts and counted in a liquid scintillation counter. All activities are expressed as U/ml. One U equals 1nmol FFA/ml/h.

GENOTYPING

The adiponectin *-11391GA* and *45TG* polymorphisms were chosen for genotyping based on earlier associations in literature with adiponectin plasma levels.[79] Genotyping was performed by means of Taqman allelic discrimication assays by design. The assays were designed and optimalised by Applied Biosystems (Foster City, California, http://store.appliedbiosystems.com). Reactions were performed on the Taqman prism 7900HT platform, according to the manufacturer's instructions.

STATISTICAL ANALYSES

SPSS version 12.0.1 was used for all analyses. Data are expressed as means ± standard error (SE). Logarithmic transformation was performed on variables that were not normally distributed (i.e. triglycerides). Comparisons between groups at baseline were performed with ANOVA for continuous variables and by χ^2 testing for categorical variables. Hardy-Weinberg equilibrium was assessed by χ^2 testing.

Bivariate correlations were calculated by Spearman's regression analyses. To correct for covariates we constructed linear regression models with adiponectin as an independent variable and baseline LPL activity, HL activity, HDL cholesterol and log-transformed triglycerides as dependent variables. Age, gender and waist-to-hip ratio (WHR) were included as covariates in all models. WHR was included based significant differences between adiponectin tertiles. Baseline log-transformed triglycerides were included in the model for HDL cholesterol. In this model additional adjustment for cholesterol ester transfer protein (CETP) mass was performed.

The study population was divided into adiponectin tertiles to study the interaction between adiponectin levels at baseline and treatment with atorvastatin. The first tertile (tertile 1) included adiponectin levels from 0 to 5.67µg/ml. Tertile 2 included adiponectin levels from 5.67 to 8.77µg/ml and tertile 3 levels from 8.77µg/ml till the maximum value. Because men and women at baseline showed the same statistically significant differences between adiponectin tertiles they were analysed simultaneously in all further analyses.

We constructed a Univariate General Linear Model with triglyceride and HDL-cholesterol levels after 30 weeks of treatment as dependent variables. Atorvastatin treatment was coded as 0 (placebo) or 1 (ator10 or ator80) and was included in the model as a fixed factor together with adiponectin tertile. Treatment dose, baseline triglyceride levels and baseline HDL-cholesterol levels were included as co-variables. In additional models, we included WHR, LPL and HL activities and adiponectin genotypes.

RESULTS

BASELINE CHARACTERISTICS

Table 1 shows baseline characteristics of the patients according to plasma adiponectin level. Baseline characteristics of the study population according to randomized treatment group have been described in detail elsewhere.[71]

The percentage of men in the lowest adiponectin tertile was significantly higher compared to the second and third tertiles. The WHR was significantly higher in patients in the lowest tertile compared to the other tertiles. Furthermore, patients with low adiponectin levels had significantly higher triglyceride levels, HL activity and lower levels of total cholesterol, HDL-cholesterol, LDL cholesterol and a borderline significant lower level of LPL activity than patients with high adiponectin levels. Analyzing these parameters separately for men and women yielded similar statistically significant results (data not shown).

Table 1. Baseline characteristics of the total study population and of adiponectin tertiles.

		Adiponectin			
	All participants	Tertile 1	Tertile 2	Tertile 3	p-value
N	194	65	66	63	
Male %	55.2	73.8	56.1	34.9	<0.001
Age (years)	59.8 ± 0.6	59.0 ± 0.9	60.8 ± 1.0	59.7 ± 0.9	NS
BMI	30.9 ± .34	31.0 ± 0.6	30.7 ± 0.6	31.0 ± 0.7	NS
WHR	0.998 ± 0.01	1.02 ± 0.01	0.99 ± 0.01	0.98 ± 0.01	0.05
HbA1c	8.7 ± 0.1	8.7 ± 0.2	8.7 ± 0.2	8.7 ± 0.2	NS
Adiponectin	7.8 ± 0.3	4.1 ± 0.1	7.1 ± 0.1	12.3 ± 0.4	<0.001
Cholesterol	6.0 ± 0.1	5.7 ± 0.1	6.1 ± 0.1	6.2 ± 0.1	0.02
Triglycerides	2.8 ± 0.1	3.1 ± 0.1	2.8 ± 0.1	2.5 ± 0.1	0.007
HDL-cholesterol	1.05 ± 0.18	0.93 ± 0.02	1.06 ± 0.03	1.14 ± 0.03	<0.001
LDL-cholesterol	3.7 ± 0.1	3.4 ± 0.9	3.7 ± 0.1	3.9 ± 0.1	0.01
LPL activity	142 ± 3	132 ± 5	144 ± 5	149 ± 5	0.05
HL activity	406 ± 11	426 ± 19	429 ± 21	362 ± 17	0.03

ADIPONECTIN GENOTYPES AND LIPASE ACTIVITIES

Table 2 shows the effects of two adiponectin polymorphisms on adiponectin levels, lipid profile and lipase activities at baseline. Both polymorphisms were in Hardy-Weinberg equilibrium. The *-11391A* allele was present in 12.7% of patients. There were no homozygous carriers of this polymorphism. For the *45G* allele there were 17.1% heterozygous and 1.9% homozygous carriers.

The *-11391A* allele was associated with higher adiponectin (GGvsGA; 7.6 ± 3.9 vs 9.4 ± 4.5, p=0.03) and LPL activity levels (GGvsGA; 139 ± 37 vs 157 ± 44, p=0.02).

Table 2. Adiponectin levels and lipid parameters and lipase activities according to adiponectin genotypes.

Polymorphism	N	Genotype	adiponectin	LPL activity	HL activity	Triglycerides	HDL cholesterol**
-11391GA	155	GG	7.6 ± 0.3	139 ± 3	411 ± 13	2.8 ± 0.1	1.05 ± 0.02
	26	GA	9.4 ± 0.9	157 ± 9	397 ± 31	2.9 ± 0.2	1.05 ± 0.04
		p-value*	0.03	0.02	0.64	0.87	0.6
45TG	153	TT	7.9 ± 0.3	140 ± 3	418 ± 13	2.8 ± 0.1	1.04 ± 0.02
	34	TG/GG	7.3 ± 0.6	147 ± 6	356 ± 23	2.9 ± 0.2	1.05 ± 0.04
		p-value*	0.33	0.71	0.04	0.6	0.9

* All p-value after adjustment for age, gender and WHR-ratio.
** additional adjustment for log-tranformed triglyceride levels

The *45G* allele was not associated with adiponectin levels, but did show a significant association with decreased HL activity levels (TT vs TG/GG; 418 ± 153 vs 356 ± 134, p=0.04). These associations were similar in analyses with and without adjusment for age, sex and WHR-ratio.

CORRELATION AND REGRESSION ANALYSES OF ADIPONECTIN AND LPL AND HL ACTIVITIES

There was a significant positive correlation between adiponectin and LPL activity levels (spearman r=0.19 p=0.012) and a significant negative correlation between adiponectin and HL activity levels (spearman r=-0.17 p=0.022). Furthermore, adiponectin correlated significantly with HDL-cholesterol (spearman r= 0.46, p<0.001) and triglyceride levels (spearman r=-0.24, p=0.002).

In table 3 the results of multiple linear regression analyses are shown. After adjustment for covariates adiponectin significantly influenced LPL activity (p=0.01), HDL-cholesterol (p<0.001) and triglycerides (p=0.002). The relation between adiponectin levels and HL activity was borderline significant (p=0.09).

Table 3. Multiple linear regression analyses of adiponectin effects on LPL and HL activity, HDL cholesterol and triglycerides.

	LPL activity		HL activity		HDL cholesterol		Triglycerides*	
Variable	B	t	B	t	B	t	B	t
Age	0.052	0.68	-0.094	-1.276	0.003	0.042	-0.194‡	-2.773‡
Gender	-0.042	-0.495	0.189†	2.333†	-0.134†	-2.01†	-0.141	-1.805
WHR	0.121	1.517	0.08	1.028	0.013	0.208	0.099	1.322
Triglycerides*					-0.432§	-7.002§		
Adiponectin	0.206†	2.595†	-0.129	-1.705	0.283§	4.491§	-0.225‡	-3.099‡

* log-transformed variable, †p<0.05, ‡p<0.01, §p<0.001

After additional adjustment for LPL and HL activities the relation between between adiponectin and triglyceride levels was not significant (p=0.14), but the relation with HDL cholesterol remained highly significant.(p=0.002). Additional adjustment for CETP mass did not change this result. (data not shown)

INTERACTION BETWEEN ADIPONECTIN LEVELS AT BASELINE AND STATIN TREATMENT.

There was no significant difference in the change of adiponectin levels in placebo, atorvastatin 10mg and atorvastatin 80mg treatment groups after 30 weeks of treatment.

Table 4 and figure 1 show the interaction of adiponectin tertiles with statin treatment. For triglyceride levels there was no significant interaction between atorvastatin and adiponectin tertiles on treatment effect. The HDL-cholesterol response was modulated significantly by adiponectin tertile at baseline. This resulted in higher increases of HDL-cholesterol in response to statin treatment with increasing adiponectin tertiles. (p for interaction =0.007). Consecutive adjustment for LPL and HL activities, adiponectin polymorphisms, WHR and change in triglyceride levels did not change this result (with p for interaction ranging from 0.005-0.009).

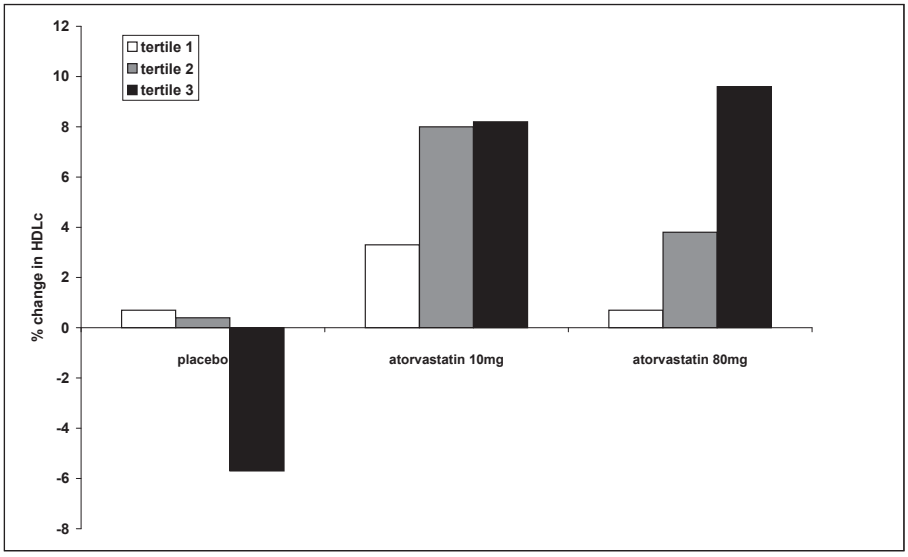

Figure 1. The effect of baseline adiponectin tertiles on the percentage of HDL cholesterol change during atorvastatin treatment

Table 4. Interaction between adiponectin tertile and treatment group on triglyceride and HDL-cholesterol treatment effects.

		Triglycerides				HDL cholesterol			
Treatment	Adiponectin tertile	Baseline	30 weeks	% Change	P for interaction	Baseline	30 weeks	% Change	P for interaction
Placebo	1	3.3 ± 0.3	3.7 ± 0.6	23.8		0.95 ± 0.04	0.98 ± 0.05	0.7	
	2	2.6 ± 0.2	2.5 ± 0.2	2.8		1.01 ± 0.03	1.01 ± 0.04	0.4	
	3	2.6 ± 0.2	2.4 ± 0.3	-3.5		1.21 ± 0.05	1.15 ± 0.06	-5.7	
Atorvastatin	1	2.8 ± 0.2	2.0 ± 0.2	-23.5		0.96 ± 0.04	0.99 ± 0.04	3.3	
10mg	2	2.8 ± 0.2	1.8 ± 0.1	-25.4		1.09 ± 0.07	1.17 ± 0.08	8.0	
	3	2.4 ± 0.1	1.6 ± 0.2	-24.5		1.09 ± 0.04	1.18 ± 0.05	8.2	
Atorvastatin	1	3.3 ± 0.2	2.1 ± 0.2	-24.3		0.9 ± 0.03	0.89 ± 0.03	0.7	
80mg	2	2.9 ± 0.3	1.4 ± 0.1	-42.8		1.1 ± 0.06	1.13 ± 0.05	3.8	
	3	2.6 ± 0.2	1.8 ± 0.4	-28.4	0.79*	1.15 ± 0.05	1.26 ± 0.07	9.6	0.007**

* p-value corrected for baseline log transformed triglyceride levels and treatment dose.

** p-value corrected for baseline HDL-levels and treatment dose.

DISCUSSION

In this study in patients with type 2 diabetes, we found that the adiponectin *-11391GA* polymorphism and adiponectin plasma levels affect LPL activity. Alternatively, statin therapy did not influence adiponectin plasma levels, but baseline adiponectin plasma levels interacted with the effect of statin therapy on HDL cholesterol levels.
The typical diabetic dyslipidemia contributes to the high burden of cardiovascular disease in patients with type 2 diabetes.[57] Because adiponectin polymorphisms and plasma levels have previously been associated with insulin resistance[80,81], cardiovascular disease[59], and dyslipidemia[61,62], it is of special interest to investigate the effects of adiponectin and the underlying mechanisms in patients with type 2 diabetes
The strengths of our study are the large number of the patients with type 2 diabetes with post heparin lipase activity measurements. This enabled us to investigate the association of adiponectin with LPL and HL activities and combine it with a molecular analysis. Moreover, the placebo-controlled intervention allowed us to investigate both the effects and interactions of statin therapy with adiponectin levels in patients with type 2 diabetes.
We have investigated the effects of two adiponectin gene polymorphisms. We cannot exclude that other polymorphisms in this gene exert effects on LPL or HL activities. Unfortunately, the population was too small for haplotype analyses of the adiponectin gene. However, the effect of the *-11391GA* polymorphism on LPL activity supports a relationship between LPL activity and adiponectin at a molecular level.
In our study, total adiponectin levels were measured. It has been argued that the high molecular weight (HMW) form of adiponectin is a better predictor of metabolic parameters.[82] However, recent evidence suggests that the low molecular forms exert metabolic effects, as well.[83,84] Because the significance of the different isoforms is unclear and because HMW and total adiponectin are highly correlated[85], we chose to measure total adiponectin levels.
Genetic factors account for a large proportion of variation in adiponectin plasma levels.[86,87] The *-11391GA* and *45TG* polymorphisms belong to the two large distinct LD blocks in the adiponectin gene.[88] We have found that the *-11391GA* polymorphism was associated with LPL activity and adiponectin plasma levels. The *45TG* polymorphism was associated with HL activity but not with adiponectin levels. Our findings are in line with a meta-analysis that included the *-11391GA* polymorphism and the *45TG* polymorphism.[88] Associations of adiponectin polymorphisms with post heparin LPL and HL activities have not been investigated previously. Despite associations with lipase activities, the polymorphisms were not associated with HDL-cholesterol or triglyceride levels. This is not surprising as these polymorphisms explained only a small part of the variation in adiponectin levels, which maximally explains a small

part of the variance lipase activities and lipoprotein phenotypes. Heid et al.[79], who investigated these relations in a large population of healthy individuals, drew a similar conclusion.

In a recent review by Lara-Castro et al.[89] LPL and HL were described as the most important enzymes through which adiponectin exerts its beneficial effects on lipoprotein metabolism. In our study, plasma adiponectin showed significant correlations with LPL and HL activities. However, these relationships were weak and the association with HL activity was not significant after adjustment for age and gender. Others have investigated the association between lipase activities and plasma adiponectin and had similar findings.[66-68] Remarkably, the associations of baseline adiponectin with HDL-cholesterol and triglyceride levels were much stronger. The association with HDL-cholesterol remained significant after correction for LPL and HL activities, whereas the effect on triglycerides disappeared. This implies that adiponectin exerts a substantial effect on HDL metabolism, independent of lipase activities, while the effect on triglyceride metabolism is largely mediated by lipase activities.

In the present study, atorvastatin treatment had large effects on lipid levels, but did not change adiponectin levels. In one group of subjects with impaired glucose tolerance, pravastatin treatment increased plasma adiponectin levels.[74] This study differs from our study due to the fact that a different statin (pravastatin as opposed to atorvastatin) was investigated and that patients with type 2 diabetes were excluded. Another study in patients with type 2 diabetes showed that atorvastatin added to rosiglitazone treatment increased adiponectin plasma levels.[72] It is difficult to compare these findings to our findings because this study was not placebo-controlled and the subjects were simultaneously treated with rosiglitazone. A number of other studies on this subject yielded results similar to ours.[75,90,91]

Although, we did not find an effect of statin treatment on adiponectin plasma levels there was a remarkable interaction of baseline adiponectin levels with the effect of statin treatment on HDL-cholesterol levels. Patients with high baseline adiponectin levels exhibited a large increase in HDL-cholesterol in response to statin treatment. This implies that some patients might benefit more from statin treatment by efficiently raising their HDL-cholesterol. This effect was LPL- and HL activity independent.

A number of potential mechanisms underlying the relationship between adiponectin plasma levels and LPL activity have been suggested. Tumor necrosis factor-α (TNFα) has been proposed as important intermediate. TNFα and adiponectin may control each others expression.[92] Thus high adiponectin could lead to less suppression of LPL synthesis in adipose tissue by TNFα.[93] Another explanation could be differences in the degree of insulin resistance between adiponectin tertiles, although de Vries

et al. have demonstrated that the association of adiponectin with LPL activity is independent of insulin resistance.[68]

In our study, adiponectin influenced HDL-cholesterol largely independent of lipase activities. In an *in vivo* kinetic study a strong negative correlation between adiponectin and apolipoprotein-A1 (Apo-A1) catabolism in persons with the metabolic syndrome was found.[94] *In vitro* experiments have shown that it increases HDL assembly in HepG2 cells.[95] An alternative explanation might be CETP activity is affected by adiponectin. High adiponectin results in lower triglyceride levels and thus less CETP activity. However, adjustment for triglyceride levels and CETP mass did not change the effects of adiponectin on HDL-cholesterol levels. Therefore, it seems unlikely that low triglyceride levels in persons with high adiponectin levels explain our results. Unfortunately, we did not measure CETP activity and therefore we could not test its involvement as an alternative explanation.

In conclusion, we have demonstrated that genetic variation in the adiponectin gene and adiponectin plasma levels influence LPL activity. The relationship between adiponectin and plasma triglyceride levels is mediated through lipase activities, while the mechanism underlying the relationship with HDL cholesterol is unknown. Although statin therapy itself does not affect adiponectin plasma levels, adiponectin plasma levels determined the HDL-cholesterol response to statin treatment. This implies that according to adiponectin plasma levels subgroups of patients with type 2 diabetes can be identified that benefit to different extents from statin therapy.

CHAPTER 5

ASSOCIATION OF AN *APOC3* PROMOTER VARIANT WITH TYPE 2 DIABETES RISK AND NEED FOR INSULIN TREATMENT IN LEAN PERSONS

M. van Hoek,
T.W. van Herpt,
A. Dehghan,
A. Hofman,
A.G. Lieverse,
C.M. van Duijn,
J.C.M. Witteman,
E. J.G. Sijbrands.

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ABSTRACT

Background: An *APOC3* promoter haplotype has been previously associated with type 1 diabetes. In our population-based study, we investigated whether *APOC3* polymorphisms increase type 2 diabetes risk and need for insulin treatment in lean participants.

Methods: In the Rotterdam Study, a population-based prospective cohort (n=7983), Cox and logistic regression models were used to analyse the associations and interactive effects of *APOC3* promoter variants (*-482C>T, -455T>C*) and BMI on type 2 diabetes risk and insulin treatment. Analyses were followed by replication in an independent case-control sample (1817 cases, 2292 controls) and meta-analysis.

Results: In lean participants, the *-482T* allele was associated with increased risk of prevalent and incident type 2 diabetes: *-482CT* OR 1.47 [1.13-1.92], *-482TT* 1.40 [0.83-2.35], p for trend 0.009; HR *-482CT* 1.35 [0.96-1.89], *-482TT* 1.68 [0.91-3.1], p for trend 0.03, respectively. These results were confirmed by replication. Meta-analyses was highly significant.(*-482T* p meta-analysis= 1.1E10-4). A borderline significant interaction was observed for insulin use among participants with type 2 diabetes. (*-482CT**BMI p=0.06, *-455TC**BMI p=0.02).

Conclusion: At a population-based level, the influence of *APOC3* promoter variant on type 2 diabetes risk varies with the level of adiposity. Lean carriers of the *-482T* allele had increased type 2 diabetes risk, while such an effect was not observed in overweight participants. Conversely, in overweight participants the *-455C* allele seemed protective against type 2 diabetes. The interaction of the variants with need for insulin treatment may indicate beta cell involvement in lean participants. Our findings suggest overlap in the genetic background of type 1 diabetes and type 2 diabetes in lean patients.

INTRODUCTION

Type 2 diabetes mellitus is characterized by overweight, impaired insulin secretion, and insulin resistance.[96] Nevertheless, a substantial proportion of patients with type 2 diabetes are lean.[97,98] These would seem to suffer predominantly from impaired insulin secretion[99-101], whereas obese patients would be more resistant to insulin.[102] Defective insulin secretion has higher heritability estimates than insulin resistance in family and twin studies[103-105] and the concordance rate for type 2 diabetes in lean twins is higher than that in obese twins.[106] This suggests higher genetic susceptibility in lean type 2 diabetes and involvement of pancreatic beta cell dysfunction.

Lean type 2 diabetes has a number of similarities to type 1 diabetes. Interestingly, type 1 diabetes is more frequent in families with type 2 diabetes and vice versa[107,108], a fact that supports the hypothesis of a genetic overlap between the two diseases. In a paper by Hokanson et al. an *APOC3* haplotype, determined by two promoter polymorphisms (*-455T>C* and *-482C>T*), was associated with type 1 diabetes.[109] ApoCIII is an apolipoprotein that is involved in triacylglycerol metabolism. It inhibits lipoprotein lipase (LPL) and mediates lipoprotein uptake by the liver.[110] Higher ApoCIII production raises triacylglycerol and non-esterified free fatty acid (NEFA) levels, and thus may affect beta cell function.[111,112] On the other hand, ApoCIII has been suggested to directly bring on beta cell death.[113] Located in an insulin/phorbol ester responsive element (IRE), the promoter polymorphisms curb the capacity of insulin to downregulate the gene.[114,115] In view of this evidence, we hypothesized that polymorphisms in the *APOC3* gene increase susceptibility of lean individuals to type 2 diabetes and that lean carriers of these polymorphisms with type 2 diabetes are more susceptible to insulin deficiency.

In the present population-based study, we investigated whether the *APOC3 -482C>T* and *-455T>C* polymorphisms influence type 2 diabetes risk in lean persons. This was followed by replication testing in an independent sample. In addition, we investigated the associations with need for insulin treatment among participants with type 2 diabetes in the Rotterdam Study. (RS1)

METHODS

STUDY POPULATION

Details of the RS1 have been described elsewhere.[116] In brief, RS1 is a prospective, population-based cohort study investigating determinants of chronic diseases in the elderly. A total of 7983 inhabitants of a Rotterdam suburb, aged 55 years or older, were included. Baseline examinations were performed with follow-up measurements

at one to three year intervals. Linkage to general practitioners records provided continuous monitoring of major disease outcomes. Municipal health authorities regularly provided information on vital status.

In accordance with the guidelines of the American Diabetes Association[116,117] and WHO[29] diabetes was defined as a fasting plasma glucose level ≥ 7.0mmol/l and/or a non-fasting plasma glucose level ≥ 11.0mmol/l and/or treatment with oral glucose-lowering medication or insulin and the diagnosis diabetes as registered by a general practitioner. Prevalent cases of diabetes were diagnosed at baseline by a non fasting or post-load glucose level (after OGTT) ≥ 11.1mmol/l and/or treatment with oral glucose-lowering medication or insulin and diagnosis of diabetes as registered by a general practitioner.

For the present study, general practitioners' records were searched for type 1 diabetes diagnoses and these participants were excluded. Data on insulin treatment were derived from interviews at baseline and the pharmacy database, which provided prospective data on prescribed insulin. Written informed consent was obtained from all participants and the Medical Ethics Committee of the Erasmus Medical Center approved the study.

For the present study, baseline data were collected between 1990 and 1993. Follow-up data were available until October 1, 2005.

REPLICATION COHORT

The Rotterdam Study Plus 1 (RSPlus1; 290 cases and 2292 controls) and the DiaGene study (1527cases) were used as a combined replication cohort (RSPlus1/DiaGene). RSPlus1 is an additional part of the prospective population based Rotterdam study and has been described in detail elsewhere.[116] The DiaGene study is an ongoing collection of type 2 diabetes cases and controls from the city of Eindhoven, the Netherlands. At the time of this replication effort, 1527 DiaGene cases were available for genotyping. RSPlus1 and DiaGene cases were diagnosed according to the guidelines of the WHO[29] and ADA[116,117] and/or based on treatment with oral glucose-lowering medication or insulin. RSPlus1 and DiaGene cases did not significantly differ in terms of age, sex and BMI and were combined in the analyses. Written informed consent was obtained from all participants and the Medical Ethics Committee of the Erasmus Medical Center approved the study

GENOTYPING

In the *APOC3* gene the *-482C>T(rs2854117)* and *-455T>C(rs2854116)* promoter polymorphisms were chosen for genotyping on the basis of the ability to tag the variation previously associated with type 1 diabetes and earlier associations with measures of glucose metabolism and localization in an IRE.[109,118-120]

Genotyping was performed with Taqman allelic discrimination assays designed and optimised by Applied Biosystems, (Foster City, CA, USA http://store.appliedbiosystems.com). Reactions were performed on the Taqman Prism 7900HT platform. The duplicates mismatch rate was 0.003 and 0.02 in RS1 and 0.018 and 0.044 in the RSPlus1/DiaGene cohort for the *-482C>T* and *-455T>C* polymorphisms, respectively. Genotype success rates for *-482C>T, -455T>C* were 0.95, 0.94 and 0.96, 0.97 in RS1 and RSPlus1/DiaGene cohorts respectively.

LABORATORY ANALYSES

In 3930 participants fasting plasma samples were available at the second follow -up visit of the study for measurement of triacylglycerols. Triacylglycerol levels were measured by enzymatic colorimetric methods (CHOD-PAP and GPO-PAP, Boehringer Mannheim, Mannheim, Germany) on a Hitachi 911 analyser (Boehringer Mannheim, Mannheim, Germany).

STATISTICAL METHODS

Analyses were performed with SPSS software version 16.0. Linkage disequilibrium was calculated with GOLD version 1.0. Continuous variables are expressed as means ± SE. Comparisons between groups were performed with independent samples t-tests and χ^2-tests for normally distributed continuous and categorical variables, respectively. Triacylglycerol levels were not normally distributed and therefore logarithmically transformed before the analyses. ANOVA was used for comparison of continuous variables between more than 2 groups. Deviations from Hardy Weinberg equilibrium were assessed by means of χ^2 testing. Genotype frequencies were compared between incident and prevalent cases and participants without type 2 diabetes.

In RS1, we tested the associations of the genotypes with type 2 diabetes and their interactions with BMI in logistic regression models and prospectively in Cox proportional hazards models for prevalent and incident type 2 diabetes, respectively. Participants with prevalent type 2 diabetes at baseline were excluded from the prospective analyses.

In the RSPlus1/DiaGene cohort we repeated the analyses described above using logistic regression. Haplotype analyses did not add any further information on analyses of the individual genotypes and are therefore not shown here.

To test the hypothesis of beta cell involvement, we tested the associations of genotypes with insulin treatment and their interactions with BMI in a logistic regression model in RS1 in patients with type 2 diabetes. Prevalent and incident insulin use was included in these analyses. Linear regression models served to analyse the

association of the polymorphisms and fasting triacylglycerol levels. All models were adjusted for year of birth and sex.
All analyses described above were repeated in two subgroups stratified for BMI using the RS1 population median. Lean persons had a BMI equal to or below 26kg/m^2 and overweight persons had BMI above 26kg/m^2.
The p-values found in RS1 and RSPlus1/DiaGene were combined by a Z-based meta-analysis using beta, standard error and number of participants of each cohort.
Combining RS1 and RSPlus1/DiaGene we had 98% power to detect an effect of OR 1.3 (MAF 0.20) in the lean group (n=802 cases, i.e. the smallest group of cases analysed for type 2 diabetes).

RESULTS

BASELINE CHARACTERISTICS RS1

Diabetes status was available for 6362 successfully genotyped persons. Participants whose genotyping was not successful or whose DNA was not available, were 5.8 years older and included 8.5% more women. Nevertheless, the genotyped and not genotyped groups had similar distributions of BMI, waist circumference and presence of type 2 diabetes.
Participants with type 2 diabetes (prevalent and incident) had significantly higher BMI and waist circumference and lower HDL-cholesterol than those without type 2 diabetes (Table 1). Prevalent cases were significantly older and had significantly lower BMI than incident cases.

POPULATION GENETICS

In the total population and BMI subgroups, the polymorphisms were in Hardy-Weinberg equilibrium. (χ^2 <3.35 df=1, p>0.07) Heterozygosity for the *-482T* allele was found in 34.6% of subjects and 5.9% were homozygous. For the *-455C* allele these percentages were 43.0 and 12.6, respectively. There was a strong linkage disequilibrium between the two polymorphisms D'=0.968 (p<0.000001) r^2=0.54. Frequencies of genotypes did not differ significantly between participants with and without type 2 diabetes or between incident and prevalent cases in the total population. (data not shown)
We identified four haplotypes, *H1* to *H4*, according to decreasing population frequency. *H1* was defined by the absence of both minor alleles, *H2* by the presence of both minor alleles, *H3* by only the presence of the *-455* minor allele and H4 by the presence of the *-482* minor allele. The allele frequencies of *H1*, *H2* and *H3* were 64.4%, 23.4% and 11.8%, respectively.

Table 1. RS1 Baseline characteristics of all participants and patients with and without type 2 diabetes.

Characteristics	All participants	Subjects without type 2 diabetes	Prevalent cases	Incident cases	p-value
	n=6362	n=5156	n=661	n=545	
Age (years)	69.4 ± 9.1	69 ± 9.1	73.5 ± 9.2	68.4 ± 7.9	<0.001[a, b]
Men (%)	40.6	40.4	39.5	44	NS
Body Mass Index (kg/m^2)	26.3 ± 3.7	26.0 ± 3.6	26.8 ± 4.1	28.1 ± 3.7	<0.001[c, a,b]
Total cholesterol (mmol/l)	6.6 ± 1.2	6.6 ± 1.2	6.5 ± 1.2	6.6 ± 1.2	NS
Triacylglycerol (mmol/l)	1.53 ± 0.01	1.47 ± 0.01	1.83 ± 0.07	1.89 ± 0.05	<0.001[a,c]
HDL-cholesterol (mmol/l)	1.34 ± 0.37	1.37 ± 0.37	1.25 ± 0.37	1.25 ± 0.34	<0.001[c, b]
Age of diagnosis	NA	NA	68.6 ± 0.50	74.8 ± 0.34	<0.001[b]

[a]p-value for comparison between prevalent cases and participants without diabetes; [b]p-value for comparison between incident cases and prevalent cases; [c]p-value for comparison between incident cases and participants without diabetes
Values are means ± standard errors unless otherwise indicated
NA: Not Applicable

TYPE 2 DIABETES RISK IN LEAN AND OVERWEIGHT PERSONS

In the total population, none of the genotypes significantly influenced the risk of type 2 diabetes. Adjustment for BMI and waist circumference did not change these results. (data not shown)

We observed an effect of the interaction of the *-482C>T* and *-455T>C* polymorphisms with BMI on prevalent type 2 diabetes (*-482C>T**BMI, p=0.01; *-455T>C**BMI p=0.01). Similar findings were observed prospectively for effect on the risk of incident type 2 diabetes (*-482C>T**BMI, p=0.002; *-455T>C**BMI p=0.03).

Table 2 shows Odds Ratios (ORs) for prevalent type 2 diabetes and Hazard Ratios (HRs) for incident type 2 diabetes according to *ApoC3* promoter genotype in lean and overweight persons. In lean participants, the *-482T* allele was associated with increased risk of diabetes (*-482CT* OR 1.47, *-482TT* OR 1.40 p for trend 0.009). Similar results were found in prospective analyses (*-482CT* HR 1.35 (0.96-1.89) *-482TT* HR1.68 (0.91-3.1); p for trend=0.03). The *-455T>C* polymorphism showed similar associations in lean participants.

In overweight participants, no significant effects of the *-482C>T* polymorphism on diabetes risk was observed while carriers of the *-455C* allele had lower prospective risk of type 2 diabetes (*-455TC* HR 0.88 (0.71-1.1), *-455CC* 0.70 (0.49-0.998), p for trend 0.04).

Table 2. HRs and ORs for incident and prevalent type 2 diabetes, replication and meta-analysis for *ApoC3* genotypes in lean and overweight participants.

Category	Polymorphism	Genotype	RS HR incident diabetes[a]	95% CI	P trend	RS OR prevalent diabetes[a]	95% CI	P trend	Replication cohort OR[b]	(95% CI)	P trend	P overall[c]
BMI≤26	*482C>T*	CC	1.0			1.0			1.0			
		CT	1.35	(0.96-1.89)		1.47	(1.13-1.92)		1.06	(0.81-1.38)		
		TT	1.68	(0.91-3.1)	0.03	1.40	(0.83-2.35)	0.009	1.99	(1.25-3.16)	0.03	1.1E-04
	-455T>C	TT	1.0			1.0			1.0			
		TC	1.27	(0.89-1.8)		1.65	(1.24-2.20)		0.86	(0.66-1.13)		
\		CC	1.39	(0.85-2.28)	0.1	1.40	(0.92-2.13)	0.01	1.09	(0.74-1.62)	0.9	0.04
BMI>26	*-482C>T*	CC	1.0			1.0			1.0			
		CT	1.04	(0.84-1.28)		0.85	(0.66-1.10)		1.04	(0.88-1.23)		
		TT	0.56	(0.32-0.98)	0.3	0.84	(0.51-1.39)	0.22	1.47	(1.08-1.99)	0.05	0.73
	-455T>C	TT	1.0			1.0			1.0			
		TC	0.88	(0.71-1.1)		0.98	(0.76-1.25)		0.90	(0.76-1.06)		
		CC	0.70	(0.49-0.998)	0.04	0.71	(0.48-1.06)	0.16	0.79	(0.62-1.02)	0.05	0.003

[a]Adjusted for year of birth and sex.
[b]Adjusted for age and sex
[c]Meta-analyses P-value combining RS1 and RSplus1/DiaGene

REPLICATION AND META-ANALYSIS

Baseline characteristics of the RSPlus1/DiaGene cohort are shown in Table 3. Cases were older (p<0.001) had higher BMI (p<0.001) and were more often men (p<0.001) than controls. In the replication analyses, we found similar results to the original cohort in which the *-482T* allele increased type 2 diabetes risk in lean persons (*-482CT* 1.06 (0.81-1.38), *-482TT* 1.99 (1.25-3.16); p for trend 0.03). No significant effects were found for the *-482C>T* and *-455T>C* polymorphisms in overweight subjects (both p for trend 0.05).

In meta-analyses the effect of the *-482T* allele in lean participants was confirmed (p-meta analysis 1.1 $E10^{-4}$). In overweight participants, a protective effect of the *-455C* allele seemed to be present (p=0.003)

Table 3. Baseline characteristics from the RSPlus1/Diagene sample

Characteristics	All participants N=4134[a]	Controls N=2292	Cases N=1817	p-value
Age (years)	65.5 ± 9.0	64.4 ± 7.8	66.8 ± 10.1	<0.001
Men (%)	48.4	44.3	53.7	<0.001
Body Mass Index (kg/m^2)	28.4 ± 4.9	26.9 ± 3.9	30.4 ± 5.3	<0.001

[a]N=25 with missing diabetes status.
Unless otherwise shown, values are means ± standard error

INSULIN TREATMENT IN LEAN AND OVERWEIGHT SUBJECTS

Table 4 shows the ORs for insulin treatment within participants with diabetes according to the *-482C>T* and *-455T>C* genotypes. None of the ORs was statistically significant in this small group of participants. However, there was a borderline significant interaction of the *-482C>T* polymorphism with BMI in the participants with diabetes. (*-482C>T**BMI p=0.06) and a significant interaction for the *-455T>C* polymorphism (*-455T>C**BMI p=0.02).

INTERMEDIATE TRAITS

The genotypes were not associated with BMI (*-482C>T* p=0.60; *-455T>C* p=0.21) or fasting triacylglycerol levels (*-482C>T* p= 0.67; *-455T>C* p= 0.73) They were also not associated with fasting triacylglycerol levels within the high and low BMI groups (data not shown).

Table 4. Odds ratios for insulin treatment in lean and overweight participants with diabetes in RS1

Category	Polymorphism	Genotype	OR[a]	(95% CI)	P for Trend
BMI ≤26	*-482C>T*	CC	reference		
(insulin treated/		CT	1.49	(0.89-2.49)	
non-insulin treated: N=83/344)		TT	1.66	(0.68-4.00)	0.11
	-455T>C	TT	reference		
		TC	1.71	(0.95-3.08)	
		CC	1.64	(0.74-3.62)	0.13
BMI>26	*-482C>T*	CC	reference		
(Insulin treated/		CT	0.94	(0.63-1.41)	
non insulin treated: n=131/575)		TT	1.26	(0.53-3.03)	0.92
	455T>C	TT	reference		
		TC	0.83	(0.56-1.25)	
		CC	0.78	(0.40-1.55)	0.34

[a]Adjusted for year of birth and sex

DISCUSSION

MAIN FINDINGS

In this population-based study, we found significant interactions between BMI and genetic variants in the *APOC3* promoter for type 2 diabetes risk. Lean participants carrying the *-482T* allele had higher risk of type 2 diabetes. These results were replicated in an independent population. Moreover, *APOC3* promoter variants showed a borderline significant interaction with BMI for the need for insulin therapy. Presence of the minor alleles showed a trend towards increased need for insulin therapy in lean participants. Conversely, in overweight participants the *-455C* allele seemed protective against type 2 diabetes.

The RS1 study design included meticulous follow-up on disease outcomes over a long time. The large number of diabetic cases enabled us to replicate the findings within our population in prevalent and incident cases. Moreover, we were able to replicate and meta-analyse the findings by means of an independent population. Information on insulin treatment provided an important intermediate phenotype for the interpretation of our findings and the understanding of pathophysiological mechanisms. To our knowledge, this is the first study that shows an interaction between *APOC3* variants and BMI to have an effect on type 2 diabetes risk.

Our results show that the effect of variation in the *APOC3* gene is context dependent. Our findings are not in line with the thrifty genotype hypothesis[121] nor with

the Barker hypothesis.[122] *APOC3* variants were not associated with BMI. The common *APOC3* variants have an intricate relationship with type 2 diabetes. We hypothesize that lean type 2 diabetes has a distinct molecular basis of its own or that it shares part of its aetiology with type 1 diabetes.

PREVIOUS STUDIES

Hokanson and colleagues found the *APOC3* promoter variants to be associated with susceptibility to type 1 diabetes.[109] Our findings in the lean type 2 diabetes group are consistent with this finding with ORs of similar magnitude and direction. Lean type 2 diabetes and type 1 diabetes may both have insulin deficiency as a main characteristic. A partly shared aetiology or at least a genetic overlap in disease susceptibility may also explain the increased frequency of lean type 2 diabetes in families with type 1 diabetes and vice versa.[107,108] Patients with type 2 diabetes, who have relatives with type 1 diabetes are leaner and have lower C-peptide concentration than those with a family history of type 2 diabetes.[123]

Our finding that lean carriers of the minor alleles may require insulin therapy more frequently, points towards an effect of *APOC3* variants on beta cell function. Nevertheless, the disease process could also involve insulin resistance. The EARS II study reported larger AUCs for glucose and insulin in carriers of the *-482T* allele after an OGTT.[118] The population of this study consisted of young, healthy, lean offspring of men with premature cardiovascular disease. It would follow therefore that *APOC3* promoter variation increases insulin resistance at a young age, possibly resulting in type 2 diabetes and an earlier need for insulin therapy. Nevertheless, caution should be exercised in extrapolating differences in insulin and glucose AUCs in young healthy participants to relevance for diabetes risk at an older age. In a study of middle-aged participants, male homozygotes of the variant promoter alleles showed less increase in insulin levels after an OGTT.[120] This is in line with our findings in lean participants.

PATHOPHYSIOLOGICAL HYPOTHESES

Strikingly, we found an opposite effect of *APOC3* promoter variation in overweight participants, in whom it would seem to protect against type 2 diabetes. Possibly, competing risks overruled the effect of the *APOC3* polymorphism and even resulted in an inverse association in overweight participants. Alternatively, there could be a pathophysiological explanation based on the metabolic differences between lean and overweight patients. In lean participants our findings could be explained as follows: The presence of the promoter polymorphisms results in insulin resistance at a molecular level; thus the insulin-resistant *APOC3* promoter shows impaired insulin-mediated downregulation.[114,115] Increased ApoCIII plasma levels have been

described in type 2 diabetes but were mostly interpreted as a consequence of the disease.[124,125] A direct toxic effect of ApoCIII on beta cells was found in in vitro studies by Junti-Berggren et al.[113] Indirect mechanisms involving lipoprotein metabolism might play a role, too. However, we did not find an association of the promoter variation with fasting triacylglycerol levels. This, however, does not exclude an effect of the polymorphisms on NEFA flux. Defective inhibition of LPL by ApoCIII might raise levels of circulating NEFAs and thus affect insulin sensitivity and beta cell function.[112,126,127]

In overweight participants, hyperinsulinemia may partly restore the insulin-mediated downregulation and may prevent beta cell toxicity. Nonetheless we do not have a clear explanation for a protective effect. A number of studies have shown that LPL metabolism may differ between lean and obese subjects. However, it is unknown how differences in lipoprotein composition[128] or post-translational regulation of LPL[129-131] influence susceptibility to type 2 diabetes. Taken together, *APOC3* promoter variation may directly affect beta cell function in lean subjects and reduce type 2 diabetes risk in overweight subjects by mechanisms that are at present unknown. Alternatively, it may affect type 2 diabetes risk in lean and obese individuals in opposing ways by mechanisms that involve LPL function.

STUDY LIMITATIONS

Some limitations of our study need to be considered. Unfortunately, we could not compare our findings with currently published genome wide association studies, since they were not publicly available for lean and overweight subjects, separately. We also cannot exclude the possibility that the observed effects are due to linkage disequilibrium with other variants in the *APOC3* gene or other genes nearby in the *ApoA1-C3-A4-A5* cluster. Nonetheless, the localization in an IRE, their defective insulin mediated downregulation in vitro, and the findings by Hokanson and colleagues in type 1 diabetes[109], make these polymorphisms likely candidates for type 2 diabetes susceptibility in lean subjects. Another limitation of the study is the relatively small group of participants in which we found a borderline significant interaction for insulin use. Although these results can help focus hypotheses on the mechanism by which *APOC3* polymorphisms affect type 2 diabetes risk, a type 1 error cannot be excluded. These results are merely an encouragement for further research and replication in independent cohorts.

CONCLUSION

In conclusion, our findings indicate that *APOC3* promoter variants significantly interact with BMI at a population-based level, increasing type 2 diabetes risk in lean and possibly decreasing type 2 diabetes risk in overweight participants. The

borderline interaction of the promoter variants with need for insulin treatment suggests beta cell function involvement in lean patients. Our results suggest a genetic overlap between type 1 and lean type 2 diabetes. A subset of type 2 diabetes has already been identified for patients with auto-antibodies, i.e. type 1.5 diabetes or Latent Autoimmune Diabetes in Adults(LADA).[132] It would seem unlikely that APOCIII is related to auto-immunity. Our findings consequently may relate to a different subgroup of lean diabetes patients showing genetic overlap with type 1 diabetes.

CHAPTER 6

A GENETIC VARIANT IN THE *IGF2BP2* GENE MAY INTERACT WITH FETAL MALNUTRITION TO AFFECT GLUCOSE METABOLISM

M. van Hoek,
J.G. Langendonk,
S.R. de Rooij,
E.J.G. Sijbrands,
T.J. Roseboom.

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ABSTRACT

Background: Fetal malnutrition may predispose to type 2 diabetes through gene programming and developmental changes. Previous studies showed that these effects may be modulated by genetic variation. Genome wide association studies discovered and replicated a number of type 2 diabetes-associated genes. We investigated the effects of such well-studied polymorphisms and their interactions with fetal malnutrition on type 2 diabetes risk and related phenotypes in the Dutch Famine Birth Cohort.

Methods: The *CDKAL1-rs7754840, CDKN2AB-rs10811661, HHEX-rs1111875, IGF2BP2-rs4402960, KCNJ11-rs5219, SLC30A8-rs13266634* and *TCF7L2-rs7903146* polymorphisms were genotyped in 772 participants of the Dutch Famine Birth Cohort Study (n=328 exposed, n=444 unexposed). Logistic and linear regression models served to analyse their interactions with prenatal exposure to famine on type 2 diabetes, Impaired Glucose Tolerance (IGT) and Area Under the Curves (AUCs) of glucose and insulin during Oral Glucose Tolerance Testing (OGTT).

Results: In the total population, the *TCF7L2* and *IGF2BP2* variants most strongly associated with increased risk for type 2 diabetes/IGT and increased AUC glucose, while the *CDKAL1* polymorphism associated with decreased AUC insulin. The *IGF2BP2* polymorphism showed an interaction with prenatal exposure to famine on AUC glucose. (β=-9.2, 95% CI –16.2;-2.1; p=0.009)

Conclusion: The *IGF2BP2* variant showed a nominal interaction with exposure to famine *in utero*, decreasing OGTT AUCs for glucose. This may provide a clue that modulation of the consequences of fetal environment depends on an individual's genetic background.

INTRODUCTION

The fetal origins hypothesis[7,133] states that malnutrition during fetal development predisposes to adverse health outcomes, such as type 2 diabetes. According to the hypothesis, fetal adaptation to a low-caloric intrauterine environment may involve programming to optimize the use of restricted nutrient supply. Programming may lead to altered gene expression profiles and eventually to disease later in life. In addition, adverse intrauterine circumstances may divert nutrients to critical organs such as the brain at the expense of organs such as the pancreas, liver or muscles.[134] Type 2 diabetes and defective insulin secretion show high heritabilities.[135] Genetic variation is likely to interact with the fetal response to an extreme nutritional situation. We found that the effects of the *Pro12Ala* polymorphism of *the PPARgamma 2* gene depend on prenatal exposure to famine[136], and several studies have shown interactions of genes with size at birth, a marker of fetal environment.[137-139] Such interactions may have consequences for development and function. Previously, the *PPARG, TCF7L2* and *KCNJ11* genes were identified by linkage and candidate gene studies as type 2 diabetes risk loci.[140-142] Recently, Genome Wide Association (GWA) studies have identified and replicated new genetic variants associated with type 2 diabetes.[14-16,19] Notably, a number of these variants are thought to be involved in the development and function of critical organs for glucose metabolism, such as the pancreatic beta cell.[143-145]

Based on the findings described above, we hypothesized that these genes interact with fetal exposure to famine. In the Dutch Famine Birth Cohort, we investigated the effects of genetic variation in these loci on type 2 diabetes and Impaired Glucose Tolerance (IGT) and Oral Glucose Tolerance Testing (OGTT) and their interaction with fetal malnutrition.

METHODS

SUBJECTS

The Dutch Famine Birth Cohort is composed of individuals born as a term singletons around the time of the Dutch famine during the Second World War II in the Wilhelmina Gasthuis in Amsterdam. Details of the study have been described elsewhere.[146] In brief, a total of 2414 singletons were born between November 1, 1943 and February 28, 1947. Members of the cohort living in the Netherlands at 1 September 2002 were invited (n=1423). Of these, 810 agreed to participate. The study was approved by the local medical ethics committee and was conducted in accordance with the declaration of Helsinki. All participants gave their written informed consent.

EXPOSURE TO FAMINE

Prenatal exposure to famine was defined as a daily food ration of the mother below 1000 calories during any 13-week period of gestation, based on the official daily food rations for the general population aged 21 years and older. Based on these data, individuals born between January 7, 1945 and December 8, 1945 were exposed to famine in utero. Initially, we defined three exposure groups of 16 weeks, dividing the period of exposure in late, mid and early gestation. However, this led to small groups in which meaningful genetic analyses were impossible. Therefore, we combined the exposed groups for the interaction analyses.

STUDY PARAMETERS

Trained research nurses conducted measurements and interviews as previously described.[146] In brief, information on medical history, lifestyle and medication was derived from a standardized interview. Information on the mother, the pregnancy and size at birth was derived from medical birth records.

The present study was limited to measures of glucose metabolism. Pre-existent diabetes was defined as the use of oral or injected glucose-lowering medication. Persons with pre-existent diabetes were excluded from OGTT. OGTT was performed after an overnight fast with a standard load of 75g of glucose at t=0. Blood samples were collected after 0, 30, 60 and 120 minutes for measurement of plasma glucose and insulin concentrations. Plasma glucose was measured by a standardized enzymatic photometric assay on a Modulator P analyser (Roche, Basel, Switzerland). Plasma insulin was measured by immunoluminometric assay on an Immulite 2000 Analyser (Diagnostic Product Corporation, Los Angeles, CA, USA). IGT was defined as a 120-min glucose level between 7.8 and 11.0 mmol/l. Diabetes based on OGTT testing was defined as a 120-min glucose level >11.0 mmol/l.

GENOTYPING

Genomic DNA was extracted from fasting blood samples. The *CDKAL1-rs7754840, CDKN2AB-rs10811661, HHEX-rs1111875, IGF2BP2-rs4402960, KCNJ11-rs5219, SLC30A8-rs13266634* and *TCF7L2*-rs7903146 polymorphisms were genotyped with Taqman allelic discrimination assays. The assays were designed and optimalized by Applied Biosystems, (Foster City, California, http://store.appliedbiosystems.com). The analyses were performed as described previously.[147] Assays were run on 90 blood bank samples to test for adequate cluster separation. Genotypes were determined in 2-ng genomic DNA. Reactions were performed on the Taqman Prism 7900HT platform. Success rates for genotyping ranged from 86.1(*IGF2BP2*) -96.7%. The duplicate mismatch rate for the *IGF2BP2* assay was 0.0. Genotyping for at least one polymorphism was successful in 772 subjects.

STATISTICAL METHODS

The primary endpoint was the Area Under the Curve (AUC) for glucose and insulin during OGTT. A secondary combined endpoint of type 2 diabetes and IGT, consisted of pre-existent type 2 diabetes and type 2 diabetes and IGT identified at OGTT testing.

Continuous variables are expressed as means ± SD. Comparisons between groups were performed with ANOVA and χ^2-tests for normally distributed continuous and categorical variables, respectively. Logarithmic transformation was performed on variables that were not normally distributed. Allele frequencies were estimated by gene counting. Hardy Weinberg equilibrium was tested by χ^2 testing.

The AUCs during OGTT were calculated by the trapezoidal rule[148]: {[15*log (gluc0min)] + [30*log (gluc30min)] + [45*log (gluc60min)] + [30*log (gluc120min)]/120} and {[15*log (ins0min)] + [30*log (ins30min)] + [45*log (ins60min)] + [30*log (ins120min)]/120} for glucose and insulin, respectively.

Binary logistic and linear regression models served to investigate the associations of the genetic variants with type 2 diabetes/IGT risk and AUC glucose and insulin. The additive model of inheritance was assumed.

Genotype by prenatal famine exposure interactions were tested by creating interaction terms for each genetic variant (coded 0, 1 or 2 for carrying the risk allele) with the exposure group (coded 0 and 1 for unexposed and exposed subjects, respectively). Unexposed subjects were the subjects born before or conceived after the Dutch famine. Genotype by birth weight interaction models were created by taking the product of birth weight (continuous) and the genetic variant (coded 0,1 and 2). All models were adjusted for sex. Subsequently, models were adjusted for BMI. Additionally, waist circumference, the mothers parity and weight at the last antenatal visit were added. Models investigating famine were additionally adjusted for birth weight and vice versa. We adjusted for multiple testing by means of Bonferroni adjustment. We had 80% power to detect interactions of ß=7.8 with gene and environmental ß=3.0 (minor allele frequency 30%, α=0.05).

RESULTS

GENERAL CHARACTERISTICS AND POPULATION GENETICS

General and maternal characteristics of 772 genotyped individuals divided into the famine exposure groups, are shown in Table 1.

Minor allele frequencies were 0.31, 0.19, 0.35, 0.29, 0.36, 0.29 and 0.30 for the *CDKAL1, CDKN2A/B, HHEX, IGF2BP2, KCNJ11, SLC30A8 and TCF7L2* polymorphisms, respectively.

Table 1. General and maternal characteristics according to time of pregnancy when exposed to the Dutch famine

	All	Born before	Late gestation	midgestation	Early gestation	Concieved after	P-value*
N	772	233	140	117	71	211	
Characteristics at age 58							
% men	45.9	46.8	44.3	39.3	42.3	50.7	NS
Age (years)	58.3 ± 0.9	59.2	58.5	58.2	58.0	57.4	NS
BMI (kg/m^2)	28.6 ± 4.8	28.7	28.3	28.1	28.0	29.0	NS
Waist (cm)	92.7 ± 8.9	93.3	92.9	90.8	91.9	93.2	NS
Current smoking (%)	24.1	20.6	27.9	25.9	31.0	23.1	NS
Birth characteristics							
Gestational age (days)	285 ± 11.1	284	283	285	289	285	NS
Birth Weight (g)	3359 ± 469	3396	3187	3204	3500	3471	<0.001
Maternal characteristics							
Age at delivery (years)	28.9 ± 6.4	28.7	31.1	28.8	27.2	28.4	NS
Primiparous (%)	33.7	36.1	20.0	33.3	42.3	37.4	0.04
Weight gain (third trimester)	2.9 ± 2.9	2.8	0.0	4.3	5.0	3.4	0.01
Weight at last antenatal visit	66.4 ± 8.7	66.4	62.8	63.6	69.0	69.3	<0.001

* P-value for comparison between exposed and non-exposed. NS means not significant

All polymorphisms were in Hardy Weinberg equilibrium (χ^2<3.3, 2df, p>0.07 for all SNPs). A total of 94 individuals with type 2 diabetes (62 prevalent and 32 based on OGTT) and 100 individuals with IGT were included in the analyses on type 2 diabetes/IGT. For the analyses on the AUC of glucose and insulin during OGTT, 102 individuals were excluded from the total population, because of pre-existent diabetes, non-fasting prior to the test or incomplete results.

ASSOCIATIONS AND INTERACTIONS WITH PRENATAL EXPOSURE TO FAMINE ON TYPE 2 DIABETES/IGT, AUC GLUCOSE AND AUC INSULIN.

Table 2 shows odds ratios(ORs) of the genetic variants for the composite outcome type 2 diabetes/IGT and the ß coefficients for AUC glucose and AUC insulin during the OGTT. The *TCF7L2* and *IGF2BP2* variants were associated with increased type 2 diabetes/IGT risk (*TCF7L2* OR 1.39 95%CI 1.08-1.79, *IGF2BP2* OR1.43 95% CI 1.11-1.85) and increased AUC glucose (*TCF7L2* β=4.5 95% CI 1.0-8.1, *IGF2BP2* β=3.6 95% CI 0.1-7.1). The *CDKAL1* variant associated with a decreased AUC insulin (β=-8.2; 95% CI -16.1;-0.41), which became less strong after adjustment for BMI. None of the other results changed considerably after additional adjustments in multivariate models.

None of the polymorphisms had a significant effect on birth weight or were related to prenatal famine exposure (data not shown).

Table 3 shows the interactions between genetic variants and exposure to famine in utero. The *IGF2BP2* showed a significant interaction on AUC glucose (β interaction –9.2 (-16.2;-2.1), p=0.009). Figure 1 shows the effects of the *IGF2BP2* polymorphism on AUC glucose in exposed and unexposed subjects. After Bonferroni correction for 21 tests, this was no longer significant. None of the polymorphisms showed a significant interaction with birth weight. (data not shown) Additional adjustments in multivariate models did not change the results.

DISCUSSION

In the present study, we investigated interactions of type 2 diabetes-associated polymorphisms in a population exposed to famine *in utero*. The *IGF2BP2* polymorphism showed a nominally significant interaction with prenatal exposure to famine on AUC for glucose during OGTT.

The Dutch Famine Birth Cohort provides a unique opportunity to directly investigate the interactions of fetal malnutrition on type 2 diabetes and related measurements. Most studies on the association of early nutrition with type 2 diabetes and related parameters have used birth weight as a marker of fetal nutrition and growth. Un-

Table 2 Associations with DM/IGT and AUC glucose and insulin in the total population

Gene-variant	major/ minor allele	OR1 DM/IGT (95% CI)	OR2 DM/IGT (95% CI)	ß1 AUC glucose	ß2 AUC glucose	ß1 AUC insulin	ß2 AUC insulin
CDKAL1 rs7754840	G/C	1.11 (0.87-1.42)	1.16 (0.90-1.49)	0.04 (-3.3;3.4)	0.7 (-2.6;4.0)	-8.2 (-16.1;-0.41)	-6.0 (-13.3;1.3)
CDKN2A/B rs10811661	T/C	1.08 (0.81-1.44)	1.03 (0.76-1.39)	-0.004 (-4.0;4.0)	-0.4 (-4.2;3.5)	-0.22 (-9.4;8.9)	-1.4 (-9.9;7.1)
HHEX rs1111875	C/T	1.09 (0.86-1.36)	0.99 (0.79-1.27)	-2.1 (-5.2;1.0)	-2.7 (-5.8;0.3)	2.43 (-4.8;9.62)	1.1 (-6.7;6.8)
IGF2BP2 rs4402960	G/T	1.43 (1.11-1.85)	1.42 (1.09-1.84)	3.6 (0.1;7.1)	3.2 (-0.2;6.5)	3.16 (-4.9;11.2)	1.66 (-5.9;9.2)
KCNJ11 rs5219	A/G	0.97 (0.76-1.23)	0.99 (0.77-1.27)	-1.4 (-4.7;1.8)	-1.4 (-4.5;1.8)	2.46 (-5.1;10.0)	2.9 (-4.1;9.9)
SLC30A8 rs13266634	C/T	0.88 (0.68-1.13)	0.91 (0.70-1.18)	0.3 (-3.1;3.8)	0.6 (-2.7;3.9)	4.26 (-3.7;12.2)	4.8 (-2.6;12.1)
TCF7L2 rs7903146	C/T	1.39 (1.08-1.79)	1.37 (1.06-1.78)	4.5 (1.0-8.1)	4.6 (1.2;8.0)	-5.5 (-13.5;2.52)	-5.3 (-12.9;2.1)

OR1 and ß1: adjusted for sex. OR2 and ß2: adjusted for sex and BMI.

Table 3 Interactions of genetic variants with prenatal exposure to famine

Gene variant	OR* interaction DM/IGT (95% CI)	P for interaction	B* interaction AUC glucose (95% CI)	P for interaction	B* interaction AUC insulin(95% CI)	P for interaction
CDKAL1-rs7754840	1.16 (0.70-1.90)	0.57	2.20 (-4.7;9.1)	0.66	7.7 (-8.2;23.7)	0.37
CDKN2A/B-rs10811661	1.37 (0.75-2.51)	0.31	3.46 (-4.8;11.7)	0.42	11.6 (-7.4;30.6)	0.24
HHEX-rs1111875	1.61 (1.01-2.58)	0.04	5.2 (-1.2;11.5)	0.08	-4.7 (-19.4;10.0)	0.55
IGF2BP2-rs4402960	0.82 (0.49-1.37)	0.45	-9.2 (-16.2;-2.1)	0.009	0.49 (-15.9;16.8)	0.94
KCNJ11-rs5219	1.22 (0.75-1.98)	0.41	-2.9 (-9.7:3.9)	0.42	-6.0 (-21.6;9.7)	0.42
SLC30A8-rs13266634	1.38 (0.82-2.32)	0.22	2.0 (-5.0-8.9)	0.62	-7.1 (-23.0-8.9)	0.40
TCF7L2-rs7903146	1.12 (0.68-1.85)	0.66	-0.1 (-7.2;7.1)	0.93	-0.8 (-15.5;17.0)	0.86

*OR and B for the interaction term: All models were adjusted for sex. P values for AUC for glucose and insulin calculated after logarithmic transformation

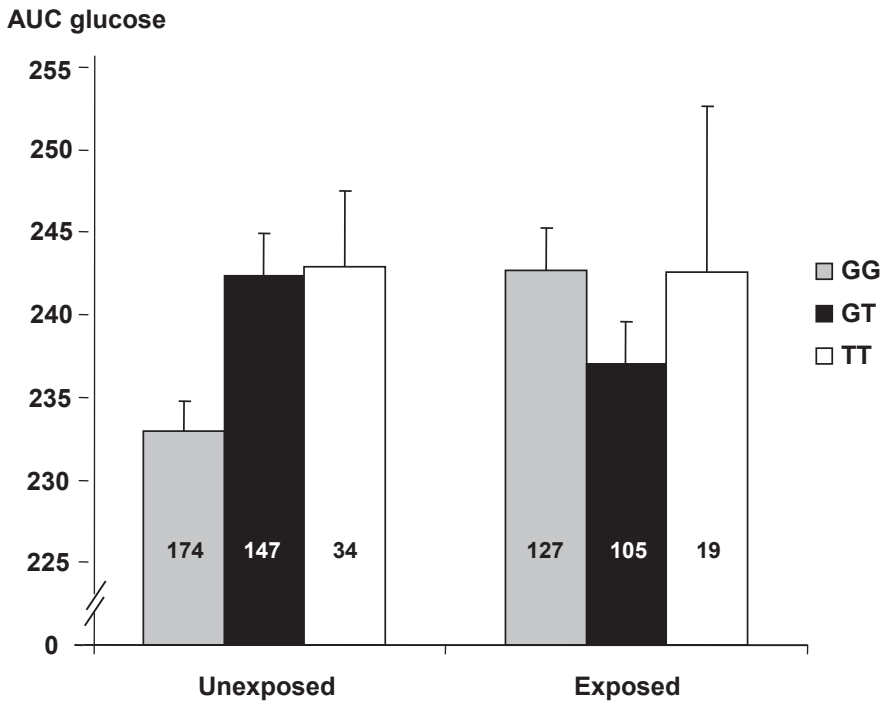


figure 1. The effect of the *IGF2BP2* genotypes on AUC glucose according to prenatal exposure to famine.
n subjects shown inside of each bar, standard errors of the mean shown on top of each bar.

fortunately, a consequent limitation is the size of our study, which limits the power to detect small effects. This especially holds for the categorical outcome type 2 diabetes/IGT. Nonetheless, effects found in the population largely corresponded to previous literature, except for the direction of the effect of the *HHEX* polymorphism, for which we have no explanation. This effect disappeared after adjustment for BMI. Due to power limitations, we were unable to investigate the interactions with timing of exposure to famine during gestation (late, mid or early gestational exposure to famine).

Several studies have identified interactions of size at birth and genetic variants on type 2 diabetes risk and measurements of glucose metabolism.[137-139] Previously, we reported an interaction between a *PPARG* gene variant and fetal malnutrition.[136] Now, we observed a nominally significant interaction of the *IGF2BP2* polymorphism with exposure to famine on glucose levels during OGTT. The effect on type 2 diabetes/IGT risk was in the same direction, but not significant. The presence of both the *IGF2BP2* risk allele and exposure to famine associated with lower AUC for glucose,

which seems counterintuitive considering the main effects of the two factors, which are both associated with increased type 2 diabetes risk. However, a similar interaction was observed in two other studies on the effect of a genetic variant in the *ACE* gene.[137,138] Subjects carrying the risk *ACE* risk allele were relatively protected from the effect of low birth weight on insulin resistance.[137,138] These findings together with the current finding on *IGF2BP2* may be explained by overefficient modulating effects of risk alleles during fetal development. This would imply that the *IGF2BP2* variant, being part of an important developmental pathway, confers a relative resistance to the detrimental consequences of fetal malnutrition on glucose tolerance at an adult age.

IGF2BP2, also referred to as *IMP-2*, is an mRNA binding protein that posttranslationally regulates IGF2, a fetal growth factor, during several developmental stages.[149] IGF2 plays a critical role during placental and fetal development.[150,151] IGFBPs have a tissue-specific expression pattern, with *IGF2BP2* being expressed in fetal lung, kidney, thymus, placenta, and having its highest expression in fetal liver.[149] Interactions between genetic variation in *IGF2BP2* and fetal malnutrition may be exerted through this IGF2 developmental pathway. However, such explanations are fully speculative and it is important to stress that our observation was no longer significant after correcting for multiple testing and should therefore be interpreted with caution. Nonetheless, it may suggest that consequences of the fetal environment depend on an individual's genetic background. Interactions of other variants investigated may unfortunately not have become apparent due to the limited power of our study. None of the polymorphisms was associated with birth weight. This is not in line with the 'fetal insulin hypothesis', which proposes the same genetic factors would alter both intrauterine growth and adult glucose metabolism.[152] The *TCF7L2* gene has consistently been associated with birth weight.[153] Unfortunately, we do not have maternal genotypes available. Mother-child pair analyses suggest that the *TCF7L2* variant effect on birth weight is a reflection of its presence in the mother.[153]

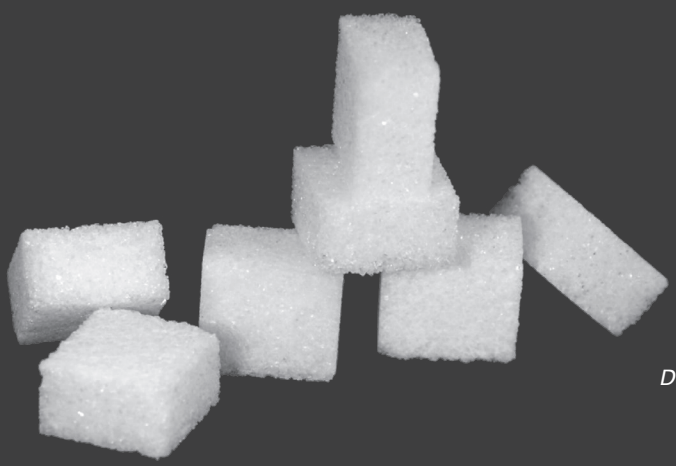
In conclusion, genetic variants involved in fetal development – like *IGF2BP2* - may influence the response to fetal malnutrition and its consequences in the adult hypercaloric environment.

PART III

THE VALUE OF GENETIC INFORMATION FOR PREDICTING TYPE 2 DIABETES

CHAPTER 7

PREDICTING TYPE 2 DIABETES BASED ON POLYMORPHISMS FROM GENOME WIDE ASSOCIATION STUDIES: A POPULATION-BASED STUDY.



M. van Hoek,
A. Dehghan,
J. C.M. Witteman,
C.M. van Duijn,
A.G. Uitterlinden
A. Hofman,
E.J.G. Sijbrands,
A.C.J.W. Janssens.

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ABSTRACT

Background: Prediction of type 2 diabetes based on genetic testing might improve identification of high-risk subjects. Genome Wide Association (GWA) studies identified multiple new genetic variants that associate with type 2 diabetes. The predictive value of genetic testing for prediction of type 2 diabetes in the general population is unclear.

Methods: We investigated 18 polymorphisms from recent GWA studies on type 2 diabetes in the Rotterdam study, a prospective, population-based study among homogeneous Caucasian individuals of 55years and older. (genotyped subjects n=6544; prevalent cases, n=686; incident cases during follow-up, n=601; mean follow-up 10.6 years). The predictive value of these polymorphisms was examined alone and in addition to clinical characteristics using logistic and Cox regression analyses. The discriminative accuracy of the prediction models was assessed by the area under the receiver operating characteristic curves (AUCs).

Results: Of the 18 polymorphisms, the *ADAMTS9, CDKAL1, CDKN2A/B-rs1412829, FTO, IGF2BP2, JAZF1, SLC30A8*, *TCF7L2* and *WFS1* variants were associated with type 2 diabetes risk in our population. The AUC was 0.60 (95% CI 0.57-0.63) for prediction based on the genetic polymorphisms; 0.66 (95% CI 0.63-0.68) for age, sex and BMI and 0.68 (95% CI 0.66-0.71) for the genetic polymorphisms and clinical characteristics combined.

Conclusion: We showed that 9 out of 18 well-established genetic risk variants were associated with type 2 diabetes in a population-based study. Combining genetic variants has low predictive value for future type 2 diabetes at a population-based level. The genetic polymorphisms only marginally improved the prediction of type 2 diabetes beyond clinical characteristics.

INTRODUCTION

Type 2 diabetes is a multifactorial disease caused by a complex interplay of multiple genetic variants and many environmental factors. With the recent Genome Wide Association (GWA) studies, the number of replicated common genetic variants associated with type 2 diabetes has rapidly increased.[14-16,18-20,154] A total of 18 polymorphisms have been firmly replicated.[14-16,18-20,154] It is unclear whether and how the currently known genetic variants can be used in practice, because the combined effect of these variants has not been investigated in a population-based study. Particularly, because most GWA studies were enriched for patients with a positive family history and early onset of the disease, association of these variants to type 2 diabetes risk in the general population, including elderly persons, remains to be determined.

Because complex diseases are caused by multiple genetic variants, predictive testing based on a single genetic marker will be of limited value.[155,156] Simulation studies suggest that the predictive value could be improved by combining multiple common low risk variants.[157-160] Several empirical studies on the predictive value of genetic polymorphisms have been conducted before the recent GWA data were available.[161-163] In a case-control study, Weedon et al showed that combining the information of three polymorphisms improved disease prediction, albeit to a limited extent.[163] Vaxillaire et al investigated 19 polymorphisms and found that the predictive value was low compared to clinical characteristics.[162]

Genetic variants associated with risk of type 2 diabetes could potentially be useful for the prediction, prevention and early treatment of the disease. We investigated whether combining the currently known and well-replicated genetic variants predicts type 2 diabetes in the Rotterdam Study, a prospective population-based follow-up study. We investigated whether these genetic variants improve prediction beyond clinical characteristics.

METHODS

SUBJECTS

The design and data collection of the Rotterdam Study have been described previously.[28] In short, the Rotterdam Study is a prospective, population-based, cohort study among 7,983 inhabitants of a Rotterdam suburb, designed to investigate determinants of chronic diseases. Participants were aged 55 years and older. Baseline examinations took place from 1990 until 1993. Follow-up examinations were performed in 1993-1994, 1997-1999 and 2002-2004. Between these exams, continuous surveillance on major disease outcomes was conducted. Information

on vital status was obtained from municipal health authorities. The medical ethics committee of the Erasmus Medical Center approved the study protocol and all participants gave their written informed consent.

DATA COLLECTION

At baseline, prevalent cases of diabetes were diagnosed by a non-fasting or post-load glucose level (after oral glucose tolerance testing) ≥ 11.1mmol/l and/or treatment with anti-diabetic medication (oral medication or insulin) and the diagnosis diabetes as registered by a general practitioner. During follow-up, diabetes was diagnosed at fasting plasma glucose levels ≥ 7.0 mmol/l and/or a non-fasting plasma glucose levels ≥ 11.0 mmol/l and/or treatment with anti-diabetic medication (oral medication or insulin)[29,117] and the diagnosis diabetes as registered by a general practitioner. Patients registered at general practitioners records with type 1 diabetes were excluded from the present analyses (n=15).

The *CDKAL1 rs7754840, CDKN2AB rs10811661, FTO rs8050136, HHEX rs1111875, IGF2BP2 rs4402960, KCNJ11 rs5219, PPARG rs1801282, SLC30A8 rs13266634 and TCF7L2 rs7903146* polymorphisms were genotyped by means of Taqman allelic discrimination assays. DNA material was available for 6,544 of the 7,983 participants for the Taqman analyses. The assays were designed and optimalized by Applied Biosystems, (Foster City, California, http://store.appliedbiosystems.com). Genotypes were determined in 2-ng genomic DNA. Reactions were performed on the Taqman Prism 7900HT platform. The analyses were performed as described previously.[147] Assays were run on 90 blood bank samples to test for adequate cluster separation. A total of 325 samples were genotyped in duplo. Success rates for Taqman genotyping ranged from 93.2% to 96.7%, with the exception of 86.1% for *IGF2BP2* and 87.4% for *HHEX.* Taqman duplicate error rates for the HHEX and IGF2BP2 polymorphisms were 1.2 and 0.6%

The *ADAMTS9 rs4411878* (proxy for *rs4607103*, r^2 =0.95), *CDC123-CAMK1D rs11257622* (proxy for *rs12779790*, r^2=0.83), *CDKN2A/B rs1412829* (proxy for *rs564398* r^2=0.97), *JAZF1 rs1635852* (proxy for *rs864745* r^2=0.97), *NOTCH2 rs1493694* (proxy for *rs10923931*, r^2=1.0), *TCF2 rs4430796, THADA rs7578597, TSPAN8-LGR5 rs1353362* (proxy for *rs7961581* r^2=0.96) and *WFS1 rs10012946* (proxy for *rs10010131*, r^2=1.0) genotypes were derived from the genotype data of the version 3 Illumina Infinium II HumanHap550 SNP chip array. From a total of 6449 subjects there was sufficient DNA material for the array. Samples with a call rate below 97.5% (n=209), excess autosomal heterozygosity >0.336 (~FDR <0.1% [n=21]), mismatch between called and phenotypic gender (n=36), or if there were outliers identified by the IBS clustering analysis with >3 standard deviations from

population mean (n=102) or IBS probabilities >97% (n=129) were excluded from the analysis; in total, 5974 samples remained for analyses.
The availability of Illumina 550K array data enabled us to compare genotype calls between Taqman and Illumina data for the *FTO, HHEX, IGF2BP2, SLC30A8, TCF7L2* and *CDKAL1* polymorphisms, as well. Concordance rates ranged between 98.6 and 99.7%. To increase success rates we merged the data and deleted pairs that were not concordant. The success rates for the polymorphisms increased to 98.4-99.4%.

STATISTICAL ANALYSES

Associations of individual polymorphisms were investigated using Cox proportional hazards models for the prediction of incident type 2 diabetes and logistic regression analyses for the prediction of prevalent and incident type 2 diabetes together. Analyses were performed crude and adjusted for age, sex and BMI. We also applied Cox proportional hazards models and logistic regression analyses to investigate the combined predictive value of (1) the 18 polymorphisms (all polymorphisms included as separate independent categorical variables) (2) a the risk allele score based on the 18 polymorphisms (assuming all effect sizes of equal weight), (3) age, sex and BMI, and (4) age, sex and BMI and all polymorphisms on type 2 diabetes risk. The risk allele score was calculated by summing up the number of risk alleles for each participant with complete genotype information, with risk alleles being the alleles associated with increased risk of type 2 diabetes.[14-16,18,19] The risk allele score assumes that all genetic variants have the same effect, i.e. minor differences in effects size are ignored. The association between the risk allele score and the predicted probabilities was quantified by the Spearman correlation coefficient.
The discriminative accuracy was evaluated by the area under the Receiver Operating Characteristic (ROC) curves. The Area under the ROC Curve (AUC) can range from 0.5 (total lack of discrimination) to 1.0 (perfect discrimination). AUCs were calculated for the predicted risks of the logistic regression model, the risk allele score, and the linear predictor values of the Cox proportional hazards models. AUCs were compared with Analyse-it® v2.11 (www.analyse-it.com), which uses the method of Hanley and McNeil for ROC curve analyses.[164,165]
The analyses were repeated for subgroups for age (cut-off 70 years of age) and BMI. (cut-off 26kg/m^2). All analyses were performed with SPSS software version 12.0.1.

SIMULATION ANALYSES

A simulation analysis was performed to quantify the expected AUC for prediction of incident type 2 diabetes based on the odds ratios (ORs) of the investigated polymorphisms in literature (ORs: 1.09 for *ADAMTS9*, 1.11 for *CDC123-CAMK1D*, 1.12 for *CDKAL1*, 1.12 for *CDKN2A/B rs1412829*, 1.20 for *CDKN2A/B rs10811661*, 1.17

for *FTO*, 1.13 for *HHEX*, 1.14 for *IGF2BP2*, 1.10 for *JAZF1*, 1.14 for *KCNJ11*, 1.13 for *NOTCH2*, 1.14 for *PPARG*, 1.12 for *SLC30A8*, 1.10 for *TCF2*, 1.37 for *TCF7L2*, 1.15 for *THADA*, 1.09 for *TSPAN8*, and 1.11 for *WFS1*).[12,14,15,19,20,154] The method of simulation has been described in detail elsewhere.[157] In brief, we simulated genetic profiles and type 2 diabetes status for 100,000 individuals, of whom 10,3% were supposed to have incident type 2 diabetes, as observed in our population. Genetic profiles were constructed from the polymorphisms based on observed allele frequencies. Under the assumption that each polymorphism has two alleles and that allele proportions were in Hardy-Weinberg equilibrium, genotype frequencies for the single polymorphisms were calculated. Assuming that the polymorphisms segregate independently, for each individual a genotype was randomly assigned. Disease risks associated with the genetic profiles were modeled using Bayes' theorem. The likelihood ratio (LR) of the genetic profile was calculated by multiplying the LRs of the single genotypes. The OR of the heterozygous genotypes compared to the homozygous non-risk genotypes were derived from the three large GWA studies.[14,15,19] Finally, disease status was modeled by a procedure that compares disease risk of each subject to a randomly drawn value between 0 and 1 from a uniform distribution. This procedure ensures that for each genomic profile, the percentage of people who will develop the disease equals the disease risk associated with that profile, when the subgroup of individuals with that profile is sufficiently large. The simulation was repeated 10 times to obtain a robust estimate of the AUC. The AUC was obtained as the c-statistic by the function somers2, which is available in the Hmisc library of R software (version 2.5.1; www.R-project.org, accessed December 2007).

RESULTS

BASELINE CHARACTERISTICS.

A total of 6544 participants were successfully genotyped for at least one polymorphism. Complete genotype information on all polymorphisms was present in 5297 subjects (of whom 490 were incident cases and 545 were prevalent cases). Age (p=0.11), sex (p=0.22), BMI (p=0.30) and presence of type 2 diabetes (p=0.20) were not significantly different between successfully genotyped persons or persons with one or more missing genotypes. General characteristics of the population are shown in Table 1. Persons with type 2 diabetes had higher BMI, higher waist circumference, more often hypertension and lower HDL cholesterol compared to persons without type 2 diabetes. All polymorphisms were in Hardy Weinberg equilibrium in the total population and in persons without type 2 diabetes. (highest χ^2 =3.58 df=2, p=0.06 for *PPARG rs1801282*)

Table 1. General characteristics of genotyped participants at study baseline by type 2 diabetes status.

Characteristics	All participants	Subjects without type 2 diabetes	Incident cases with type 2 diabetes	Prevalent cases with type 2 diabetes
	n=6,544	n=5,221	n=601	n=686
Age (years)	69.5 ± 0.11	69.0 ± 0.13	68.2 ± 0.32*	73.6 ± 0.35†
Men (%)	40.7	40.4	44.3	39.8
Body Mass Index (kg/m^2)	26.3 ± 0.05	26.0 ± 0.05	28.0 ± 0.15†	26.8 ± 0.15†
Waist circumference (cm)	90.5 ± 0.14	89.6 ± 0.16	94.7 ± 0.45†	93.8 ± 0.46†
Systolic blood pressure (mmHg)	139.3 ± 0.3	137.9 ± 0.31	143.5 ± 0.85†	146.8 ± 0.93†
Diastolic blood pressure (mmHg)	73.7 ± 0.1	73.6 ± 0.2	75.5 ± 0.5†	73.1 ± 0.5
Hypertension (%)	33.4	30.5	46.9†	52.9†
Total cholesterol (mmol/l)	6.6 ± 0.02	6.6 ± 0.02	6.6 ± 0.05	6.5 ± 0.05
HDL-cholesterol (mmol/l)	1.34 ± 0.005	1.37 ± 0.005	1.25 ± 0.01†	1.25 ± 0.01†
Current smoking (%)	22.1	22.5	25.5	22.1
Former Smoking (%)	40.7	42.0	42.8	39.0

Continuous variables are expressed as means ± standard error. Type 2 diabetes status was missing for 36 individuals.
*p<0.05, †p<0.001 for comparison with subjects without type 2 diabetes. Comparisons between groups were performed using ANOVA for continuous variables and χ^2-test for categorical variables.

INDIVIDUAL EFFECTS OF CLINICAL CHARACTERISTICS AND POLYMORPHISMS ON TYPE 2 DIABETES RISK.

Table 2 shows the effect of each polymorphism on type 2 diabetes risk (prevalent and incident type 2 diabetes). The minor alleles of the *CDKAL1, FTO, IGF2BP2 and TCF7L2* variants were associated with increased risk of type 2 diabetes. The *ADAMTS9, CDKN2A/B rs1412829, JAZF, SLC30A8 and WFS1* minor alleles decreased type 2 diabetes risk. Cox regression analyses restricted to incident type 2 diabetes results gave similar results. Adjustment for age, sex and BMI did not materially change the results, except for the *FTO* polymorphism, for which the effect on type 2 diabetes risk disappeared after adjustment for BMI.
In a Cox regression analysis, age (HR 1.02, 95% CI1.01-1.03), sex (HR 0.67, 95% CI 0.57-0.79) and BMI (HR 1.14, 95% CI 1.12-1.16) affected prospective type 2 diabetes risk.

RISK ALLELE SCORE AND RISK OF TYPE 2 DIABETES

Figure 1 shows the ORs associated with increasing risk allele scores compared to the reference group (0-12 risk alleles) in a logistic regression model. Persons carry-

Table 2. Individual effects nine polymorphisms on type 2 diabetes risk, with and without adjustment for covariates.

Gene variant	Risk allele	Genotype (n)	% Cases		% Controls	All cases	Incident type 2 diabetes	
			Prevalent	Incident		OR (95% CI)	Crude HR (95% CI)	Adjusted HR * (95% CI)
ADAMTS9	*C*	*CC*(3450)	61.9	61.1	57.4	1.0	1.0	1.0
rs4411878†		*CT*(2159)	32.5	34.7	37.1	0.84 (0.74-0.97)	0.88 (0.73-1.05)	0.87 (0.73-1.04)
		TT(321)	5.6	4.2	5.5	0.84 (0.62-1.13)	0.68 (0.45-1.04)	0.68 (0.45-1.04)
CDC123/	*C*	*TT*(3952)	64.4	67.4	67.9	1.0	1.0	1.0
CAMK1D		*TC*(1758)	31.5	28.8	29.6	1.04 (0.90-1.20)	0.98 (0.82-1.19)	0.98 (0.81-1.18)
rs11257622‡		*CC*(213)	4.2	3.8	3.5	1.18 (0.84-1.64)	1.07 (0.69-1.67)	1.05 (0.68-1.63)
CDKAL1	*C*	*GG* (3097)	47.1	45.4	48.7	1.0	1.0	1.0
rs7754840		*GC* (2692)	41.1	43.0	41.9	1.05 (0.93-1.20)	1.09 (0.92-1.29)	1.10 (0.93-1.31)
		CC (633)	11.8	11.6	9.4	1.31 (1.07-1.61)	1.31 (1.001-1.72)	1.38 (1.06-1.80)
CDKN2A/B	*A*	*AA* (1915)	31.3	35.2	32.1	1.0	1.0	1.0
rs1412829§		*AG* (2910)	47.7	50.4	49.2	0.97 (0.84-1.12)	0.94 (0.78-1.13)	0.89 (0.74-1.08)
		GG (1098)	21.0	14.5	18.7	0.93 (0.77-1.12)	0.72 (0.56-0.94)	0.70 (0.54-0.92)
CDKN2A/B	*T*	*TT* (4131)	72.0	63.1	66.0	1.0	1.0	1.0
rs10811661		*TC* (1865)	24.7	34.5	30.1	0.95 (0.83-1.09)	1.17 (0.99-1.39)	1.13 (0.95-1.34)
		CC (232)	3.2	2.4	3.9	0.70 (0.49-1.02)	0.64 (0.37-1.08)	0.68 (0.40-1.17)

FTO	*A*	*CC* (2526)	38.3	38.4	39.8	1.0	1.0	1.0
rs8050136		*CA* (2944)	44.0	46.3	46.3	1.01 (0.88-1.16)	1.03 (0.86-1.23)	1.00 (0.84-1.20)
		AA (927)	17.7	15.3	14.0	1.23 (1.02-1.47)	1.12 (0.88-1.43)	1.06 (0.83-1.36)
HHEX	*C*	*CC* (2235)	36.1	36.3	34.8	1.0	1.0	1.0
rs1111875		*CT* (3097)	50.1	46.1	48.4	0.96 (0.84-1.10)	0.91 (0.76-1.09)	0.93 (0.78-1.12)
		TT (1052)	13.7	17.6	16.8	0.89 (0.74-1.07)	1.01 (0.80-1.27)	1.01 (0.79-1.28)
IGF2BP2	*T*	*GG* (3101)	46.9	47.7	49.4	1.0	1.0	1.0
rs4402960		*GT* (2650)	41.8	41.5	42.0	1.04 (0.91-1.18)	1.01 (0.85-1.19)	1.01 (0.85-1.20)
		TT (575)	11.3	10.8	8.6	1.35 (1.09-1.66)	1.24 (0.95-1.63)	1.23 (0.94-1.62)
JAZF1	*T*	*TT*(1646)	31.0	29.8	27.1	1.0	1.0	1.0
rs1635852[‖]		*TC*(2927)	46.5	48.6	49.8	0.85 (0.73-0.98)	0.88 (0.73-1.07)	0.85 (0.70-1.04)
		CC(1357)	22.5	21.6	23.1	0.85 (0.71-1.02)	0.86 (0.68-1.09)	0.84 (0.66-1.06)
KCNJ11	*G*	*AA* (2394)	37.7	39.9	39.2	1.0	1.0	1.0
rs5219		*AG* (2925)	48.3	46.6	47.8	1.00 (0.88-1.15)	0.97 (0.81-1.16)	0.97 (0.81-1.16)
		GG (807)	14.0	13.5	13.0	1.07 (0.88-1.30)	1.02 (0.79-1.32)	1.02 (0.79-1.32)
NOTCH2	*T*	*CC*(4670)	77.8	79.0	78.8	1.0	1.0	1.0
rs1493694[¶]		*CT*(1168)	20.7	19.7	19.6	1.04 (0.89-1.22)	1.03 (0.84-1.27)	1.07 (0.86-1.32)
		TT(92)	1.4	1.3	1.6	0.86 (0.50-1.49)	0.75 (0.35-1.57)	0.74 (0.35-1.56)

Table 2. Continued

Gene variant	Risk allele	Genotype (n)	% Cases		% Controls	All cases	Incident type 2 diabetes	
			Prevalent	Incident		OR (95% CI)	Crude HR (95% CI)	Adjusted HR * (95% CI)
PPARG	*C*	*CC* (4888)	79.7	76.9	77.1	1.0	1.0	1.0
rs1801282		*CG* (1322)	19.1	21.7	21.1	0.95 (0.81-1.10)	1.03 (0.84-1.25)	0.99 (0.81-1.21)
		GG (108)	1.2	1.4	1.8	0.70 (0.41-1.20)	0.76 (0.38-1.54)	0.66 (0.33-1.34)
SLC30A8	*C*	*CC* (3176)	49.8	54.1	48.8	1.0	1.0	1.0
rs13266634		*CT* (2709)	43.7	38.2	42.4	0.91 (0.80-1.04)	0.82 (0.69-0.97)	0.84 (0.70-0.99)
		TT (546)	6.6	7.7	8.8	0.75 (0.59-0.96)	0.80 (0.59-1.09)	0.84 (0.62-1.14)
TCF2	*G*	*AA*(1560)	25.9	24.1	26.8	1.0	1.0	1.0
rs4430796		*AG*(2924)	48.1	50.7	49.6	1.06 (0.91-1.24)	1.11 (0.90-1.36)	1.14 (0.92-1.40)
		GG(1417)	26.1	25.2	23.6	1.16 (0.97-1.39)	1.16 (0.91-1.47)	1.16 (0.91-1.48)
TCF7L2	*T*	*CC* (3292)	43.7	47.5	52.6	1.0	1.0	1.0
rs7903146		*CT* (2587)	42.4	42.3	39.7	1.23 (1.08-1.41)	1.19 (1.01-1.41)	1.20 (1.01-1.42)
		TT (554)	13.9	10.2	7.7	1.82 (1.48-2.24)	1.48 (1.12-1.95)	1.62 (1.22-2.14)
THADA	*T*	*TT*(4608)	79.9	78.8	77.3	1.0	1.0	1.0
Rs7578597		*TC*(1227)	19.0	19.0	21.1	0.88 (0.75-1.03)	0.91 (0.73-1.12)	0.90 (0.72-1.12)
		CC(94)	1.1	2.2	1.6	1.01 (0.60-1.67)	1.32 (0.74-2.34)	1.51 (0.85-2.67)

TSPAN8/	*C*	*TT*(3018)	50.4	48.3	51.4	1.0	1.0	1.0
LGR5		*TC*(2409)	40.3	41.8	40.6	1.05 (0.92-1.20)	1.10 (0.92-1.31)	1.08 (0.90-1.29)
Rs1353362#		*CC*(493)	9.3	9.9	8.0	1.25 (0.99-1.57)	1.29 (0.96-1.73)	1.21 (0.90-1.62)
WFS1	*C*	*CC*(2179)	40.7	40.4	35.8	1.0	1.0	1.0
*Rs1412829***		*CT*(2801)	44.6	45.7	47.8	0.83 (0.73-0.96)	0.86 (0.72-1.04)	0.87 (0.73-1.05)
		TT(949)	14.7	13.9	16.4	0.77 (0.63-0.93)	0.77 (0.59-1.00)	0.76 (0.58-0.99)

* Hazard ratio (HR) adjusted for age, sex and BMI; † proxy for rs4607103, r^2 =0.95; ‡proxy for rs12779790; § proxy for rs564398 r2=0.97: ||proxy for rs864745 r^2=0.97, ¶proxy for rs10923931, r^2=1.0, #proxy for rs7961581 r^2=0.96, **proxy for rs10010131, r^2=1.0

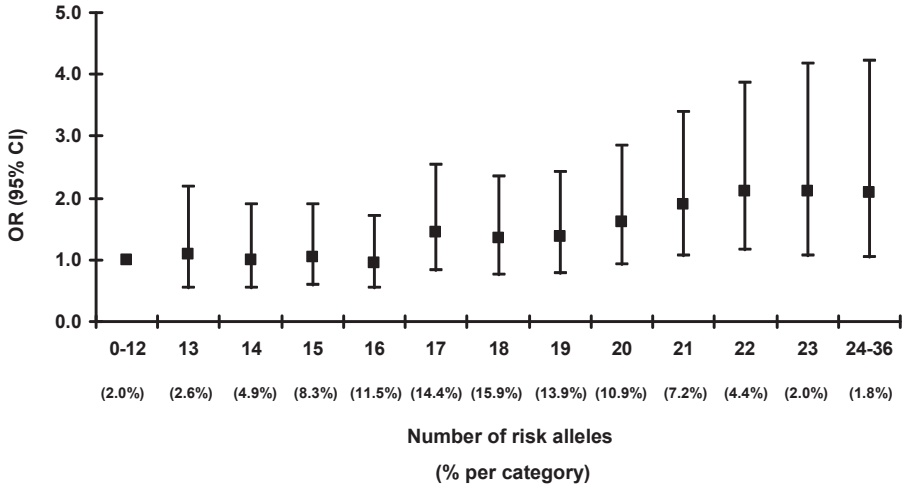


Figure 1. Odds ratios for type 2 diabetes according to the number of risk alleles carried.

ing 21 risk alleles or more (14.4% of the population) had significantly higher type 2 diabetes risk. (7.2% of the population carried 21 alleles: OR 1.90 95%CI 1.07-3.40; 4.4% had 22 alleles: OR 2.11 95%CI 1.15-3.86; 2.0% had 23 alleles: OR 2.11 95%CI 1.07-4.18; 1.8% had 24-32 alleles: OR 2.10 95%CI 1.04-4.22) compared with the reference group of 0-12 alleles (N=109; 2.0% of the population) In a Cox regression analysis on incident cases of diabetes, this figure was similar (data not shown). The per allele HR was 1.04, 95%CI 1.02-1.07 (p=0.001).

RISK ALLELE SCORE

Figure 2 shows the predicted type 2 diabetes risks from the logistic regression model that included all 18 genetic polymorphisms in relation to the risk allele score. The Spearman correlation coefficient was 0.60, indicating a wide range of predicted risks for each value of the risk allele scores. When analyzing only incident type 2 diabetes, this figure was similar (Spearmen rho 0.59, figure not shown).

ANALYSES OF DISCRIMINATIVE ACCURACY

Figure 3 shows the ROC curves for the prediction of incident type 2 diabetes based on the genetic polymorphisms, clinical characteristics and both. The AUC was 0.60 (95% CI 0.57-0.63) for prediction based on the genetic polymorphisms, 0.66 (95% CI 0.63-0.68) for age, sex and BMI and 0.68 (95% CI 0.66-0.71) for the genetic polymorphisms and clinical characteristics combined. The difference between the AUCs for clinical characteristics with and without the genetic polymorphisms was significant (p<0.0001). The AUC of the risk allele score was 0.56 (95% CI 0.53-0.59). When including only the significantly associated genetic variants of the current

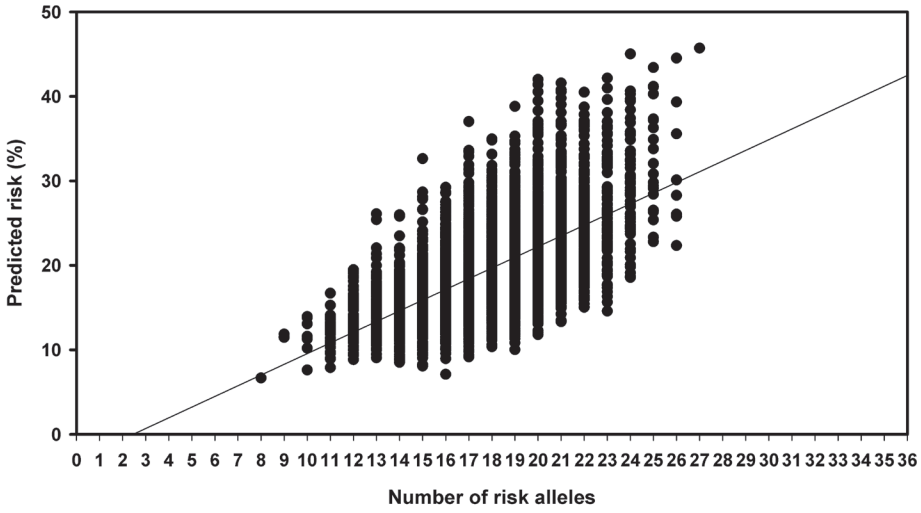


Figure 2. Correlation of predicted type 2 diabetes risks with the risk allele score. Predicted risks of type 1 diabetes were obtained from the logistic regression model with 18 genetic polymorphisms as independent categorical variables.

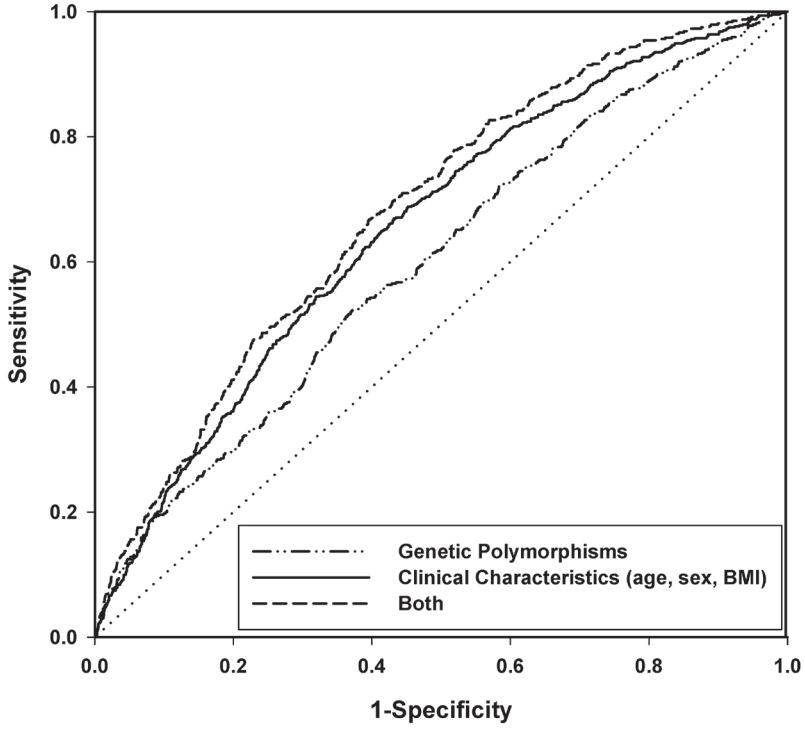


Figure 3. ROC curves for prediction of incident type 2 diabetes based on 18 genetic polymorphisms, clinical characteristics (age, sex and BMI), and both.

study (*ADAMTS9, CDKAL1, CDKN1412829, FTO, IGFBP2, JAZF1, TCF7L2, SLC30A8* and *WFS1*) the AUC was 0.58 (95% CI 0.56-0.61). Combining the *KCNJ11, PPARG* and *TCF7L2* variants resulted in an AUC of 0.53 (95% CI 0.50-0.55). Based on the simulation study, the expected AUC for all genetic polymorphisms using the effect sizes described in literature was 0.57. When combining incident and prevalent type 2 diabetes cases, the AUC of all polymorphisms was 0.60 (95%CI 0.58-0.62).
In subgroup analyses, the AUC of all polymorphisms was 0.62 (95%CI 0.58-0.67) in the low BMI group and 0.59 (95%CI 0.56-0.62) in the high BMI subgroup. The AUC was 0.61 (0.59-0.65) in the low age-group and 0.63 (0.58-0.67) in the high age-group.

DISCUSSION

We investigated the predictive value of 18 type 2 diabetes risk polymorphisms from the recent GWA studies for the prediction of type 2 diabetes in a large, prospective, population-based study of elderly persons. Our study shows that combining information of these 18 well-replicated variants has relatively low discriminative accuracy for the prediction of type 2 diabetes in a general population (AUC = 0.60). The 18 genetic variants identified to date did not substantially improve the discriminative accuracy of disease prediction based on clinical characteristics.
In line with the results of the GWA studies[14-16,18,19,166], the *ADAMTS9, CDKAL1, CDKN2A/B, FTO, IGFBP2, JAZF1, SLC30A8, TCF7L2* and *WFS1* were associated type 2 diabetes risk in our population. Some of these effects were slightly stronger than the effects described previously. In contrast to most previous studies, the *KCNJ11* polymorphism was not associated. The ORs of the other polymorphisms were similar to previously published results, but not statistically significant, which may be explained by the smaller number of type 2 diabetes cases and therefore smaller power to reach significance in our prospective study.
A risk allele score, obtained by counting the number of risk alleles, can be used as a simple proxy of the combined effect of multiple polymorphisms. Risk allele scores ignore the effect sizes of the individual genetic variants, but a previous simulation study has shown that this has limited impact on the discriminative accuracy.[158] In contrast, we found a wide range of predicted risks for each value of the risk allele score, suggesting that differences in the variants carried result in substantial differences in actual disease risks. The risk allele score associated with modest increases in disease risk and the AUC for prediction was 0.56. When taking effect size differences between polymorphisms into account the AUC was 0.60, showing that the risk allele score predicted less accurately. Other empirical studies have not

investigated the differences in diagnostic accuracy between simple risk allele scores and predicted risks obtained from regression models.[161-163,167]

The discriminative accuracy of predictive genetic testing in complex diseases depends on the number of genes involved, the risk allele frequencies and the size of the associated risks.[157] The maximum discriminative accuracy is determined by the heritability of the disease.[157] Based on previously published effect sizes for the 18 polymorphisms, we predicted that the AUC would be 0.57, and based on our empirical data we found that it was 0.60. This difference is explained by the fact that some polymorphisms had a slightly larger effect in our population than described in literature.[12,14-16,19,20,154,163] Nonetheless, the discriminative accuracy of all known replicated type 2 diabetes susceptibility variants to date was rather low. However our analysis was based on 18 common variants with relatively small effects. The heritability of type 2 diabetes is estimated to range from 30 to 70% depending on the population investigated[168] and many common variants with small effects or fewer rare variants with stronger effects are still to be discovered. These may further improve the discriminative accuracy of predictive genetic testing for type 2 diabetes.

Several previous studies have investigated the predictive value of multiple genetic variants in type 2 diabetes, either alone or in addition to clinical characteristics. An overview of the studies performed so far in Caucasian populations and the number of genes investigated is provided in Table 3. Weedon et al. investigated three variants that were also in our study and reported an AUC of 0.58[163] where we found an AUC of 0.53 for the same polymorphisms. The population of Weedon et al consisted for a large part of patients who had early onset of type 2 diabetes or a positive family history of type 2 diabetes, whereas our population included elderly subjects from the general population. The percentage of variance of the disease explained by genetic factors is expected to be higher in populations with a positive family history for the disease. And this may lead to a higher diagnostic accuracy for genetic variants. Recently, Chauci et al investigated 15 genetic variants that

Table 3. Overview of diagnostic accuracies obtained from earlier empirical studies on genetic risk variants and type 2 diabetes.

Study	No. of genetic variants	AUC Genetic variants	AUC Clinical characteristics	AUC Combined
Weedon et al.[163]	3	0.58	NR	NR
Lyssenko et al.[161,169]	2	NR	0.68*	0.68
Vaxillaire et al.[162]	3	0.56	0.82†	0.84
Cauchi et al.[167]	15	NR	NR†	0.86

NR: not reported

*clinical characteristics: Fasting plasma glucose and BMI

†clinical characteristics: age, sex and BMI

were associated in GWA analyses in a French case-control study.[167] The AUC of the genetic variants together with age, sex and BMI was 0.86. Unfortunately, the AUC was not calculated for genes and clinical characteristics separately to assess the additive value of genetic information. Some of the included variants were specifically identified in this case-control study and had relatively large effects on type 2 diabetes risk compared to effects found in meta-analysed GWA studies.[14,15,19,20] It is therefore difficult to generalize these findings to other populations and we expect that our population-based prospective study yielded more realistic estimates. Two other studies investigated the improvement of the discriminative accuracy by adding genetic test results to clinical characteristics.[161,162] Even though we included the 18 firmly replicated polymorphisms to date and they predominantly tested other genetic variants, our findings were similar demonstrating no substantial added value of genetic information beyond clinical characteristics.[161,162,169]

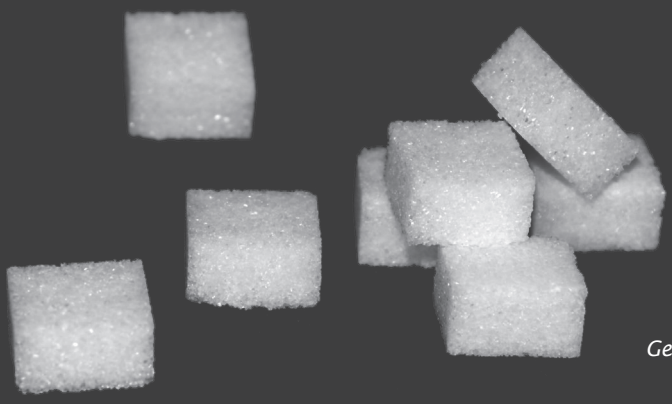
We can only speculate on the reasons why these genetic variants have little added value beyond clinical characteristics. First, the effects of the genetic variants on type 2 diabetes risk could be exerted through clinical characteristics such as BMI, which implies that including both genes and intermediate factors in the regression model will reduce the effect of the gene. However, adjustment for clinical characteristics did not substantially change the effect of the genetic variants on type 2 diabetes risk (Table 2). Second, the effects of age, sex and BMI on type 2 diabetes risk in our population may outweigh the contribution of the genetic variants. Such an effect was illustrated in an earlier study, which showed that a genetic predisposition became apparent in subjects with less other risk factors.[170] In our elderly population, one may expect that non-genetic risk factors are more prevalent compared to younger populations. However, the AUC for the genetic variants was higher than expected from the simulation analyses. This makes an underestimation of the contribution of the genetic variants in our population unlikely.

Obvious strengths of our study are the large size of the study population, the population-based design and the long period of follow-up. Despite these advantages, the number of incident type 2 diabetes cases was still relatively low to demonstrate statistically significant effects of low-risk susceptibility genes. Furthermore, we had insufficient statistical power to formally investigate gene-gene and gene-environment interactions. In age and BMI subgroup analyses the estimates for prediction based on the genetic variants were similar and showed overlapping confidence intervals. However, due to smaller numbers of cases in the subgroups these results should be interpreted with caution. Cauchi et al. reported gene-gene interactions of recently discovered loci, but did not report on the effects of these interactions on the AUC.[167] Earlier studies found no evidence for strong gene-gene interactions.[162,163] Taking into account these interactions may further improve the discriminative accuracy.

In conclusion, we showed that nine out of 18 currently well-established genetic risk variants were associated with type 2 diabetes in a population-based study. The currently known and replicated genetic variants found in GWA studies contributed modestly to the prediction of type 2 diabetes in a population-based setting and marginally improved the risk prediction when added to clinical characteristics. Future research should aim at identifying and replicating new genetic susceptibility variants, gene-gene and gene-environment interactions to approach levels of discriminative accuracy that enable the identification of at-risk groups.

CHAPTER 8

EVALUATION OF RISK PREDICTION UPDATES FROM COMMERCIAL GENOME WIDE SCANS



R. Mihaescu,
M. van Hoek,
E.J.G. Sijbrands,
A.G. Uitterlinden,
J. C.M. Witteman,
A. Hofman,
C.M. van Duijn,
A.C.J.W. Janssens.

Genet Med. 2009 Aug;11(8):588-94.

ABSTRACT

Background: Commercial internet-based companies offer genome-wide scans to predict the risk of common diseases and personalize nutrition and lifestyle recommendations. These risk estimates are updated with every new gene discovery.

Methods: To assess the benefits of updating risk information in commercial genome-wide scans, we compared type 2 diabetes risk predictions based on *TCF7L2* alone, 18 polymorphisms alone, and 18 polymorphisms plus age, sex, and body mass index. Analyses were performed using data from the Rotterdam study, a prospective, population-based study among individuals aged 55 years and older. Data were available from 5297 participants.

Results: The actual prevalence of type 2 diabetes in the study population was 20%. Predicted risks were below average for carriers of the *TCF7L2* CC genotype (predicted risk 17.6%) and above average for the *CT* and *TT* genotypes (20.8% and 28.0%). Adding the other 17 polymorphisms caused 34% of participants to be reclassified (i.e., switched between below and above average): 24% of the CC carriers changed to increased risk, 52% and 6% of the CT and TT carriers changed to decreased risk. Including information on age, sex, and body mass index caused 29% to change categories (27%, 31%, and 19% for CC, CT, and TT carriers, respectively). In total, 39% of participants changed categories once when risk factors were updated, and 11% changed twice, i.e., back to their initial risk category.

Conclusion: Updating risk factors may produce contradictory information about an individual's risk status over time, which is undesirable if lifestyle and nutritional recommendations vary accordingly.

INTRODUCTION

The accelerating rate of genomic discoveries is rapidly increasing our understanding of the genetic basis of common diseases. Recent genome-wide association studies have identified novel susceptibility variants for type 2 diabetes, age-related macular degeneration, cancer, and many other common diseases.[171] These discoveries have fueled expectations about applications of predictive genetic tests in preventive and clinical health care.[172,173] It is envisioned that genetic tests will personalize medicine through targeted treatment for patients with common diseases and individualized lifestyle and dietary recommendations for high-risk individuals.[174,175]

Although genome-based clinical and public health applications still await empirical evidence, several companies already offer online genetic tests to predict an individual's risk of common diseases.[176] These tests are based on single susceptibility genes (e.g., DNA direct[177]); based on genetic profiles using a limited number of variants (e.g., Sciona[178] and Genovations[179]), or genome-wide scans (e.g., 23andMe[180], Navigenics[181], and de-CODEme[182]); and based on whole genome sequencing (e.g., Knome[183]). It is widely acknowledged that testing single susceptibility genes is uninformative for predicting common diseases as, on their own, they only minimally affect disease risk[156,184,185] and most currently offered profiles based on a few selected variants are uninformative as they lack a firm scientific basis for the polymorphisms included.[176]

Companies that offer genome-wide scans take a more rigorous approach in the selection of the variants, but the clinical validity and utility of their results may also be limited at present, as susceptibility genes for common diseases are still being discovered. Because of this, risk predictions from genome-wide scans frequently become outdated when scientific knowledge progresses. Therefore, commercial companies offer updates of the risk predictions when new susceptibility genes are discovered. Given that single new variants only have a minor contribution to disease risk, we might expect that risk predictions change minimally at each update. However, as many individuals will have disease risks that are only slightly higher or lower than average[186], even minor updates may reclassify people from below to above average disease risk or vice versa, and lifestyle and nutrition recommendations may vary accordingly.

We investigated the extent to which updating of risk predictions leads to reclassification of individuals from below to above average disease risk or vice versa. Taking type 2 diabetes as an example, we compared risk predictions based on a single gene, on multiple polymorphisms and on multiple polymorphisms combined with age, sex, and body mass index (BMI). Analyses were performed using data from the Rotterdam Study, a population-based cohort of individuals aged 55 years and older.

METHODS

SUBJECTS

The design and data collection of the Rotterdam Study was been described elsewhere.[28] In short, the Rotterdam Study is a prospective, population-based, cohort study among 7983 inhabitants of a Rotterdam suburb, designed to investigate determinants of chronic diseases. Participants were aged 55 years and older. Baseline examinations took place from 1990 until 1993, and follow-up examinations were performed in 1993–1994, 1997–1999, and 2002–2004. Among these examinations, continuous surveillance on major disease outcomes was conducted. The medical ethics committee of the Erasmus Medical Center approved the study protocol, and all participants gave their written informed consent.

DATA COLLECTION

The following polymorphisms[187] were genotyped: *TCF7L2 rs7903146* (MIM 602228), *CDKAL1 rs7754840 (MIM 611259), CDKN2A/B rs10811661 (MIM 600160, MIM 600431), FTO rs8050136 (MIM 610966), HHEX rs1111875 (MIM 604420), IGF2BP2 rs4402960 (MIM 608289), KCNJ11 rs5219 (MIM 600937), PPARG rs1801282 (MIM 601487), SLC30A8 rs13266634 (MIM 611145), ADAMTS9 rs4411878 (MIM 605421), CDC123-CAMK1D rs11257622 (MIM 607957), CDKN2A/B rs1412829, JAZF1 rs1635852 (MIM 606246), NOTCH2 rs1493694 (MIM 600275), TCF2 rs4430796 (MIM 189907), THADA rs7578597 (MIM 611800), TSPAN8-LGR5 rs1353362 (MIM 600769, MIM 606667),* and *WFS1 rs10012946 (MIM 606201).*[14-16,18,19] Details on genotyping techniques, genotype success and odds ratios for the genotyped variants have been published elsewhere.[188]

At baseline, diagnostic criteria for prevalent cases of diabetes were a nonfasting or a postload glucose level (after oral glucose tolerance testing) >=11.1 mmol/L and/or treatment with glucose-lowering medication (oral medication or insulin) with a diagnosis of diabetes recorded by a general practitioner. During follow-up, incident cases of diabetes were diagnosed at fasting plasma glucose levels >=7.0 mmol/L, and/or nonfasting plasma glucose levels >=11.1 mmol/L, and/or treatment with glucose-lowering medication (oral medication or insulin[29,117]), with a diagnosis of diabetes recorded by a general practitioner. Patients with a recorded diagnosis of type 1 diabetes were excluded from the present analyses (n = 15). BMI was calculated as weight (kg) divided by height (m) squared. Age and BMI were obtained from the baseline assessment.

STATISTICAL ANALYSES

Predicted risks were obtained using logistic regression analyses with type 2 diabetes (prevalent and incident cases) as the dependent variable. All polymorphisms were entered as categorical variables in the analyses, allowing effect sizes to differ between heterozygous and homozygous carriers of the risk alleles. To evaluate how risk predictions change after adding more information, we compared first risk predictions based on the strongest genetic predictor of type 2 diabetes, *TCF7L2*, alone, 18 polymorphisms including *TCF7L2*, and 18 polymorphisms plus age, sex, and BMI. Second, we compared risk predictions based on clinical factors, clinical factors and *TCF7L2*, and clinical factors plus all 18 polymorphisms. Predicted risks from the three models were evaluated by comparing risk distributions and discriminative accuracy and by examining reclassification. To evaluate how risk predictions change when each polymorphism is added individually, we simulated 1000 random permutations of all possible orderings of the added polymorphisms. Discriminative accuracy, measured as the area under the receiver operating characteristic curve (AUC), indicates the degree to which the predicted risks can discriminate between individuals who will develop the disease and those who will not. AUC can range from 0.50 (equal to tossing a coin) to 1.00 (perfect discrimination). Reclassification was calculated as the percentage of individuals who switched from being at increased to being at decreased risk, when compared with the average risk in the population or vice versa.[189] Reclassification was assessed in individuals with complete genotype and clinical information. Analyses were performed using the SPSS software version 15.0.1 and R programming language version 2.8.0.

RESULTS

GENERAL CHARACTERISTICS

A total of 6544 participants were successfully genotyped for at least one polymorphism. Complete genotype information on all polymorphisms was available from 5297 participants, of whom 490 were incident and 545 were prevalent cases of type 2 diabetes (i.e., 20% had type 2 diabetes). Of those with complete genotype information, 41% were men, mean age was 69.5 years (standard deviation 9.1 years), and mean BMI was 26.3 kg/m^2 (standard deviation 3.7 kg/m^2). Complete information on genotype, age, sex, and BMI was available from 5111 participants. The average risk of type 2 diabetes in the population was defined as the actual prevalence (i.e. 20%).

IMPROVING RISK PREDICTION AT A POPULATION LEVEL

Prediction based on the 18 polymorphisms and clinical characteristics yielded more differentiation in predicted risks than prediction based on one or multiple polymorphisms alone (Figure 1), which means that adding more risk factors yielded more extreme risk predictions. For example, the 5% of the population indicated to be at highest risk had a predicted risk of 28.0% based on *TCF7L2* but predicted risks of at least 29.7% based on the 18 polymorphisms and at least 36.8% based on the polymorphisms plus clinical factors. This increased differentiation is also reflected in higher AUCs. The AUC was 0.55 (95% CI: 0.53–0.57) for prediction based on *TCF7L2*, 0.60 (95% CI: 0.58–0.62) for prediction based on 18 polymorphisms, and 0.66 (95% CI: 0.64–0.68) for prediction based on 18 polymorphisms plus age, sex, and BMI. At the average risk, the main improvement in model performance was reflected in the increase in specificity. The specificity was 51.8% for prediction based on *TCF7L2*, 62.7% for prediction based on 18 polymorphisms, and 64.5% for prediction based on 18 polymorphisms plus age, sex and BMI (Table 1).

Table 1. Measures of model performance for dichotomizing predicted risk at average risk

	Model based on *TCF7L2*	Model based on 18 polymorphisms	Model based on 18 polymorphisms, age, sex and body mass index
Sensitivity	55.7%	50.8%	57.2%
Specificity	51.8%	62.7%	64.5%
PPV	21.8%	24.7%	28.0%
NPV	82.9%	84.1%	86.2%

Measurements are based on individuals with complete genotype and clinical information. PPV indicates positive predictive value. NPV indicates negative predictive value.

RECLASSIFICATION

Predicted risks were lower than average for carriers of the *TCF7L2* CC genotype (predicted risk 17.6%) and higher than average for the CT and TT genotypes (20.8% and 28.0%, respectively; Figure 2). As indicated by the larger standard deviations predicted risks diverged after adding novel risk factors, leading to reclassification. Based on testing the 18 polymorphisms, 33.5% of the participants were reclassified: 23.6% of noncarriers switched to the increased risk category and 43.6% of the heterozygous and homozygous carriers (51.6% and 5.6%, respectively) switched to the decreased risk category (Table 2). Based on all polymorphisms, age, sex, and BMI, 28.5% of participants switched their risk category compared with prediction based on the 18 polymorphisms alone; the proportion of switchers was 26.5%, 31.4%, and 19.1% for carriers of the CC, CT and TT genotypes, respectively (data not shown). Overall, predicted risks changed from above to below average or vice versa in 50% of all individuals: 39% switched once and 11% switched twice, i.e., back to their initial risk category (Figure 3).

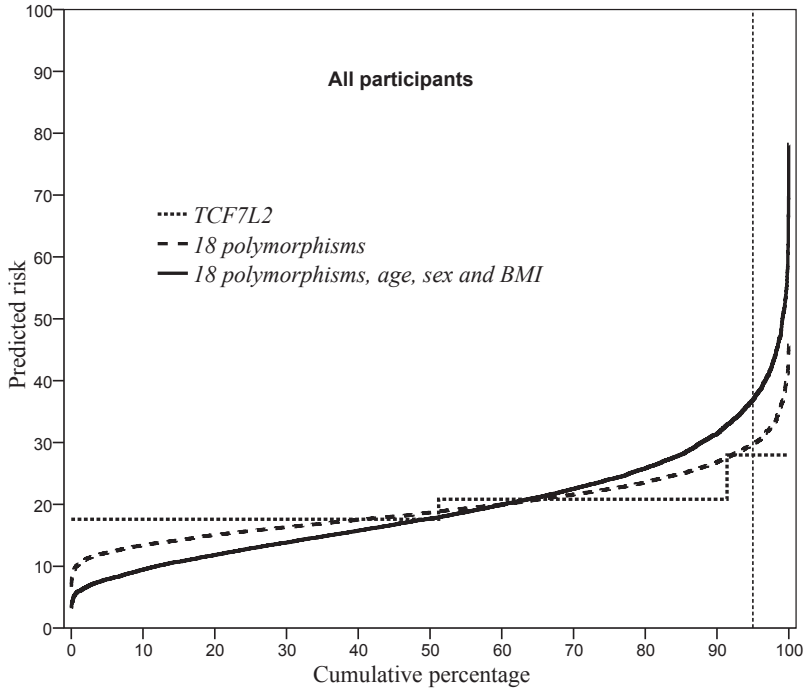


Figure 1a

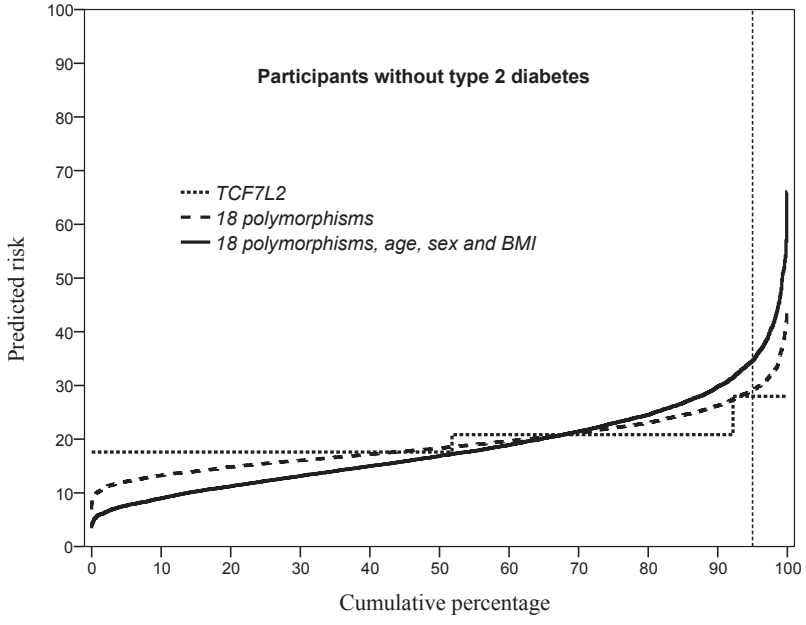


Figure 1b

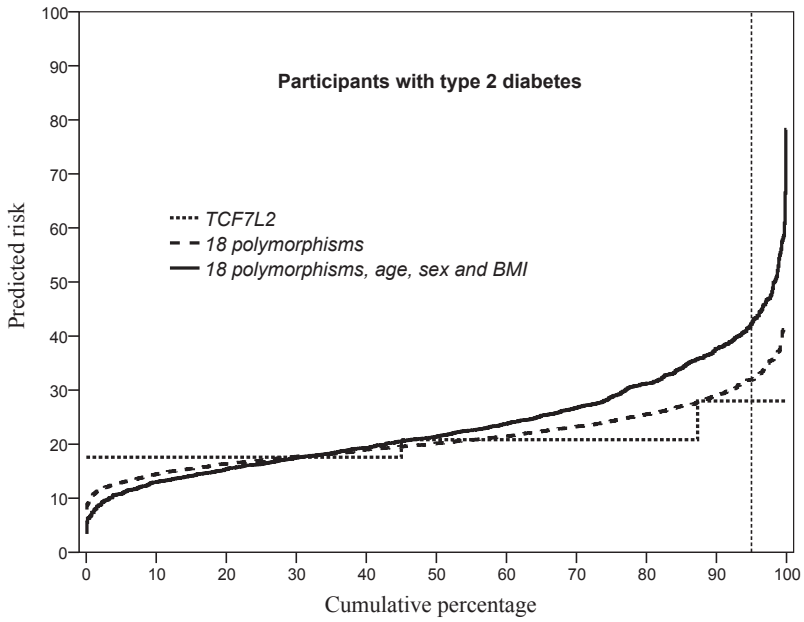


Figure 1c

Figures 1a, 1b, 1c. Predictiveness curves for *TCF7L2* alone, 18 polymorphisms alone, and 18 polymorphisms plus age, sex and body mass index
Legend: Predicted risks were obtained using logistic regression analysis. Cumulative percentage indicates the percentage of the population that has a predicted disease risk equal or lower than the risk value. E.g. based on genetic testing of 18 polymorphisms 90% (x-axis) of the individuals have a predicted risk lower than 26.8% (y-axis). Figure 1a shows the predictiveness curves for all participants, 1b for participants without type 2 diabetes, and 1c for participants with type 2 diabetes. BMI indicates body mass index (calculated as weight in kilograms divided by height in meters squared).

UPDATING A MODEL STARTING FROM AGE, SEX, AND BMI

The AUC was 0.63 (95% CI: 0.61–0.65) for prediction based on age, sex, and BMI, 0.64 (95% CI: 0.62–0.66) for prediction based on age, sex, BMI, and *TCF7L2*, and 0.66 (95% CI: 0.64–0.68) for prediction based on age, sex, and BMI plus 18 polymorphisms. Starting from predictive testing based on age, sex, and BMI, 13.2% of participants were reclassified after updating by *TCF7L2*. Based on age, sex, BMI, and all polymorphisms, 16.3% of participants switched their risk category compared with prediction based on age, sex, BMI, and *TCF7L2*. Overall, predicted risks changed from above to below average or vice versa in 25.6% of all individuals: 21.7% switched once and 3.9% switched twice, i.e., back to their initial risk category.

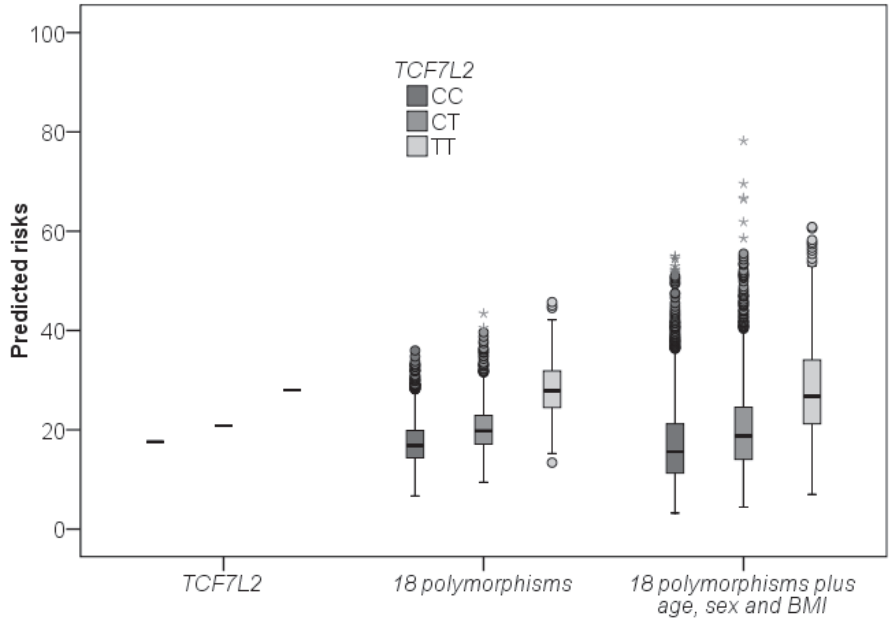


Figure 2. Predicted risk of type 2 diabetes based on *TCF7L2* alone, 18 polymorphisms alone, and 18 polymorphisms plus age, sex and body mass index
Legend: Predicted risks were obtained using logistic regression analysis. The bold line shows the median, the boxes indicate the interquartile ranges (25%-75% range), and the whiskers present 1.5 times the interquartile range. The points represent outliers and the asterisks represent extreme outliers that have values more than three times the interquartile range. BMI indicates body mass index (calculated as weight in kilograms divided by height in meters squared).

UPDATING BY ADDING EACH POLYMORPHISM INDIVIDUALLY

Finally, we considered risk updating by each additional polymorphism individually to the model that was based on testing *TCF7L2* alone, up to the model based on all 18 polymorphisms. Using 1000 random orderings in which the 17 polymorphisms can be added to the profile, we calculated that on average 47% (standard deviation 1.2%) of the participants ultimately switched at least once when risks were updated after every single polymorphism (Figure 4). Seventeen percent switched once, and 30% switched multiple times (range 2–15) from below to above average disease risk or vice versa. When *TCF7L2* was also added in a random order, on an average, 71% (standard deviation 8.3%) of the participants switched at least once (data not shown).

Table 2. Risk stratification table for the pairwise comparison of two consecutively updated prediction models

	Model based on 18 polymorphisms		Total		Model based on 18 polymorphisms, age, sex and body mass index		Total
Model based on *TCF7L2*	Below average	Above average		Model based on 18 polymorphisms	Below average	Above average	
Below average				Below average			
n (%)	1966 (76.4)	607 (23.6)	2573 (100)	n (%)	2366 (77)	706 (23)	3072 (100)
% of total	38.5	11.9	50.4	% of total	46.3	13.8	60.1
Cases, n	298	141	439	Cases, n	303	185	488
Observed risk(%)	15.2	23.2	17.1	Observed risk (%)	12.8	26.2	15.9
Above average				Above average			
n (%)	1106 (43.6)	1432 (56.4)	2538 (100)	n (%)	717 (35.2)	1322 (64.8)	2039 (100)
% of total	21.6	28.0	49.6	% of total	14.7	25.9	39.9
Cases, n	190	363	553	Cases, n	122	382	504
Observed risk(%)	17.2	25.3	21.8	Observed risk(%)	17.0	28.9	24.7
Total				Total			
n (%)	3072 (60.1%)	2039 (39.9)	5111 (100)	n (%)	3083 (60.3)	2028 (39.7)	5111 (100)
% of total	60.1%	39.9	100	% of total	60.3	39.7	100
Cases, n	488	504	992	Cases, n	425	567	992
Observed risk(%)	15.9%	24.7	19.4	Observed risk(%)	13.8	28.0	19.4

Reclassification was calculated in participants for whom all data were available to avoid part of the reclassification being caused by differences in the average risk rather than differences in predicted risks.

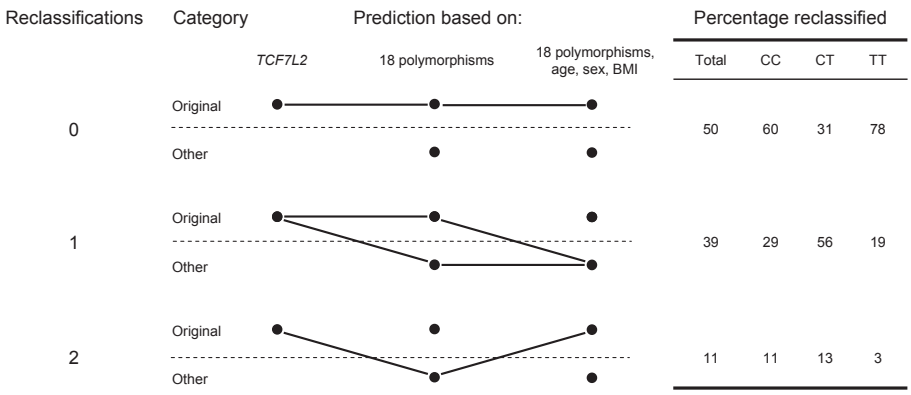


Figure 3. Patterns of reclassification that result from updating risk predictions
Legend: The number of reclassifications represents how many times a person switched between risk categories based on the three prediction models. E.g. a person did not reclassify (reclassification is 0) if they had above average or below average risks according to all three models. A person reclassified once (reclassification is 1) if they switched risk categories from the model based on *TCF7L2* to the model based on 18 polymorphisms, or from the model based on 18 polymorphisms to the model including clinical factors. The table explains what percentage of people reclassifies 0, 1 or 2 times, overall and by *TCF7L2* genotype. BMI indicates body mass index (calculated as weight in kilograms divided by height in meters squared).

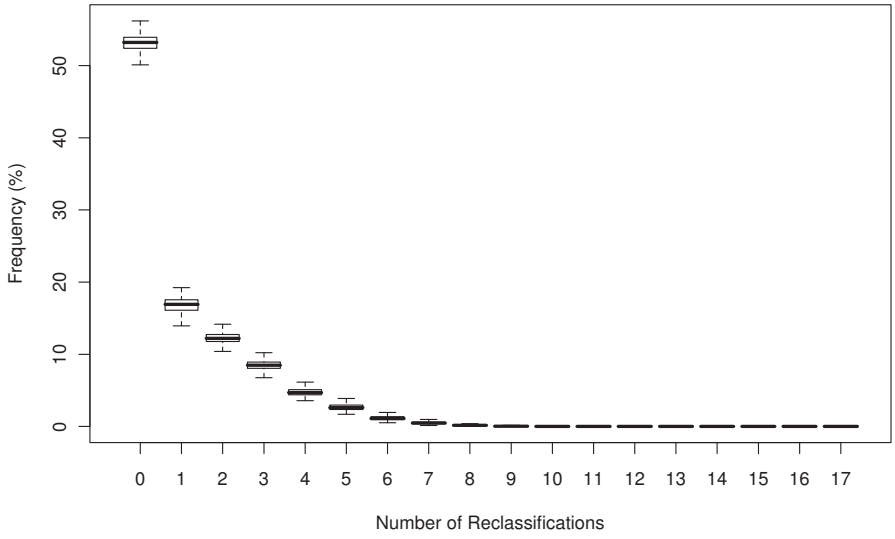


Figure 4. Median percentage of reclassification in step by step update of prediction based on *TCF7L2* to prediction based on 18 polymorphisms
Legend: The plot is obtained from a simulation of 1000 permutations of single polymorphism updates. Numbers on the x axis represent no reclassification (i.e. number of reclassification is 0) up to reclassification at each step (i.e. number of reclassification is 17). The bold line shows the median, the boxes indicate the interquartile ranges (25%-75% range), and the whiskers present 1.5 times the interquartile range.

DISCUSSION

Using type 2 diabetes as an example, we showed that updating risk predictions by including more polymorphisms, age, sex, and BMI improved risk prediction at the population level as reflected in the higher AUC values. However, at the individual level, we found that 34% of the participants switched between risk categories when risks were updated from 1 to 18 polymorphisms and that 29% switched when age, sex, and BMI were taken into consideration. In total, 39% of the participants switched risk categories once and 11% switched twice.

Before interpreting the public health relevance of these results, two methodological issues of our study, that may affect the degree of reclassification, should be pointed out. First, although we investigated 18 established type 2 diabetes polymorphisms, only about half were statistically significantly associated with type 2 diabetes risk in our population.[188] This is in line with other studies that investigated the combined predictive value of the 18 polymorphisms, which also found that not all polymorphisms were statistically significantly associated with the disease.[162,190-192] If the effect sizes of all polymorphisms in our study had been the same as in the original studies that had identified their associations, we would have observed a larger variation in predicted risks (Figure. 2) and likely also more reclassification.

Second, we focused on changes in risk prediction based on *TCF7L2* with that based on 18 polymorphisms, age, sex, and BMI, which reflects the practice of commercial companies. However, in clinical settings, it is more logical to update a model based on recognized clinical risk factors. We showed that when risks were updated from age, sex, and BMI to age, sex, BMI, and *TCF7L2*, and further to age, sex, BMI, and all 18 polymorphisms, 22% of individuals switched risk categories once, and 4% twice. We compared risk prediction based on *TCF7L2* with that based on 18 polymorphisms, age, sex, and BMI, but we considered only two risk updates: one based on adding 17 polymorphisms and one on adding age, sex, and BMI. The percentage of reclassification was even higher when we considered risk updating by each additional polymorphism individually, as is done by the companies. The exact percentage of reclassification then varies with the order in which polymorphisms are added, and for 1000 random orderings of the 17 polymorphisms, we calculated that 47% of the participants would have switched at least once when risks were updated after every single polymorphism, when compared with 34% of the participants when the 17 polymorphisms were added in a single update.

The reason why people switch between risk categories is that the added polymorphisms may have different effects on disease risk compared with the polymorphisms already considered. Figure 2 showed that individuals who are at increased risk according to their *TCF7L2* genotype may be at decreased risk of type 2 diabetes,

when all 18 polymorphisms are considered if they inherited protective genotypes on many other polymorphisms. In our analyses, the risk increase conferred by *TCF7L2* risk alleles was counterbalanced by protective effects of other alleles in 6% of the individuals and was counterbalanced by young age, male sex, and normal BMI in 19% of the individuals.

Our final prediction model included 18 polymorphisms, age, sex, and BMI, but it is important to realize that risk predictions can be further improved. Even with our current understanding of genomic factors, prediction of type 2 diabetes risk can be improved by also considering family history and fasting plasma glucose levels,[192] factors that are currently not considered by the companies that offer genome-wide scans. In the future, risk prediction may be improved by the addition of novel genetic factors, novel biomarkers, and with gene-gene and gene-environment interactions if these are demonstrated in future genetic epidemiologic studies.[193,194] Thus, the risk predictions presented in this article are not final and individuals may be subject to further reclassification as science advances.

Commercial companies assert that genome-wide scans will help consumers to learn their likelihood of developing a disease, but it is widely agreed that risk predictions and results from genetic tests are difficult for the lay public to understand.[195,196] To facilitate the interpretation of risk estimates, companies present the predicted risks together with the average risk of the disease for the total population or for a sex- and age-matched population. Individuals can thereby learn whether they are at higher or lower risk than others and, based on this information, may decide to make lifestyle and dietary changes. However, individuals differ in the way they value the information gained from genetic testing. Some may find a slight increase in predicted risk sufficiently motivating to adopt or maintain healthy behaviors, whereas others may not even change their behavior when they learn that their risk is markedly increased. A systematic review of the psychological and behavioral impact of genetic testing for hereditary nonpolyposis carcinoma, hereditary breast, and ovarian cancer, and Alzheimer disease reported that, generally, 12 months after testing, perceived risk in carriers decreased to the pretest level or even below it.[197] A study on the harms and benefits of *APO-E* genotyping in first-degree relatives of patients with Alzheimer disease reported that disclosure of genotype status increased the motivation for risk reduction activities.[198] Note that previous studies mainly addressed psychological and behavioral impact of genetic testing for monogenic and major gene disorders, and these findings cannot be directly translated to the impact of low-risk susceptibility genetic testing.

If individuals are informed that they have switched categories from above to below average risk of disease, or vice versa, their perceptions about the need for health behavior changes may vary accordingly. In current commercial genome scans, risks

are updated on every new gene discovery and individuals may frequently reclassify over time. To date, it is unknown how individuals respond to variations in risk predictions over time and how it affects their perceptions about the threat of being at increased risk.[199-201] Because health behavior changes are difficult to achieve, we might expect that individuals will become insensitive to risk information if they learn that their risk status may change over time, even without any lifestyle changes. Also, reclassification primarily focuses on changes in risk compared with the average risk and less on the absolute risks of disease. The absolute risk should be important as well in decision making about healthy behavior, and it is of interest to find out whether absolute or comparative risk information influences health behavior change. Such potentially adverse consequences of updating risk predictions warrant further investigation.

The companies that offer genome-wide scans or whole genome sequencing for the prediction of multiple diseases take a higher scientific standard for the selection of susceptibility variants than those previously reviewed.[176] They include only variants that have been consistently associated in multiple studies, and transparently present the polymorphisms that constitute genetic profiles for each disease, including references to scientific studies demonstrating their impact on disease risk. Nevertheless, with scientific advance their risk predictions may further improve, as causation of disease is better understood, and the benefit of these updates at the individual level are unclear. This does not imply that the introduction of genome-based applications in health care should wait until we completely understand the etiology of diseases, but we need to recognize that a premature introduction may have adverse effects.

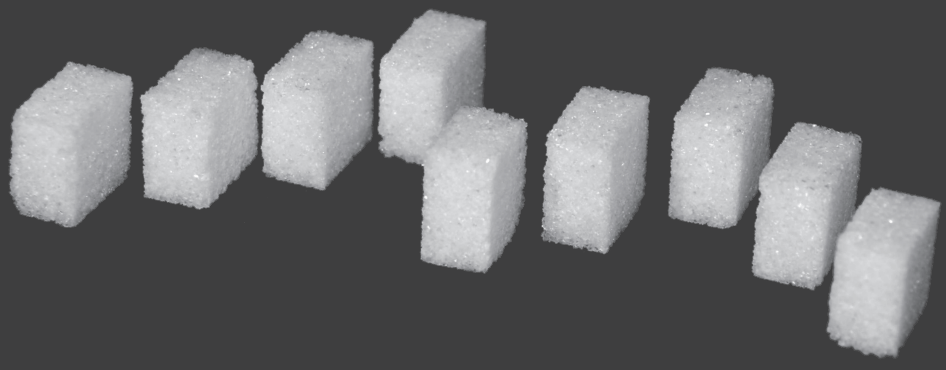
PART IV

GENERAL DISCUSSION

General Discussion Chapter 9

CHAPTER 9

GENERAL DISCUSSION



In this thesis, the associations and interactions of several candidate genes in relation to type 2 diabetes risk were investigated. Furthermore, the predictive ability of currently known type 2 diabetes risk-associated genes were explored. In this section, I will discuss main findings, methodological issues and implications for clinical practice and future research.

9.1 MAIN FINDINGS

PART II: ASSOCIATIONS AND INTERACTIONS

Type 2 diabetes is a multifactorial disease probably caused by intricate interactions between genes and environmental factors.

In Part II of this thesis, we have investigated several genes and their interactions with specific environments. Variants in adipokines may be relates to type 2 diabetes. First, we investigated a genetic variant in *RBP4* (chapter 3), a recently identified adipokine, and its interaction with vitamin A on type 2 diabetes risk. Then, we explored the effects of adiponectin polymorphisms and plasma levels on lipid metabolism and statin treatment effects in patients with type 2 diabetes (chapter 4).

Genes involved in lipid and fat metabolism may also affect type 2 diabetes risk. We investigated promoter variants in *ApoC3*, an apolipoprotein, and its interaction with body mass index on type 2 diabetes risk (chapter 5).

Finally, we have investigated a set of genetic variants (identified by the first wave of GWAs on type 2 diabetes) and their interactions with fetal environment on the risk of type 2 diabetes (chapter 6).

RBP4; INTERACTIONS WITH VITAMIN A (CHAPTER 3)

RBP4 is originally known as the principal plasma transport protein for retinol.[24] However, in more recent years, *RBP4* was appreciated as an adipokine as well.[23] A Mongolian case-control study identified a functional variant associated with *RBP4* levels and type 2 diabetes risk.[27] In our population-based prospective study, we found increased type 2 diabetes risk in homozygotes for the minor allele of this variant, in line with the previous study. Two other studies did not support a similar association, possibly due to differences in population characteristics and size.[34,35]

The possible mechanisms of action of *RBP4* are unknown and may be mediated by the protein itself as well as retinoids carried by it. Vitamin A has long been known to be involved in metabolism and retinoids are regulators of gene transcription through a large number of nuclear receptors.[36] In our study, we did not find associations or interactions of the polymorphism with retinol intake and vitamin A plasma levels on type 2 diabetes risk.

Whether *RBP4* primarily affects insulin resistance or insulin secretion is under investigation. Most studies have pointed towards a mechanism related to insulin resistance through decreased insulin signaling in muscle and adipose tissue and increased hepatic gluconeogenesis through upregulation of PEPCK.[23] However, one study found that *RBP4* levels were negatively associated with insulin secretion.[202] Alternatively, retinol may be related to beta cell function through transthyretin. In our large population based study we could not perform such functional studies. However, we did not find an association of the *RBP4* polymorphisms with need for insulin therapy within patients in our population (unpublished data). Although limited by power, our data do not support an insulin secretion mediated mechanism. These findings should be replicated by an independent population.

ADIPONECTIN; INTERACTIONS WITH STATIN TREATMENT (CHAPTER 4)

Adiponectin is an adipokine that is thought to be involved in multiple metabolic pathways, including both glucose and lipid metabolism. Type 2 diabetes is often accompanied by metabolic derangements such as dyslipidemia, adding up to the increased risk of cardiovascular complications. Statins are a very effective treatment for a number of dyslipidemias. Unfortunately, low HDL-cholesterol, one of the main characteristics of diabetic dyslipidemia, responds poorly to statin treatment. We investigated whether adiponectin influences statin treatment effects in patients with type 2 diabetes and found that adiponectin tertiles prior to treatment were related to the HDL raising effect of statins. Moreover, we investigated the relationships between adiponectin and lipase activities, that were described in previous studies.[65,66,68] We found that adiponectin polymorphisms showed a significant association with lipoprotein lipase and hepatic lipase activities, thereby providing evidence for the direction of this effect.

It is an ongoing discussion whether adiponectin has a causal relation to metabolic disease or is merely a reflection of a insulin resistant state. Although causal evidence in mice is present[203-205], it is largely lacking in humans. The relationship between lipase activities and adiponectin is possibly bidirectional. Our finding of adiponectin polymorphisms associated with lipase activities supports a causal relation in which adiponectin affects the lipase activities. However, these are non-replicated findings with marginal statistical significance.

We do not have a biological explanation for the association of adiponectin levels with statin treatment effect. Lipase activities did not explain the effect of adiponectin on statin treatment. Whether causal or not, this finding emphasizes that separate groups of patients may benefit differently from specific treatment.

APOC3; INTERACTIONS WITH BODY MASS INDEX (CHAPTER 5)

Patients with type 2 diabetes show a large range of phenotypes, for example a wide range of body mass indices. Generally, overweight is considered one of the main risk factors for type 2 diabetes, but a substantial proportion of patients are lean. Based on an association found between *APOC3* variants and type 1 diabetes[109], we investigated if lean type 2 diabetes might show genetic similarities to type 1 diabetes. In two independent cohorts, lean carriers of the risk genotype showed increased type 2 diabetes risk, while this effect was not observed in overweight subjects. Moreover, the risk variants were associated with need for insulin therapy, suggesting a beta cell related mechanism.

ApoCIII is an apolipoprotein involved in triglyceride metabolism. The mechanism behind the interaction might involve lipid related or lipid unrelated mechanisms. The polymorphisms responsible for the interaction are located in an insulin responsive element, and might lead to defective insulin mediated downregulation of the gene.[115] ApoCIII may impair triglyceride catabolism by inhibition of LPL mediated lipolysis. Triglyceride and free fatty acids level have been linked to diabetes through both insulin resistance and decreased insulin production[112]. In addition, a direct effect of ApoCIII on beta cell function has been found *in vitro.*[113]

Mechanisms through insulin downregulation, LPL function or both, might explain the interaction with BMI. Both insulin and LPL may quantitatively and functionally differ between lean and overweight individuals.[206,206,207] This study underlines the possibility that subgroups of diabetes may exist within the current clinically defined entities.

IGF2BP2; INTERACTIONS WITH FETAL ENVIRONMENT (CHAPTER 6)

Fetal malnutrition may predispose to type 2 diabetes through mechanisms described in the Barker hypothesis[7,133], i.e. fetal programming of gene expression and impaired organogenesis. It is likely that genetic variation may interact with these mechanisms. In the Dutch Famine Birth Cohort, we investigated seven firmly established type 2 diabetes risk variants[14-16,19] and their interactions with fetal malnutrition. The Dutch Famine Birth Cohort confers a unique opportunity to investigate fetal malnutrition through experimental conditions that were unintentionally created during World War II.[146] We found a nominal significant interaction for an *IGF2BP2* variant with fetal malnutrition on OGTT test results.

IGF2BP2 is a posttranslational modulator of IGF2[149], which plays a critical role during fetal development and is therefore an excellent candidate for interacting with fetal programming. The direction of the interaction found was counterintuitive, resulting in the best OGTT results in subjects exposed to both famine and to the *IGF2BP2* risk allele. However, a similar finding has been reported by others regarding an interac-

tion between the *ACE* gene and birthweight.[137,138] These findings may be interpreted as an overefficient modulating effect, where the genetic variant confers resistance to detrimental circumstances. Notably, a recently performed study in Goto-Kakizaki diabetic rats supports our finding, showing that intrauterine malnutrition increases IGF-2 production and thereby improves the development of beta cell mass in the fetus.[208]

A similar set of genetic variants was investigated in the Helsinki Birth cohort for interactions with birth weight. No interaction with birth weight on type 2 diabetes risk for the *IGF2BP2* variant was found, although significant interaction were described for *HHEX, CDKN2A/2B* and *JAZF1* gene variants.[209] In our study, none of the polymorphisms showed a significant interaction with birth weight. It must be taken into consideration that birth weight and fetal malnutrition are not identical. Birth weight is a surrogate endpoint that might influenced by other factors besides nutritional status, such as genetic predisposition of both the mother and the fetus for body stature and weight. Moreover, animal studies indicate that substantial effects of fetal malnutrition may be present without affecting birth weight.[210]

Overall, the findings of our study support the fact that interactions may exist between an individuals fetal environment and genetic background. This may involve epigenetic changes and effects on organogenesis and development.

PART III: PREDICTION

Accurate prediction of diseases is one of the main goals of translational genetic research. Because the genetic make-up of an individual is more or less stable throughout life, this would provide the advantage of testing disease risk early in life, before clinically apparent and potentially irreversible risk factors have emerged. Thereby, early intervention for prevention of disease may be facilitated.

In part III of this thesis, we investigated the predictive ability of 18 firmly established genetic risk variants in a large population-based study (chapter 7) and the effect of updating genetic risk predictions for commercial use (chapter 8).

PREDICTION OF TYPE 2 DIABETES BASED ON 18 GENETIC VARIANTS (CHAPTER 7)

Type 2 diabetes is a polygenic disease and a large number of established genetic variants should be combined to get an adequate prediction model. Moreover, the prediction based on genetic variants should be compared and combined with conventional risk factors to test for any added value.

In our prospective population-based study, we investigated the predictive capacity of 18 genetic variants that were firmly established at the time of our study. We found that the predictive capacity of the genetic variants was modest and added little to clinical characteristics alone. We found that adding 18 genetic variants to

clinical characteristics only marginally improved disease risk prediction. Our study was in line with a number of other studies.[161-163,167,190-192]
The low predictive ability of genetic variants in our study is not surprising, since simulation studies already have suggested that either models containing hundreds of genetic variants of the currently known effect sizes or a number of variants with much larger effects are needed to accurately predict a common multifactorial disease as type 2 diabetes.[157] Moreover, it is a complex, only partially heritable disease, caused by an intricate interplay between genes and environment. Interactions between genes, and between genes and environment are currently unknown and therefore not taken into account in prediction models.

UPDATING RISK PREDICTIONS WITH INCREASING GENETIC KNOWLEDGE (CHAPTER 8)
Despite the fact that prediction with the current genetic knowledge is probably insufficient for use in clinical practice, companies have developed commercial genome-wide scans that are publicly available. The risk estimates of these commercial tests are frequently updated when scientific knowledge has progressed. In chapter 8, we investigated the extent to which updating of risk predictions results in reclassification of individuals from increased to decreased disease risk and vice versa. We investigated the effects on reclassification of updating a prediction model from 1 genetic variant to 18 genetic variants and finally to 18 genetic variants combined with age, sex and BMI. By performing these updates a total of 50% of individuals switched risk category from above to below average risk and vice versa. (39% switched once, 11% switched twice). The analyses described involve only 3 steps of updating risk, while in practice companies perform updates with every new genetic discovery. One can imagine that getting opposing risk estimates and consequent lifestyle advices with every update may lead to confusion or even incompliance to the advice given. As an example, an individual might at some point get the advice to lose weight because of high type 2 diabetes risk, while months later by increased genetic knowledge, he/she will get a reassuring test result, not being at increased risk anymore, without any need for a change in lifestyle.
These frequent reclassifications are a reflection of the fact that genome-wide commercial diagnostics are currently not fit for individual use.

9.2 METHODOLOGICAL ISSUES

With regard to the research described in this thesis, a number of methodological issues should be addressed.

STUDY POPULATIONS AND STATISTICS

THE ROTTERDAM STUDY

The Rotterdam study is a prospective population-based cohort with a long period of follow up and meticulous identification of incident cases of diabetes.[28] The major strength of such a study design is the low risk of selection bias in this random sample from the general population. A consequent disadvantage is the relative small number incident cases which reduces power.

The Rotterdam study is a relatively old population, since its aim is to study diseases in the elderly. Findings in this study may not be totally generalizible to other, notably younger, populations. On the other hand, gene discoveries from GWA studies showed similar risk estimates in our study compared to the original discovery studies.

THE DUTCH FAMINE BIRTH COHORT

The Dutch Famine Birth Cohort is a unique cohort that provides the opportunity to study the effects of fetal malnutrition. In comparison with other studies, it has the advantage of an objective measure of fetal malnutrition instead of a surrogate measure such as birth weight. A consequent disadvantage of the study is its relative small size. Another disadvantage of the study size is that it did not allow us to investigate the interaction of genetic variants within groups of separate gestational age. Previous findings in this cohort indicate that the effects of exposure to fetal malnutrition may depend on the timing of exposure.[211] However, exposure to famine during any stage of gestation was associated with glucose intolerance.[146,212]

Selection issues should also be considered. Women are less fertile during famine. Women that did conceive (especially those exposed during early gestation) may have had a more favorable constitution than others. However, correcting for maternal characteristics possibly related to fertility (weight, parity) did not alter our results. Infant mortality may have induced selection, as well. Nonetheless, the health outcomes in the most seriously affected groups were homogeneous, indicating that such an effect cannot have been large. Finally, selection through participation of the fittest individuals and prior mortality may be present. This effect was kept to a minimum by performing home visits, but cannot be excluded and may have led to an underestimation of malnutrition effects found in the study. All genetic variants studied were in Hardy-Weinberg equilibrium, making a specific selection based on these variants unlikely.

Besides the Barker hypothesis, an alternative hypothesis on metabolic diseases in relation to birth weight and disease risk is the fetal insulin hypothesis[213], which proposes that low birth weight and consequent risk of disease is a reflection of the

genetic profile of both the fetus and its parents. Unfortunately, we did not have access the maternal and paternal genotypes, meaning that we could not investigate such effects. Notably, the *IGF2BP2* polymorphism was not related to birth weight in our study, which makes an effect via the fetal insulin hypothesis unlikely.

DALI STUDY

The Dali study is a double blind randomized controlled trial for the effects of statin treatment in patients with type 2 diabetes.[71] This design is a major strength of the study. The participants were selected based on a mild hypertriglyceridemia, which is frequent in type 2 diabetes. This means that we have investigated statin treatment effects in a subset of patients, i.e. the ones clearly suffering from dyslipidemia. Laborious measurements of post heparin lipase and hepatic lipase were available. A limitation of the study is its size, which limits power for the genetic analyses.

GENETIC ASSOCIATION AND INTERACTION STUDIES

Candidate gene studies as described in chapter 3, 4, 5 and 6 have been subject to criticism. The main pitfalls of the method being lack of power and replication. Our studies may have suffered from these pitfalls, although we have made substantial efforts to reduce this risk. We have specifically chosen loci within candidate genes of which the biological function suggests presence in the causal path. Moreover, we preferred variants with localization in genetic regions with a strong indication of having biological impact on gene function or transcription.

In the *RBP4* gene, we have chosen the *-803GA* polymorphism located in an hepatic nuclear factor1α (HNF1α) binding domain. *In vivo* the A allele showed increased *RBP4* levels for the A allele, and *in vitro* this allele exerted increased binding of HNF1α, greater transcriptional activity and enhanced expression in adipocytes.[27,214]

In the Dali study, we have investigated two polymorphisms in the adiponectin gene in relation to lipase activities. At the time of our study, these polymorphisms were two of the most frequently associated genetic variants with metabolic syndrome and cardiovascular disease in other studies, although they have been subject to difficulties in replication, as is the case for all adiponectin polymorphisms. They are located in 2 separate haploblocks of the gene and were chosen based on their strong association with adiponectin plasma levels in a large Caucasian study.[79] The -11391GA polymorphism is located in the promoter region of the gene. In a subsequent meta-analysis this variant was significantly associated with adiponectin plasma levels.[88] The +45Tg polymorphism was chosen based on its location in exon 2, in the second LD block of the gene. Although many studies have found associa-

tions of this variant with adiponectin plasma levels, type 2 diabetes and coronary artery disease, these results were unfortunately inconsistent.[88]

The *APOC3* gene was chosen as a candidate gene based on its involvement in lipid metabolism, as well as, a recent association of promoter variants with type 1 diabetes. The *-482CT* and *-455TC* variants in the promoter gene are located in an insulin responsive element. The polymorphisms have been shown to impair insulin binding and subsequent downregulation.[115]

The choice of genetic variants in chapters 6, 7 and 8 was based on GWA findings.[14-16,19,20] These are firmly established type 2 diabetes risk variants. We have investigated the interactions of these variants with fetal malnutrition in chapter 6 and applied them in genetic prediction models in chapters 7 and 8.

PREDICTION BASED ON GENETIC VARIANTS

In chapter 7, we evaluated the predictive capacity of 18 firmly established type 2 diabetes risk variants in a prospective population-based study and their added value upon clinical characteristics (age, sex and BMI).

The power of the Rotterdam study for effects of individual polymorphisms used in the prediction models described in chapters 7 was limited. For prediction purposes, the effect sizes of the individual polymorphisms are important, irrespective of their statistical significance. The effect sizes of the polymorphisms tested in our study, were largely comparable to the effect sizes in their discovery studies.[15,19,20] The confidence intervals of the AUCs did not contain the value 0.50, indicating that there was sufficient power to show an effect significantly different from prediction by chance.

In the studies described in chapters 7 and 8, risk prediction may improve when updated with the current new wave of firmly established type 2 diabetes risk variants. However, as we have learned from previous simulation studies, with the currently observed effect sizes, this improvement is expected to be marginal.[157]

A number performance measures of prediction models should be considered, as well.[215]

The overall performance of prediction in terms of Nagelkerke R^2 was 0.029, 0.049 and 0.079 for models containing all genetic variants, clinical characteristics and the combination of both, respectively. This means that, although the explained variability increases after adding more information to the model, the maximum explained variation is still only 7.9%. Further evaluation in terms of calibration using

the Hosmer-Lemeshow 'goodness of fit' test showed that the genetic prediction models were adequately calibrated (p>0.05 unpublished data).
An accurate prediction model should discriminate between those who will and those who will not develop the disease. The discriminative ability was 0.60, 0.66 and 0,68 for prediction based on the genetic variants, clinical characteristics and clinical characteristics combined with the genetic variants, respectively. The difference in AUC between these models was statistically highly significant, but the small improvement is not clinically relevant.

In the past few years, new measures have been proposed to assess the performance of prediction models, such as reclassification tables, net reclassification improvement (NRI) and integrated discrimination improvement (IDI). NRI is a measure of the direction of reclassification and IDI is a measure of correct net reclassification over all categories.
Using reclassification, predicted risk are categorized using risk cut-off thresholds. When novel risk factors are added to a prediction model, individuals may reclassify to a different risk category. Assignment based on prediction to a certain risk category may result in a medical decision.
Subsequent research[216] has shown that that the choice of a cut-off value is of utmost importance and should represent a value with meaningful clinical implications. Unfortunately, based on current knowledge, it is unclear what an optimal cut-off value for diabetes risk should be.
A second finding from this study is, that it is unlikely that an increase in reclassification without an increase in AUC improves clinical utility of a test.
This implies that assessing improvement of prediction by changes in AUC is a suitable method at the current stage of genetic prediction of type 2 diabetes.

9.3 CLINICAL IMPLICATIONS

The primary goal for performing genetic research is gaining insight in disease pathophysiology, and thereby potentially improving prediction, prevention, diagnosis and treatment. At the moment, the clinical use of genetic information in type 2 diabetes is limited. The studies presented in this thesis do not contain results that are directly applicable in clinical practice. However, they do provide a number of insights that may have important consequences.

Currently, a wide range of phenotypes are captured under the clinical entity 'type 2 diabetes'. In addition to glucose level criteria and the exclusion of other types

of diabetes, there is no clear cut diagnostic test that provides verification that all people allocated to this group suffer from the exact same disease. Genetic research as presented in chapters 4, 6 and especially chapter 5 illustrate that the current concept of type 2 diabetes may contain subgroups of distinct disease entities. The recognition and characterization of these subgroups provides the opportunity to develop targeted treatment and preventive measures. In the past, this has proven successful for a subgroup of monogenetic diabetes, Mature Onset Diabetes of the Young (MODY), a Mendelian form of diabetes.[217] The challenge will be to also identify subgroups based on polygenic backgrounds.

An important insight from the current thesis is that commercially available genome wide scans are not reliable and that people should not be encouraged for their use at this stage. Genetic knowledge at the moment is not sufficient to accurately predict the disease in the general population. Progressing genetic knowledge will improve prediction in the future. However, considering the complexity of the disease, the fact that the disease is only partly heritable and has intricate interactions with the environment, makes it unsure if it soon will be clinically applicable. Nonetheless, it is imaginable that certain groups may have more benefit from genetic prediction than others. For instance, genetic testing at a younger age or in groups with a strongly positive family history may be more effective than testing the general population.

9.4 DIRECTIONS FOR FUTURE RESEARCH

Although much progress has been made in the field of type 2 diabetes genetics, both in the genetic field and in the biological field, many unanswered questions remain.

GENETICS; MISSING HERITIBILITY

Type 2 diabetes, with to date a total of approximately 40 associated variants, is one of the best examples of the success of GWA studies.[218] Nonetheless, the variants identified only explain a small proportion of heritability of the disease. The reasons for this 'missing heritability' are divers.

First, the currently identified variants might not reflect the actual effect size of the true causal variants of a locus. It has proven to be difficult to find these true causal variants and further fine-mapping has to date been unsuccessful for most currently known variants. Secondly, genetic studies, in particular GWAs have been powered and designed to detect common variants with small effect sizes. Rare variants with large effect sizes or structural changes (inversions, deletions, copy number varia-

tions) are not easily detected. Third, gene-gene and gene-environment interactions have so far been unaccounted for.
New techniques (e.g. whole genome sequencing at large scale) and databases (e.g. the 1000 genomes project) are being developed to tackle the challenges posed above and provide the opportunity to identify at least part of the missing heritability that is currently present.

PHENOTYPES
An important step for future research is accurate phenotyping and performing research into different phenotype subgroups. Currently, type 2 diabetes compromises a disease with a large range of phenotypes. Identification and research in specific phenotype subgroups, such as BMI and age of onset subgroups, may yield important new biological and genetic insights. This may substantially improve future diagnosis and treatment by means of identifying gene-environment interactions, or even by identification of separate clinical entities of the disease.

EPIGENETICS
The different hypotheses on the origin of diabetes and other diseases may all hold truth and are possibly even interacting, resulting in an individuals disease risk. Epigenetic research has been evolving through development of new techniques for assessing genome-scale DNA methylation. Going forward, it is critical to develop genome-wide tools to determine the relationship between genetic variation, epigenetic variation and disease simultaneously. This will provide the basis for the field of epigenetic epidemiology.

FROM GENETIC ASSOCIATION TO BIOLOGICAL MECHANISM
The information explosion in the genetic field has not resulted in true understanding of biological mechanisms yet. Although some of the established genetic risk variants are linked to biologically plausible pathways, for many of the identified variants this is not the case. For a large part it is even unsure through which gene a so far unknown biological effect is exerted. This will require extensive genetic and genomic research
Targeted research to identify biological mechanisms is an important next step. This may involve *in vitro* models of beta cells and beta cell expression profiles, genetically manipulated rodent models, but also *in vivo* studies of insulin secretion, beta-cell function and insulin resistance in human subjects carrying specific genotypes.
Moreover, systems biology may subsequently improve insights by combining diverse types of biological and genetic information in computer-based models that integrate information and allow the uncovering of underlying system principles.

PREDICTION

Genetic prediction of type 2 diabetes will certainly improve by the discovery of more genetic risk variants, variants with larger effect sizes and improved knowledge of gene-gene and gene-environment interactions. However, besides these improvements, future research could also be directed at identification of specific risk groups in which genetic prediction might be more useful than in others as opposed to genetic prediction designed for a general population (e.g. young, lean subjects with strong positive family history).

10.5 CONCLUDING REMARKS

The current thesis presents a set of genetic association and interaction studies in relation to type 2 diabetes. In addition, the predictive ability of genetic risk variants has been explored. In this chapter, I gave an overview of the results, methodological issues and clinical implications of these studies and proposed some directions for future research. This overview is by no means exhaustive but does show the rapid developments in this field, each of them resulting in many new challenges ahead.

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SUMMARY

Type 2 diabetes is a common complex disease with a polygenic background. Although, type 2 diabetes is a growing healthcare issue, the pathophysiology of the disease is still incompletely understood. In the past few years, great progress has been made in the field of type 2 diabetes genetics. Nonetheless, much of the genetic risk is still unexplained.
Part I of the thesis starts with a general introduction on type 2 diabetes in chapter 1. This chapter gives an overview on the currently known risk factors, etiological hypothesis, genetic research and genetic research challenges. Subsequently, the aims and scope of this thesis are summarized in chapter 2.

In part II of the thesis, we investigated several genetic variants, their associations with type 2 diabetes and their interactions with environmental factors.
Chapter 3 contains the results of a genetic association study on a promoter polymorphism in *RBP4* on type 2 diabetes risk. *RBP4* is a newly identified adipokine, but is also the only known retinol transport protein. We found that homozygotes for the minor allele had increased risk of type 2 diabetes at a population based level. The association was not explained or modulated by retinol intake or vitamin A plasma levels, which did not allow differentiation between a retinol-dependent or -independent mechanism underlying this association.
In chapter 4, we report the results of a study on adiponectin, lipase activities and statin treatment effects in a randomized controlled trial in patients with type 2 diabetes. Adiponectin and lipase activities have been correlated in the past, but the direction of the effect was unknown. First, we found an association of the *-11391GA* polymorphism in the adiponectin gene with lipoprotein lipase activities and an association of the *45TG* polymorphism with hepatic lipase activities. Although borderline significant, these association support the hypothesis of adiponectin influencing lipase activities. Second, we found that adiponectin tertiles positively influenced HDL-cholesterol increase in response to statin treatment. We conclude that specific groups may benefit differently from statin treatment.
Lipid metabolism and glucose metabolism are tightly interrelated. In chapter 5 we investigate genetic variants in the *APOC3* promoter and their association with type 2 diabetes and need for insulin treatment in lean and overweight subjects. The genetic variants investigated were previously associated with risk of type 1 diabetes by others. We found that the *-482CT* promoter variants was associated with type 2 diabetes risk in lean subjects and need for insulin treatment, suggesting a beta cell related mechanism. These results were confirmed in an independent cohort. We

conclude that lean type 2 diabetes might show similarities to type 1 diabetes and may encompass a genetically distinct group of diabetes.
Chapter 6 presents the results of an interaction analyses between a set of well-replicated type 2 diabetes risk variants and fetal malnutrition in the Dutch Famine Birth cohort. We found an interaction between a genetic variant in *IGF2BP2* and fetal malnutrition on OGTT test results. Carriers of the risk variant were relatively protected from the detrimental effects of fetal malnutrition on glucose metabolism. This is a counterintuitive result, however, considering the important role of IGF2 in fetal development, it may represent an overefficient modulating effect. We conclude that modulation of the consequences of fetal malnutrition may depend on the individuals genetic background.

In part III of the thesis, the prediction of type 2 diabetes and updating of prediction models with new genetic information were explored.
Prediction of type 2 diabetes based on genetic testing might improve identification of high risk subjects. In chapter 7, we investigated the predictive value of 18 well replicated genetic type 2 diabetes risk variants when added to clinical risk factors (age, sex and BMI). We found that the AUC was 0.6 for the genetic variants, 0.66 for the clinical characteristics and 0.68 for the combination of both. We conclude that the currently tested genetic polymorphisms had low predictive value and only marginally improved prediction of type 2 diabetes beyond clinical characteristics.
Commercial companies offer genetic risk prediction estimates on the internet and provide consequent personalized lifestyle advice. These estimates are updated as genetic knowledge increases. Chapter 8 assesses the effect of updating risk estimates for type 2 diabetes based on 1 polymorphism, 18 polymorphisms and all polymorphisms combined with clinical characteristics. We found that in total 50 % of individuals changed risk category at least once. We conclude that updating genetic risks based on current knowledge may provide confusing contradictory information about an individuals risk over time, which may lead to contradictory lifestyle advice.

Finally, part IV of the thesis contains the general discussion in chapter 9. Here, I discuss the main findings in the context of ongoing research, methodological issues, clinical implications and directions for future research. The main conclusions of this thesis are: 1) Based on genetic information, subgroups of patients may be identified that have a distinct genetic etiology and/or may benefit differently from certain treatment. 2) Currently established genetic knowledge is insufficient for accurate prediction of type 2 diabetes risk. 3) Commercial online genetic scans may provide contradictory risk estimates in a large proportion of subjects when updated with new information and should therefore not be encouraged for use.

Major challenges lie ahead for establishing new genetic discoveries, unraveling the biological mechanisms associated with known variants and exploring ways of improving the use of prediction models for clinical practice.

SAMENVATTING

Type 2 diabetes is een veel voorkomende complexe ziekte met een polygenetische achtergrond. Ondanks het feit dat type 2 diabetes een groeiend gezondheidsprobleem is, is de onderliggende pathosfysiologie grotendeels onbegrepen. In de afgelopen jaren is er grote vooruitgang geboekt op het gebied van type 2 diabetes genetica. Desondanks is ook het erfelijke risico van de ziekte nog grotendeels onverklaard.

Deel I van dit proefschrift begint met een algemene introductie over type 2 diabetes in hoofdstuk 1. Dit hoofdstuk geeft een overzicht van de huidige kennis met betrekking tot risicofactoren, etiologische hypotheses, genetisch onderzoek en de daaraan verbonden uitdagingen. Vervolgens worden het doel van het proefschrift uiteengezet in hoofdstuk 2.

In Deel II van dit proefschrift hebben wij verscheidene genetische varianten en hun associaties met type 2 diabetes en interacties met omgevingsfactoren onderzocht.
Hoofdstuk 3 bevat de resultaten van een genetische associatie studie naar de effecten van een *RBP4* promoter polymorfisme op type 2 diabetes risico. RBP4 is een recent ontdekte adipokine, maar tevens het enige bekende transporteiwit voor retinol. Wij vonden dat homozygoten voor het minor allel op populatieniveau een verhoogd risico hebben op type 2 diabetes. Deze associatie werd niet verklaard of gemoduleerd door retinol consumptie en plasma spiegels van vitamine A. Hierdoor konden wij geen onderscheid maken tussen een retinol-afhankelijk of –onafhankelijk mechanisme achter deze associatie.
In hoofdstuk 4 presenteren wij de resultaten van een studie naar adiponectine, lipase activiteiten en de effecten van statine behandeling in een gerandomiseerde, gecontroleerde studie in patiënten met type 2 diabetes. Adiponectine en lipase activiteiten zijn in het verleden met elkaar gecorreleerd, echter de richting van de invloeden in deze relatie was onbekend. In deze studie vonden we ten eerste een associatie van het adiponectine *–11391GA* polymorfisme met lipoproteine lipase activiteiten en een associatie van het *45TG* polymorfisme met hepatisch lipase activiteiten. Hoewel deze bevindingen, zoals te verwachten, klein zijn en net significant, ondersteunt dit de hypothese dat adiponectine invloed heeft op lipase activiteiten. Ten tweede vonden wij dat de basale adiponectine tertielen voorafgaand aan behandeling een positieve relatie hadden met de toename van HDL cholesterol tijdens behandeling met een statine. Wij concluderen dat specifieke patiëntgroepen in verschillende mate baat hebben van statine behandeling.

Lipiden- en glucose metabolisme zijn sterk met elkaar gerelateerd. In hoofdstuk 5 hebben we genetische varianten in de *APOC3* promoter en hun associatie met type 2 diabetes en insuline gebruik geanalyseerd in slanke personen en personen met overgewicht. Deze genetische varianten waren eerder door anderen in verband gebracht met type 1 diabetes. Wij vonden dat de *-482CT* promoter variant gepaard gaat met type 2 diabetes in slanke individuen en met insuline gebruik. Wij konden deze bevinding bevestigen in een onafhankelijke studie. Wij concluderen hieruit dat type 2 diabetes in slanke individuen overeenkomsten kan vertonen met type 1 diabetes en dat er zelfs sprake zou kunnen zijn van een nieuwe genetische subgroep binnen diabetes.
Hoofdstuk 6 toont de resultaten van een studie naar de interacties tussen een set van bewezen type 2 diabetes risicovarianten en foetale ondervoeding in het hongerwintercohort. Wij vonden een interactie tussen een genetische variant in het *IGF2BP2* gen en foetale ondervoeding met een effect op de uitkomst van een OGTT. Dragers van het risicoallel waren relatief beschermt tegen de ongunstige effecten van foetale ondervoeding op glucose metabolisme. Dit resultaat lijkt niet overeen te komen met de verwachting, echter de belangrijke rol van IGF2 in de foetale ontwikkeling doet vermoeden dat er sprake kan zijn van een overefficiënt modulerend effect. Wij concluderen hieruit dat modulatie van de effecten van foetale ondervoeding afhankelijk kan zijn van de genetische achtergrond van een individu.

In Deel III van het proefschrift worden genetische voorspelling van type 2 diabetes en het actualiseren van genetische voorspellingsmodellen met nieuwe genetische informatie onderzocht.
Voorspelling van type 2 diabetes op basis van genetische tests zou mogelijk de opsporing van individuen met een hoog risico kunnen verbeteren. In hoofdstuk 7 hebben wij de voorspellende waarde van 18 bekende, gerepliceerde type 2 diabetes risicovarianten onderzocht wanneer deze worden toegevoegd aan klinische risicofactoren en parameters (leeftijd, geslacht, BMI). Wij vonden dat de AUC 0.60 was voor de genetische risicovarianten, 0.66 voor de klinische factoren en 0.68 voor de combinatie van beiden. Wij concluderen dat de polymorfismen geringe voorspellende waarde hebben en slechts een marginale – niet klinisch relevante – verbetering geven boven klinische risicovoorspellers.
Desalniettemin bieden commerciele bedrijven genetische risicovoorspellingen inclusief leefstijladviezen aan via internet. De risicovoorspellingen worden geactualiseerd wanneer een progressie is van genetische kennis. Hoofdstuk 8 brengt de effecten in kaart van het updaten van risicovoorspelling van 1 polymorfisme naar 18 polymorfismen en naar 18 polymofismen in combinatie met klinische informatie. Wij vinden dat 50% van de individuen tenminste 1 maal van risicocategorie wisselt

ten gevolge van deze updates. Wij concluderen dat het updaten van genetische risicovoorspellingen gebaseerd op de huidige kennis verwarrende en tegenstrijdige informatie oplevert over het risico van een individu door de tijd heen. Dit kan leiden tot tegenstrijdige leefstijladviezen.

Tot slot bevat Deel IV van dit proefschrift de algemene discussie in hoofdstuk 9. Hier worden de belangrijkste bevindingen in de context van voortgaande wetenschappelijke inzichten, methodologische kwesties, klinische implicaties en mogelijkheden voor toekomstig onderzoek besproken. De belangrijkste conclusies van dit proefschrift zijn:

1) Gebaseerd op genetische achtergrond kunnen mogelijk subgroepen van patiënten worden geïdentificeerd, die een specifieke etiologie van type 2 diabetes hebben of op eigen wijze voordeel hebben van bepaalde behandelingen;
2) De huidige genetische kennis is nog onvoldoende om een accurate voorspelling te verkrijgen van het risico op type 2 diabetes;
3) Online commerciële genetische scans kunnen tegenstrijdige risicovoorspellingen geven in een groot aantal personen wanneer zij worden geactualiseerd met de nieuwste genetische informatie en moeten daarom in dit stadium niet worden aangeraden.

Er zijn nog grote uitdagingen voor het doen van nieuwe genetische ontdekkingen: het ontrafelen van de onderliggende biologische mechanismen en het exploreren van manieren om genetische risicovoorspellingen te verbeteren voor gebruik in de klinische praktijk.

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CURRICULUM VITAE

Mandy van Hoek was born on October 13th, 1980, in Rotterdam, the Netherlands. In 1999, she started studying Medicine at the Erasmus University in Rotterdam. During her medical study, she got involved in doing research at the Departments of Internal Medicine and Biochemistry of the Erasmus University Medical Center. In the summer of 2005, she obtained her qualification as a medical doctor cum laude and started working on her thesis as a PhD student at the Department of Internal Medicine under the supervision of prof. dr. E.J.G. Sijbrands. As a part of this thesis, she spend three months working at the Wellcome Trust Center for Human Genetics, University of Oxford, United Kingdom under supervision of prof. M.I. McCarthy and dr. E. Zeggini. For this research she received a grant from the Albert Renold Fellowship of the European Foundation for the Study of Diabetes (EFSD). On May 1st 2009 she started her residency in Internal Medicine (Supervisors: prof. dr. J.L.C.M. van Saase, Erasmus University Medical Center Rotterdam and dr. H.E. van der Wiel, IJsselland Ziekenhuis, Capelle aan den IJssel).

ABBREVIATIONS

APOCIII:	Apolipoprotein C-III
AUC:	Area Under the Curve
BMI:	Body Mass Index
CETP:	Cholesterol Ester Transfer Protein
GWA:	Genome Wide Association
HL:	Hepatic Lipase
HMW:	High Molecular Weight
HNF1α:	Hepatic nuclear factor 1α
HR:	Hazard Ratio
IGT:	Impaired Glucose Tolerance
IRE:	Insulin/phorbol ester responsive element
LPL:	Lipoprotein Lipase
NEFA:	Non-Esterified Free Fatty Acid
OGTT:	Oral Glucose Tolerance Test
OR:	Odds Ratio
RBP4:	Retinol Binding Protein 4
RE:	Retinol Equivalent
ROC:	Receiver Operator Characteristic
RS1:	Rotterdam Study
RSPlus1:	Rotterdam Study Plus 1
RXR:	Retinoid Acid X Receptor
SFFQ:	Semiquantitative Food-Frequency Questionnaire
TNFα:	Tumor Necrosis Factor α
WHR:	Waist-to-Hip Ratio

PHD PORTFOLIO

GENETICS OF TYPE 2 DIABETES: ASSOCIATION, INTERACTION, PREDICTION

Mandy van Hoek

Erasmus MC department:	Internal Medicine
Research School:	Cardiovascular research school Erasmus University Rotterdam (COEUR)
Promotor:	Prof.dr. E.J.G. Sijbrands
Co-promotor:	Dr. A. Dehghan
PhD period:	2005-2011

PhD training	**year**	**ECTS**
General academic skills		
Biomedical English writing and communication-NIHES	2006	3.0
N.W.O talent classes- creative thinking	2006	0.3
Research skills		
Classical methods for data analyses –NIHES	2005	6.0
Erasmus Summer Programme 2007:		
Genome Wide association analyses, genetic epidemiology	2007	4.5
Coeur courses and research seminars	2005-2009	5.7
MOLMED SNPs and Human diseases	2005, 2006, 2007	4.5
KNAW and MOLMED special colloquium	2006	0.75
Young cardiovascular master class	2005, 2006	1.2
Wellcome Trust Center, Oxford, research seminars	2008	0.3
Symposia and conferences		
European Society for the Study of Diabetes (Athens, Rome)	2005,2008	2.4
Symposium vascular medicine	2006, 2007	0.3
Wetenschapsdagen inwendige geneeskunde	2007*,2008*	1.2
Dutch atherosclerosis Society (DAS)	2006	0.6
Ned Ver Diabetes Onderzoek (NVDO) Meeting	2006	0.6
Symposium dyslipidemia, new aspects in diabetes	2007	0.3
Internistendagen	2008, 2010, 2011	1.8
ENGAGE/MORGAM workshop, Krakow, Poland	2008**	0.6
American Society of Human Genetics (ASHG)	2009*	1.2

Dutch lipoprotein club	2005**-2009	1.2
Rotterdamse internistendag	2007	0.3

Teaching

Junior Med School	2006,2007	0.3
Supervising Medical students in extracurricular research	2007	2.4

Grants

Albert Renold Fellowship	2008
Stichting Simonsfonds travel grant	2008

* poster presentation, **oral presentation