Genetic determinants of diabetes and vascular complications

A population-based approach

Norbert Vaessen
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Genetic determinants of diabetes and vascular complications

A population-based approach

Genetische determinanten van diabetes en vasculaire complicaties

Een populatie onderzoek

Proefschrift

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Financial support by the Dutch Diabetes Foundation and the Netherlands Heart Foundation for the publication of this thesis is gratefully acknowledged.
The history of modern knowledge is concerned in no small degree with man's attempt to escape from his previous concepts.

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CHAPTER 1

GENERAL INTRODUCTION
Introduction

Diabetes mellitus is the most common metabolic disorder in the Western world, affecting more than 120 million people worldwide. The chronic hyperglycemia of diabetes mellitus is associated with damage to various organs, leading to severe complications such as blindness, end-stage renal failure, and coronary heart disease. The increased lifespan of individuals, the growing world population and the global change in lifestyle towards a more 'Western' way of living will lead to an explosion in the number of patients with diabetes mellitus within the next decade. Epidemiological studies predict that the prevalence of diabetes mellitus in the Westernized countries will rise from 3 to 10% at present to a total of 30% in 2025, which makes it a major health problem worldwide. A better understanding of the specific factors that contribute to the development of diabetes will ultimately lead to improved diagnosis, treatment and prevention, and thereby help to stop or even reverse the predicted rise in the prevalence of this disorder.

This thesis focuses on the identification of specific genetic factors that may be involved in the etiology of diabetes mellitus and the development of vascular complications. Identification of these genetic factors may increase our understanding of the pathogenic pathways involved through the identification of proteins (gene products) involved in the disease. In this introduction, a brief overview is given of the clinical characteristics and pathophysiology of diabetes mellitus and the current knowledge of the etiology, with a major emphasis on the role of genetic factors.

Clinical characteristics and pathophysiology of diabetes mellitus

Diabetes mellitus is a heterogeneous group of metabolic disorders with chronic hyperglycemia as a common feature. The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus of the American Diabetes Association recently advised to categorize the vast majority of cases of diabetes mellitus into three broad categories: (1) type 1 diabetes mellitus, formerly known as insulin-dependent diabetes mellitus (IDDM) or juvenile diabetes mellitus, and
characterized by an absolute deficiency of insulin secretion; (2) type 2 diabetes mellitus, also known as non-insulin dependent diabetes mellitus (NIDDM) and characterized by an imbalance between insulin secretion, insulin sensitivity and hepatic glucose production, and (3) other specific types of diabetes, including specific monogenetic disorders, genetic syndromes associated with diabetes and drug-induced forms of diabetes \(^2\). Although it is recognized that this division is in a sense artificial, as an extensive overlap in the etiology and clinical expression of these disorders exists, this classification will also be used throughout this thesis.

This thesis focuses on the two main categories, type 1 and type 2 diabetes mellitus. Type 1 diabetes mellitus usually occurs with acute symptoms like thirst, polyuria, weight loss and ketoacidosis, and is lethal if patients are not treated with insulin. It is the most common form of diabetes in children, with a peak age of onset of 12 years and a prevalence of 0.2 to 0.4 percent in the Western World \(^3\). Type 2 diabetes mellitus is often asymptomatic in its early stages and can remain undiagnosed for many years. This disease is mostly seen in adults and has a prevalence of 6 to 10 percent in most Western countries \(^2\). Type 2 diabetes mellitus is strongly associated with several cardiovascular risk factors, including hypertension, obesity, hyperinsulinemia and serum lipid and lipoprotein abnormalities, altogether also indicated as the metabolic syndrome \(^4\).

The chronic hyperglycemia of diabetes mellitus is associated with long-term damage and dysfunction of various organ systems, especially the eyes, kidneys, nerves, heart and blood vessels \(^5,6\). The clinical presentation of these complications occurs after a period of time following onset (diagnosis) of diabetes, ranging from a few years to a few decades. The increased morbidity and mortality of diabetic patients mainly results from cardiovascular complications \(^7\). Both type 1 and type 2 diabetes mellitus are strong risk factors for atherosclerotic vascular disease and coronary heart disease. Coronary heart disease mortality and morbidity rates are two to four times higher in diabetic patients than in non-diabetic subjects and of adult diabetic patients 75-80% die from cardiovascular complications \(^7\).
From a pathophysiological perspective, diabetes mellitus, whether type 1 or type 2, results from an inadequate mass of functional pancreatic beta-cells. In type 1 diabetes mellitus, the beta-cell mass is reduced by the autoimmune destruction of the beta-cells. In type 2 diabetes mellitus, there is a relative shortage of functional beta-cells, resulting in incomplete compensation of insulin production in situations of increasing insulin demands, such as insulin resistance, obesity or pregnancy. Although a (relative) lack of insulin is the common feature in the pathophysiology of type 1 and type 2 diabetes mellitus, also other hormonal disturbances can be observed in these diseases. Both in type 1 and in type 2 diabetes mellitus, low levels of insulin-like growth factor-I (IGF-I) are often seen. IGF-I is a hormone that shares structural and functional homologies with insulin and evidence is accumulating that this hormone may play a role in the regulation of glucose homeostasis and in the development of diabetic vascular complications. We and others have investigated the role of IGF-I in the pathogenesis of diabetes mellitus and vascular pathology by focusing on circulating IGF-I levels. A problem with these biochemical studies is that IGF-I levels may change due to the presence of (early) diabetes related pathology. This problem may be circumvented by studying genetic polymorphisms involved in the expression of the IGF-I gene. In this thesis, a genetic polymorphism in the regulatory region of the IGF-I gene is studied to elucidate the role of IGF-I in the pathogenesis of diabetes mellitus and in the development of cardiovascular pathology.

**Etiology of diabetes mellitus**

In the vast majority of patients, the occurrence of diabetes mellitus is a result of a combination of several risk-increasing genetic factors and environmental factors. Several environmental factors have been implicated in the etiology of type 1 diabetes mellitus, including viral infections and exposure to cow’s milk. The exact nature of the relation between type 1 diabetes mellitus and these putative environmental factors is still unclear however. Numerous (epidemiological) studies have focused on environmental factors influencing the risk for type 2
diabetes mellitus. The major risk factors identified in these studies are increasing age, obesity and lack of physical activity \(^\text{17}\).

The genetic basis of diabetes mellitus is demonstrated by many studies showing an increased prevalence of diabetes mellitus in individuals who have a first degree relative with the disease compared with individuals who do not have an affected first degree relative. This familial aggregation has been demonstrated for type 1 and type 2 diabetes mellitus in a variety of populations \(^{18-22}\). The extent to which familial aggregation of diabetes mellitus is due to inheritance or shared environmental factors can be assessed by comparing the extent to which monozygotic twins are concordant for the disease with the concordance of dizygotic twins. Since monozygotic twins are genetically identical while dizygotic twins share on average half of their genes, the difference between the concordance rates signifies the contribution of genetic factors to the etiology of diabetes mellitus. Many studies have shown that the concordance rates for type 1 and for type 2 diabetes mellitus are higher for monozygotic than for dizygotic twins \(^{18-22}\). A recent Danish population-based study reported a concordance rate for type 1 diabetes mellitus of 70% in monozygotic twins and 13% in dizygotic twins \(^{23}\). For type 2 diabetes mellitus concordance estimates in European studies range from 20 – 90 % for monozygotic twins and 10-23% for dizygotic twins \(^{21,22,24}\).

**Genetic risk factors for type 1 and type 2 diabetes mellitus**

It has been recognised that several haplotypes at the human leukocyte antigen (HLA) loci are associated with an increased risk to develop type 1 diabetes mellitus \(^{25}\). More than 90% of type 1 diabetes mellitus patients of European ancestry have at least one copy of the HLA-DR3 or DR4 allele (variants at the class II HLA-DRB1 locus on chromosome 6p21.3), compared to 45% in the general population. Almost 40% of type 1 diabetes patients have both alleles, while 3% is expected based on the frequency of the general population \(^{26}\). Since the identification of the HLA-DRB1 predisposition, numerous studies have found several other genotypes within the HLA-region to be associated with type 1 diabetes mellitus. The specific predisposing genes in the HLA region, altogether
designated as IDDM1, are not yet fully delineated. Different combinations of HLA-DRB, DQA and DQB alleles may confer varying degrees of susceptibility or resistance to type 1 diabetes mellitus. However, the total contribution of genes in the HLA-region to the familial clustering of type 1 diabetes mellitus is estimated to be 44%, indicating that other non-HLA genes are involved.

One of these non-HLA genes is the insulin gene on chromosome 11p15, designated IDDM2. Although even the largest linkage study of type 1 diabetes mellitus could not detect significant evidence for linkage to this region (maximum lodscore 0.6), significant association (linkage disequilibrium) has been demonstrated in many studies. The frequency of the short ‘class I’ alleles of a variable number of tandem repeat (VNTR) marker in the 5' flanking region of the insulin gene (INS) is increased in subjects with type 1 diabetes mellitus in various populations. This VNTR appears to influence insulin transcription, but the mechanism through which this modifies susceptibility to type 1 diabetes mellitus is still unclear.

**Table 1:** Genomic regions outside the HLA (IDDM1) and insulin gene region (IDDM2) demonstrating at least suggestive evidence for linkage to type 1 diabetes mellitus (lodscore > 2.0). Loci are ordered by the highest lodscore observed.

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Lodscore</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>10p</td>
<td>4.7</td>
<td>Mein et al, 1998</td>
</tr>
<tr>
<td>6q</td>
<td>4.5</td>
<td>Luo et al, 1996</td>
</tr>
<tr>
<td>14q</td>
<td>4.0</td>
<td>Field et al, 1996</td>
</tr>
<tr>
<td>11q</td>
<td>3.9</td>
<td>Luo et al, 1996</td>
</tr>
<tr>
<td>6q</td>
<td>3.6</td>
<td>Luo et al, 1996</td>
</tr>
<tr>
<td>16q</td>
<td>3.4</td>
<td>Mein et al, 1998</td>
</tr>
<tr>
<td>1q</td>
<td>3.3</td>
<td>Concannon et al, 1998</td>
</tr>
<tr>
<td>2q</td>
<td>3.3</td>
<td>Morahan et al, 1996</td>
</tr>
<tr>
<td>2q</td>
<td>3.2</td>
<td>Nisticco et al, 1996</td>
</tr>
<tr>
<td>2q</td>
<td>2.6</td>
<td>Owerbach et al, 1995</td>
</tr>
<tr>
<td>15q</td>
<td>2.5</td>
<td>Field et al, 1994</td>
</tr>
<tr>
<td>6q</td>
<td>2.3</td>
<td>Concannon et al, 1998</td>
</tr>
</tbody>
</table>
In the past 7 years, a number of centers throughout the world have performed genome-wide linkage screens using families containing multiple siblings affected with type 1 diabetes mellitus (multiplex families) 32-43. To date, the largest genome-wide linkage screens for type 1 diabetes mellitus genes are those of Concannon et al (1998) and Mein et al (1998) 44,45. These and other studies have resulted in the localization of more than 20 genomic regions containing putative type 1 diabetes-predisposing genes (see table 1) 32-43. However, it remains to be shown in independent studies whether all these regions contain type 1 diabetes genes or whether some of these findings represent false positive findings.

Studies for loci influencing susceptibility to type 2 diabetes mellitus have been conducted in a number of populations. The first genome scan, conducted on 330 Hispanic American sibling pairs, found linkage on chromosome 2q37 46. The evidence for linkage was recently found to be explained by a common polymorphism within the calpain-10 (CAPN10) gene 47. One SNP (UCSNP-43) in intron 3 of CAPN10 showed an increased frequency of the common G allele in patients compared with controls. In addition, two haplotypes within the CAPN10 locus were significantly associated with an increased risk (threefold) of type 2 diabetes mellitus in both Mexican Americans and Europeans 47. The G/G genotype of UCSNP-43 has recently been associated with lower levels of CAPN10 mRNA in skeletal muscle and increased insulin resistance due to lower rates of insulin-stimulated glucose oxidation 48. Together, the genetic and functional studies suggest that CAPN10 has a role in determining the normal response of skeletal muscle to the effects of insulin.

A variety of polymorphisms in other genes have been associated with type 2 diabetes mellitus including genes within the HLA-region, the glycogen synthetase gene, the glucagon receptor gene, the sulfonylurea receptor gene, the insulin receptor substrate-1 gene, the glycogen targeting subunit of the type 1 protein phosphatase gene, a Trp-Arg missense mutation in the beta-3 adrenergic receptor gene and the frataxin gene 49-57. In most of these cases, however, there are also populations in which there is no association of diabetes mellitus with these polymorphisms.
Numerous other genomic regions have demonstrated at least suggestive evidence for linkage (lodscore > 2), but only a few regions have shown significant evidence for linkage (lodscore > 3. See table 2). As is true also for type 1 diabetes mellitus, the positive signals in many of these regions need to be confirmed using additional linkage datasets or using alternate approaches. In this thesis, linkage disequilibrium mapping is used as an alternative approach to identify susceptibility genes for type 1 and type 2 diabetes mellitus.

Table 2: Genomic regions demonstrating at least suggestive evidence for linkage to type 2 diabetes mellitus or related disorders (lodscore > 2.0). Loci are ordered by the highest lodscore observed.

<table>
<thead>
<tr>
<th>Chrom.</th>
<th>Trait studied</th>
<th>Lodscore</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>11q</td>
<td>Obesity and diabetes</td>
<td>4.9</td>
<td>Hanson et al, 1998</td>
</tr>
<tr>
<td>3q</td>
<td>Diabetes or glucose intolerance &lt; 45yr</td>
<td>4.7</td>
<td>Vionnet et al, 2000</td>
</tr>
<tr>
<td>1q</td>
<td>Abnormal glucose homeostasis</td>
<td>4.3</td>
<td>Elbein et al, 1999</td>
</tr>
<tr>
<td>2q</td>
<td>Diabetes</td>
<td>4.3</td>
<td>Huang et al, 1996</td>
</tr>
<tr>
<td>3p</td>
<td>Abnormal glucose homeostasis</td>
<td>4.2</td>
<td>Elbein et al, 1999</td>
</tr>
<tr>
<td>10q</td>
<td>Age of onset of diabetes</td>
<td>3.7</td>
<td>Duggirala et al, 1999</td>
</tr>
<tr>
<td>6q</td>
<td>Diabetes</td>
<td>3.3</td>
<td>Hegele et al, 1999</td>
</tr>
<tr>
<td>16</td>
<td>Diabetes</td>
<td>3.2</td>
<td>Hegele et al, 1999</td>
</tr>
<tr>
<td>17p</td>
<td>Insulin resistance</td>
<td>3.2</td>
<td>Watanabe et al, 2000</td>
</tr>
<tr>
<td>3p</td>
<td>Fasting C-peptide/ glucose level</td>
<td>3.1</td>
<td>Watanabe et al, 2000</td>
</tr>
<tr>
<td>10p</td>
<td>Acute insulin response</td>
<td>3.1</td>
<td>Watanabe et al, 2000</td>
</tr>
<tr>
<td>18q</td>
<td>Abnormal glucose homeostasis</td>
<td>3.1</td>
<td>Elbein et al, 1999</td>
</tr>
<tr>
<td>20p</td>
<td>Diabetes</td>
<td>3.1</td>
<td>Gosh et al, 1999</td>
</tr>
<tr>
<td>1q</td>
<td>Lean diabetes</td>
<td>3.0</td>
<td>Vionnet et al, 2000</td>
</tr>
<tr>
<td>X</td>
<td>Diabetes</td>
<td>3.0</td>
<td>Ehm et al, 2000</td>
</tr>
<tr>
<td>13q</td>
<td>2-hours insulin level</td>
<td>2.9</td>
<td>Watanabe et al, 2000</td>
</tr>
<tr>
<td>19q</td>
<td>Insulin resistance</td>
<td>2.8</td>
<td>Watanabe et al, 2000</td>
</tr>
<tr>
<td>12q</td>
<td>Abnormal glucose homeostasis</td>
<td>2.7</td>
<td>Ehm et al, 2000</td>
</tr>
<tr>
<td>20q</td>
<td>Diabetes</td>
<td>2.7</td>
<td>Gosh et al, 1999</td>
</tr>
<tr>
<td>1q</td>
<td>Young-onset diabetes</td>
<td>2.5</td>
<td>Hanson et al, 1998</td>
</tr>
<tr>
<td>3p</td>
<td>Age of onset of diabetes</td>
<td>2.5</td>
<td>Duggirala et al, 1999</td>
</tr>
<tr>
<td>10p</td>
<td>Diabetes</td>
<td>2.4</td>
<td>Ehm et al, 2000</td>
</tr>
<tr>
<td>2p</td>
<td>Diabetes</td>
<td>2.3</td>
<td>Vionnet et al, 2000</td>
</tr>
<tr>
<td>20q</td>
<td>Diabetes</td>
<td>2.2</td>
<td>Gosh et al, 2000</td>
</tr>
<tr>
<td>2q</td>
<td>Abnormal glucose homeostasis</td>
<td>2.1</td>
<td>Elbein et al, 1999</td>
</tr>
<tr>
<td>4q</td>
<td>Lean diabetes or glucose intolerance</td>
<td>2.1</td>
<td>Vionnet et al, 2000</td>
</tr>
<tr>
<td>9p</td>
<td>Age of onset of diabetes</td>
<td>2.1</td>
<td>Duggirala et al, 1999</td>
</tr>
</tbody>
</table>
Chapter 1

Aim of this thesis
Diabetes mellitus is a growing epidemic. The etiology of the two common types, type 1 and type 2 diabetes mellitus, and the etiology of diabetic vascular complications are still unclear. Genetic studies may enable to unravel the etiology of these diseases through identification of genes and therewith the proteins involved. The aim of this thesis is to identify genes that play a role in the etiology of type 1 and type 2 diabetes mellitus and to elucidate the role of genetic variation in the gene for insulin-like growth factor-I in the pathogenesis of type 2 diabetes mellitus and diabetic vascular complications. In Chapter 2, a genome scan approach is used to identify genes involved in the pathogenesis of diabetes mellitus in the recently genetically isolated population of the Genetic Research in Isolated Populations (GRIP) study. In chapter 2.1 and 2.2 the results of whole genome scans for type 1 and type 2 diabetes mellitus are presented. In Chapter 3, a candidate gene approach is used to study the pathogenesis of type 2 diabetes mellitus and cardiovascular pathology in the Rotterdam Study; a large population based cohort study. We chose the gene for insulin-like growth factor-I (IGF-I) as a candidate gene. In chapter 3.1 to 3.4 we studied the functional properties of a genetic polymorphism in the promoter region of the IGF-I gene and investigated its relationship with type 2 diabetes mellitus, vascular pathology and fetal growth. Finally, Chapter 4, provides a more general view on the opportunities for population-based studies of complex genetic disorders. Before these are addressed an integrated overview is given of the main findings, with a brief discussion of their clinical relevance.
References:


CHAPTER 2

SCREENING THE GENOME
A GENOME-WIDE SEARCH FOR LINKAGE-DISEQUILIBRIUM WITH TYPE 1 DIABETES MELLITUS IN A RECENT GENETICALLY ISOLATED POPULATION FROM THE NETHERLANDS


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Summary
It has long been recognized that type 1 diabetes mellitus (DM1) has a substantial genetic component. Although there is consistent evidence for a susceptibility locus in the HLA-DR/DQ region on chromosome 6p and the insulin gene region on chromosome 11p, other genes must be involved. Genome scans aiming to localize these genes have identified more than 18 other genomic regions that may harbor putative DM1 genes. However, evidence for most of these regions varies in different data sets, probably due to a weak effect of susceptibility genes, genetic heterogeneity or random variation. Given the genetic heterogeneity of DM1, studies in homogeneous genetically isolated populations may be more successful in mapping susceptibility loci than studies in complex outbred populations. Here we describe the results of a genome-wide search in a recently isolated population in the Netherlands. We identified 43 DM1 patients that could be traced back to a common ancestor within 15 generations and performed a genome-wide scan by using a combined linkage and association based approach. In addition to the HLA locus, evidence for DM1 loci was observed on chromosome 8q with marker D8S1128 and on chromosome 17q with marker D17S2059. Both the 8q and 17q localization are supported by allele sharing at adjacent markers in affected individuals and by the observation that the risk of diabetes increased with the number of copies of the associated allele for each of these markers. Further, statistical evidence for a conserved ancestral haplotype was found around the locus on chromosome 8q. These findings confirm the localization of a previously identified DM1 locus on chromosome 8q and suggest the presence of a new DM1 locus on chromosome 17q.
Type 1 diabetes mellitus (DM1), also known as insulin-dependent diabetes mellitus, is the second most common form of diabetes. The disease is characterized by an absolute insulin deficiency due to autoimmune destruction of the insulin producing beta-cells in the pancreas. Although the exact mechanism of the beta-cell destruction in DM1 is not known, both genetic and environmental factors appear to be involved. Studies in the past three decades have suggested a large number of genes or genomic regions that may be implicated in the pathogenesis of DM1. The HLA class II region (also known as IDDM1) on chromosome 6p21 and a locus in the insulin region (IDDM2) on chromosome 11p15.5 account for approximately 50% of the total familial clustering. Both these susceptibility regions were initially identified using association analysis of candidate genes. Since then progress in mapping additional genes has been very slow. Several genome-wide linkage studies have identified more than 18 other genomic regions that may harbor susceptibility loci for DM1, however, most findings could not be reproduced in other studies. Although this may suggest that some of the initial linkage findings have been false positive results, it may also reflect the genetic heterogeneity of DM1 in diverse ethnic groups. Given the presumed genetic heterogeneity, genetic studies in homogeneous genetically isolated populations may be more successful in localizing susceptibility loci than studies in complex outbred populations. Here we describe the results of a genome-wide search for linkage-disequilibrium with type 1 diabetes mellitus in a recently genetically isolated population in the Netherlands.

We ascertained 46 patients with DM1 in an isolated village in the Southwest of the Netherlands. This village has been founded by approximately 150 individuals in the middle of the 18th century and until the last decades descendants of these founders have lived in social isolation, with minimal immigration (less than 5%). From the year 1848 the population has expanded from 700 up to 20,000 inhabitants. Genealogical information was collected for all 46 patients and revealed that 43 patients (93%) could be traced back to a common ancestor within 15 generations. The genealogical information within these 15 generations was complete for more than 90% of the patients. In figure 1, the
Figure 1: Genealogical lineages of 43 DM1 patients that could be traced back to a common ancestor within 15 generations. The figure is based on the shortest number of meioses separating each person from this common ancestor.
genealogical lineages of these patients are shown, based on the shortest number of meioses separating them from a common ancestor. This figure represents a simplified example of the genealogical structure of this population. In reality most patients are related with each other in several ways via multiple common ancestors. To ensure a homogeneous study sample, the analyses in this study are restricted to these 43 patients that are related within 15 generations. We confirmed the diagnosis of diabetes obtained from the treating physician using the criteria of the American Diabetes Association. A diagnosis of DM1 was made if diabetes was diagnosed before the age of 30 years and if patients were insulin-dependent within 1 year following diagnosis. All patients completed a questionnaire on family and medical history and gave blood samples for DNA-extraction and detection of autoantibodies. The HLA-DR3 and DR4 allele were genotyped in all participants. GAD and IA2 antibodies were tested by immunoprecipitation as previously described. For each patient, at least 2 first-degree relatives were asked to donate a blood sample for the reconstruction of haplotypes. Additional clinical information of these relatives was not collected. Characteristics of the patients are given in table 1. Only 33 patients (73.3 %) carried the HLA-DR3 or DR4 allele and only 8 (17.8 %) were heterozygous for the DR3/DR4 allele. These frequencies were lower than the expected frequencies of 90% (p < 0.00006) and 35% (p = 0.017) which are found in most Caucasian populations, suggesting that other DM1 genes play a more important role in this population.

<table>
<thead>
<tr>
<th>Table 1: Characteristics of 46 type 1 diabetes patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
</tr>
<tr>
<td>Mean age at time of diagnosis</td>
</tr>
<tr>
<td>Presence of detectable GAD - antibodies</td>
</tr>
<tr>
<td>Presence of detectable IA2 - antibodies</td>
</tr>
<tr>
<td>Presence of both antibodies</td>
</tr>
<tr>
<td>Frequency of high-risk HLA genotypes:</td>
</tr>
<tr>
<td>HLA-DR3 and/or HLA-DR4 allele</td>
</tr>
<tr>
<td>Heterozygous HLA-DR3/DR4</td>
</tr>
</tbody>
</table>

Data are percentages or means + range
We performed a genome scan using the 43 patients and 86 first-degree relatives. The genome screen was performed using 391 markers covering the whole genome, with an average spacing of 10 cM and an average heterozygosity of 0.75 (version 6 of the Weber lab screening set, see http://research.marshfieldclinic.org/genetics/sets/combo.html).

To evaluate statistical evidence for a DM1 locus we used a combined linkage and association-based approach \textsuperscript{20,21}. In isolated populations, linkage disequilibrium (allelic association) is expected in the vicinity of a disease gene, as a high proportion of affected individuals are likely to share the chromosomal segment containing the gene identical by descent (IBD) from a common ancestor \textsuperscript{13,14,22,23}. Therefore, genome regions where marker allele frequencies differ between affected individuals and the background population likely harbor disease loci. When such deviation is based on a substantially increased frequency of one or a few alleles in affected individuals, the region is likely inherited IBD with the disease gene from a common ancestor. In our genome scan, we used a modification of the procedure of Terwilliger to evaluate statistical evidence for linkage and excess sharing of founder alleles in our patient population \textsuperscript{24,20,21}. The test assumes that one marker allele will be over-represented on chromosomes that carry the disease mutation. The proportion of disease chromosomes with this ancestral allele is represented by the parameter $\lambda$. This parameter is estimated for all markers that have a lodscore $> 0$. It is not a priori known which marker allele will be the over-represented allele, and therefore the test considers each of the marker alleles separately as potential founder alleles. Consequently, a total likelihood is obtained by computing the likelihood for the data for each potential founder allele, and summing those likelihood's, weighted for the population frequency of the respective founder allele. In our study, a $p$ value $< 0.016$ (corresponding to a LOD-score $> 1.0$) was used as a threshold to select markers for further analysis. We found evidence for combined linkage and association with 3 markers: Marker D6S1014 in the HLA-region on chromosome 6p ($p = 0.009, \lambda = 0.46$), marker D8S1128 on chromosome 8q ($p = 0.003, \lambda = 0.45$) and marker D17S2059 on chromosome 17q ($p = 0.012, \lambda = 0.65$).
### Table 2: Case-control analysis of flanking markers on chromosome 8q

<table>
<thead>
<tr>
<th>Marker</th>
<th>cM</th>
<th>Allele (bp)</th>
<th>Genotype</th>
<th>Frequency in controls</th>
<th>Frequency in cases</th>
<th>Risk (95%CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>D8S592</td>
<td>125</td>
<td>150 0</td>
<td></td>
<td>69.7 %</td>
<td>45.6 %</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td>15.2 %</td>
<td>40.0 %</td>
<td>3.8 (1.1 - 12.6)</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td>15.2 %</td>
<td>11.4 %</td>
<td>1.1 (0.3 - 4.7)</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>trend</td>
<td>1.4 (0.7 - 2.8)</td>
<td></td>
<td>0.16</td>
</tr>
<tr>
<td>D8S1179</td>
<td>136</td>
<td>181 0</td>
<td></td>
<td>46.9 %</td>
<td>20.6 %</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td>50.0 %</td>
<td>55.8 %</td>
<td>2.7 (0.9 - 8.1)</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td>3.1 %</td>
<td>20.6 %</td>
<td>14.9 (1.5 - 146.2)</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>trend</td>
<td>3.3 (1.4 - 146.2)</td>
<td></td>
<td>0.008</td>
</tr>
<tr>
<td>D8S1128</td>
<td>142</td>
<td>243 0</td>
<td></td>
<td>84.4 %</td>
<td>45.8 %</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td>15.6 %</td>
<td>45.9 %</td>
<td>5.8 (1.7 - 19.2)</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td>0 %</td>
<td>9.4 %</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>trend</td>
<td>6.4 (2.0 - 19.7)</td>
<td></td>
<td>0.0005*</td>
</tr>
<tr>
<td>D8S1100</td>
<td>160</td>
<td>192 0</td>
<td></td>
<td>43.8 %</td>
<td>29.4 %</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td>42.9 %</td>
<td>47.1 %</td>
<td>1.6 (0.5 - 4.7)</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td>12.3 %</td>
<td>23.8 %</td>
<td>2.8 (0.7 - 11.5)</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>trend</td>
<td>1.7 (0.8 - 3.3)</td>
<td></td>
<td>0.08</td>
</tr>
</tbody>
</table>

Results of the case-control analysis for markers on chromosome 8q. Results are based on the ancestral allele as identified in the initial screen. * P-value calculated by Fisher exact test.

### Table 3: Case-control analysis of flanking markers on chromosome 17q

<table>
<thead>
<tr>
<th>Marker</th>
<th>cM</th>
<th>Allele (bp)</th>
<th>Genotype</th>
<th>Frequency in controls</th>
<th>Frequency in cases</th>
<th>Risk (95%CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>D17S809</td>
<td>85</td>
<td>242 0</td>
<td></td>
<td>75.0 %</td>
<td>54.5 %</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td>21.9 %</td>
<td>30.3 %</td>
<td>1.9 (0.6 - 6.0)</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td>3.1 %</td>
<td>15.2 %</td>
<td>6.7 (0.7 - 62.1)</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>trend</td>
<td>2.2 (1.0 - 5.1)</td>
<td></td>
<td>0.03</td>
</tr>
<tr>
<td>D17S1290</td>
<td>91</td>
<td>172 0</td>
<td></td>
<td>100 %</td>
<td>83.3 %</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td>0 %</td>
<td>13.9 %</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td>0 %</td>
<td>2.8 %</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>trend</td>
<td>-</td>
<td></td>
<td>0.01*</td>
</tr>
<tr>
<td>D17S2059</td>
<td>106</td>
<td>253 0</td>
<td></td>
<td>48.6 %</td>
<td>13.2 %</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td>37.1 %</td>
<td>52.6 %</td>
<td>5.2 (1.5 - 17.7)</td>
<td>0.008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td>14.3 %</td>
<td>34.2 %</td>
<td>8.8 (2.1 - 37.1)</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>trend</td>
<td>3.1 (1.5 - 6.3)</td>
<td></td>
<td>0.0005</td>
</tr>
<tr>
<td>D17S1301</td>
<td>116</td>
<td>154 0</td>
<td></td>
<td>64.5 %</td>
<td>30.3 %</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td>25.8 %</td>
<td>51.5 %</td>
<td>4.3 (1.4 - 13.2)</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td>9.7 %</td>
<td>18.2 %</td>
<td>4.0 (0.8 - 19.4)</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>trend</td>
<td>2.5 (1.2 - 5.4)</td>
<td></td>
<td>0.01</td>
</tr>
</tbody>
</table>

Results of the case-control analysis for markers on chromosome 17q. Results are based on the ancestral allele as identified in the initial screen. * P-value calculated by Fisher exact test. The risk for marker D17S1290 could not be calculated because the ancestral allele was not present in controls.
To confirm that these non-HLA regions represent true positive findings, three additional analyses were performed. First, the risk for DM1 was determined for the associated alleles of markers D8S1128 and D17S2059 as well as for flanking markers in these regions. These analyses were restricted to the putative ancestral allele that was identified in the initial genome scan. The non-transmitted alleles of parents and alleles of partners were used as controls. We hypothesized that, if the initial findings represent true positive findings, flanking markers in these regions should also be associated with an increased risk for diabetes. Although the flanking markers in the 8q region did not show a p-value < 0.016 in the initial genome scan, the ancestral alleles of markers D8S592, D8S1179, D8S1128 and D8S1100 were all associated with an increased risk for DM1 (table 2). Also in the 17q region, allele sharing at additional markers by affected individuals was observed. The ancestral alleles of the adjacent markers D17S809, D17S1290, D17S2059 and D17S1301 were all associated with a highly increased risk for DM1 (table 3). Second, following this observation, a trend analysis was performed for each of these markers. We hypothesized that, if the 8q and 17q localization’s represent true susceptibility loci, the risk for diabetes should increase with the number of ancestral alleles present for each marker. For chromosome 8, the odds ratio for trend reached a maximum of 6.4 at D8S1128 (p = 0.0005), while the lowest odds ratio was observed for marker D8S592 (OR 1.4, p = 0.16) and D8S1100 (OR 1.7, p = 0.08) located at approximately 18 cM on either side of D8S1128 (table 2). This suggest that a susceptibility gene is probably located around D8S1128. For chromosome 17, a significant trend was observed for all 4 markers, in particular for D17S2059 (OR 3.1, p = 0.0005, table 3). Finally, we investigated whether affected individuals showed evidence for an ancestral haplotype surrounding the markers identified in the initial screen. Haplotypes within the 8q region were associated with a highly increased risk for DM1, supporting the presence of a true susceptibility locus inherited from a common ancestor (table 4). In the 17q region, the distribution of two-marker haplotypes was not significantly different between cases and controls (data not shown).
Table 4: Haplotype analysis chromosome 8q

<table>
<thead>
<tr>
<th>Marker</th>
<th>cM</th>
<th>Allele (bp)</th>
<th>Haplotype</th>
<th>Frequency in controls</th>
<th>Frequency in cases</th>
<th>Risk (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>D8S592</td>
<td>125</td>
<td>150</td>
<td>Heterozygous</td>
<td>48 %</td>
<td>15.3 %</td>
<td>3.5 (0.9 - 13.4)</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Homozygous</td>
<td>0 %</td>
<td>0 %</td>
<td>trend</td>
<td>-</td>
</tr>
<tr>
<td>D8S179</td>
<td>136</td>
<td>181</td>
<td>Heterozygous</td>
<td>1.6 %</td>
<td>12.1 %</td>
<td>9.0 (1.1 - 74.0)</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Homozygous</td>
<td>0 %</td>
<td>3.6 %</td>
<td>trend</td>
<td>9.5 (1.2 - 73.4)</td>
</tr>
<tr>
<td>D8S128</td>
<td>142</td>
<td>243</td>
<td>Heterozygous</td>
<td>32 %</td>
<td>10.6 %</td>
<td>3.7 (0.7 - 18.4)</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Homozygous</td>
<td>0 %</td>
<td>1.5 %</td>
<td>trend</td>
<td>1.0 (1.0 - 18.5)</td>
</tr>
<tr>
<td>D8S100</td>
<td>160</td>
<td>192</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results of the case-control analysis for haplotypes on chromosome 8q. Haplotypes are based on the ancestral alleles as identified in the initial screen. *P-value calculated by Fisher exact test.

To study a possible interaction between the 8q or 17q region and the high-risk HLA alleles, a stratified analysis was performed. In this analysis the distribution of ancestral alleles, as identified in the initial genome scan, was assessed in individuals with or without the HLA-DR3 or DR4 allele. The ancestral alleles of marker D8S179 (p = 0.05) and D8S128 (p = 0.0005) were more frequently observed in patients with the high risk HLA-DR4 allele, compared to patients without the HLA-DR4 allele, suggesting an interaction between the susceptibility gene on chromosome 8q and the HLA-DR locus. No difference in the distribution of the ancestral alleles in the 17q region was observed between the different HLA-subgroups (data not shown).

Although convincing evidence has been collected for DM1 susceptibility genes in the HLA region and the insulin region, these regions do not account for all the risk attributable to genetic factors. Linkage studies for other loci influencing the risk for DM1 have been conducted in a number of populations. However, only a few of the significant findings have been replicated in other studies. One reason for these discrepancies might be that DM1 is a genetically
heterogeneous disease. In our study we aimed to reduce the impact of this problem by screening patients with DM1 derived from a homogeneous, recently isolated population. Because of the recent isolation, this population is likely to show more resemblance with the general Caucasian population than other frequently studied older isolates, such as Sardinia or Finland. Therefore, we believe that the loci identified in this study may be relevant for the general Dutch population and possibly also for other Caucasian populations.

Due to the limited number of founders and the low immigration rate, the genetic variability of this population has been reduced. The lower frequency of the high risk HLA-DR3 and DR4 allele makes this population very suitable for mapping of new DM1 genes. The chances of finding new genes are further improved by the extensive relationship between cases as shown in the pedigree. By screening the genome with a relatively sparse marker set we were able to detect combined evidence for linkage and association with three regions in the genome, on chromosome 6p, 8q and 17q. Both the 8q and 17q localization are supported by statistical trend analysis and by allele sharing at additional markers in affected individuals. There have been two previous reports of linkage to a locus on chromosome 8q, approximately 7 to 25 cM centromeric to the location in our study. Although linkage to this region could not be replicated in a follow-up analysis by Cucca et al, our study in a homogeneous population confirms the initial findings. No evidence for linkage to chromosome 17q has been reported before. While our findings suggest a DM1 locus on 17q, no ancestral haplotype could be identified with the current sparse marker set. Therefore, this locus still remains to be confirmed.

Although it has recently been argued that old genetic isolates will not prove to be more valuable than outbred populations for linkage-disequilibrium mapping of common variants underlying complex disease, our study in a recently isolated population has yielded promising findings. The identification of two susceptibility loci for DM1 on chromosome 8q and 17q demonstrates the practical value of linkage-disequilibrium studies of complex genetic disorders in recent genetically isolated populations.
Acknowledgements

This work was supported by a grant of the Dutch Diabetes Foundation. We would like to thank A Jacobs and E Wauters for their help in genotyping and P Veraart and E Boeren for the collection of genealogical information. For the help in collection of clinical information we thank R.P Rutten of Stichting Huisartsen Laboratorium Breda and the local physicians G. Dröge, Th.W.C. Snieders, P. van Wouw, M. Kraanen, W.C.J. van Broekhoven and P. Biemond. Above all we would like to thank the patients and relatives from the GRIP population and the municipality that made this work possible.
References

A GENOME-WIDE SEARCH FOR LINKAGE-DISEQUILIBRIUM WITH TYPE 2 DIABETES MELLITUS AND FASTING GLUCOSE CONCENTRATION IN A RECENT GENETICALLY ISOLATED POPULATION FROM THE NETHERLANDS


* Genetic Epidemiology Unit, Department of Epidemiology & Biostatistics, Department of Clinical Genetics. Erasmus Medical Center Rotterdam, The Netherlands.
† Oxagen limited, UK

Due to a publication delay, parts of this chapter have been anonymized
Summary
Type 2 diabetes mellitus (DM2) is the most frequent disorder of glucose homeostasis and is characterized by a combination of resistance to insulin action and an inadequate compensatory insulin secretory response. It is well recognized that DM2 has a substantial genetic component. Although several genomic regions have been identified that may harbor susceptibility loci for DM2, few linkage findings have yielded significant results, and failure to reproduce published results is endemic. Given the presumed genetic heterogeneity of DM2, genetic studies in homogeneous genetically isolated populations may be more successful in localizing susceptibility loci than studies in complex outbred populations. Several genome scans have focused on sibling pairs or extended families in populations with prolonged isolation, such as the Finnish, Mexican-Americans or the Pima Indians. Here we describe the results of a genome-wide search in a population of more recent isolation, which is likely to show more resemblance with the general Caucasian population. We identified 79 nuclear families with at least one DM2 patient that could be traced back to a common ancestor within 13 generations and performed a 770 marker genome-wide scan by searching for shared founder alleles. Nine markers yielded a lod score over 1.0 and the strongest evidence for a DM2 locus was observed with marker 8b on chromosome **p. Of these nine markers, marker 4b on chromosome #q and 8b on ++q, showed additional evidence for association with fasting glucose levels in an independent set of first-degree relatives. Both loci are supported by sharing of additional markers by affected individuals in this region. Further, we observed that flanking markers and haplotypes on chromosomes #q and ++p were significantly associated with fasting glucose concentration in first-degree relatives of patients. The replication of the findings of the initial genomic scan in patients with type 2 diabetes with glucose levels in relatives suggests that we localized two susceptibility loci for type 2 diabetes.
CHAPTER 2.2

We ascertained 128 patients with DM2 in the GRIP population (genetic research in isolated populations), an isolated village in the Southwest of the Netherlands. This village has been founded by approximately 150 people in the middle of the 18th century and up until the last decades descendants of these founders have lived in social isolation, with minimal immigration (less than 5%). From the year 1848 on the population has expanded from 700 up to 20,000 inhabitants. We identified 128 patients with DM2, derived from 86 nuclear families. Genealogical information was collected up to 15 generations for all 86 families and revealed that 79 families (117 patients; 92% of all patients) could be traced back to a common ancestor within 13 generations. The total pedigree structure of these 117 patients and ancestors consists of 876 persons within 13 generations. In figure 1, the genealogical lineages of these patients are shown, based on the shortest number of meioses separating them from this common ancestor. To ensure a homogeneous study population, the analyses in this study are restricted to these 79 families that are related within 13 generations. We confirmed the diagnosis of DM2 obtained from the treating physician using the American Diabetes Association criteria for the diagnosis of diabetes 13. Individuals, who were diagnosed with diabetes before the age of 25 years, were pregnant at time of diagnosis or individuals who were insulin-dependent within 1 year following diagnosis were assigned a diagnosis of “unknown”. For each patient, at least 2 first-degree relatives were asked to participate in the study to be able to reconstruct haplotypes. For 84 first-degree relatives (parents, siblings and children) glucose concentration was determined in a fasting blood sample. Clinical characteristics of the patients and these 84 first-degree relatives are given in table 1.

We performed a whole genome scan on these 117 DM2 patients and 202 first-degree relatives. To evaluate statistical evidence for a DM2 locus we used a combined linkage and association based approach. In isolated populations, linkage disequilibrium (allelic association) is predictably present in the vicinity of a disease gene, as a high proportion of affected individuals are likely to share the chromosomal segment containing the gene identical by descent (IBD) from a
Fig. 1: Genealogical lineages of 117 DM2 patients that could be traced back to a common ancestor within 13 generations. The figure is based on the shortest number of meiosis separating each person from this common ancestor.
### Table 1: Characteristics of type 2 diabetes patients and first degree relatives

<table>
<thead>
<tr>
<th></th>
<th>Patients</th>
<th>Relatives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>117</td>
<td>84</td>
</tr>
<tr>
<td>Male / female</td>
<td>47 / 70</td>
<td>21 / 63</td>
</tr>
<tr>
<td>Age at examination</td>
<td>63.5 (13.0)</td>
<td>44.5 (13.3)</td>
</tr>
<tr>
<td>Age at onset</td>
<td>57.9 (12.3)</td>
<td>-</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>29.8 (4.8)</td>
<td>27.5 (4.3)</td>
</tr>
<tr>
<td>Medication: non / oral / insulin</td>
<td>25 / 72 / 20</td>
<td>84 / 0 / 0</td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>8.7 (3.2)</td>
<td>5.8 (1.7)</td>
</tr>
</tbody>
</table>

In total 202 relatives participated. Clinical characteristics were available for 84 first degree relatives (parents, siblings or children). Values are given as total numbers or as means (SD).

*Including subjects using glucose lowering medication.*

### Table 2: Markers with LOD-score >1 in the initial genome scan

<table>
<thead>
<tr>
<th>Marker</th>
<th>Associated allele (bp)</th>
<th>Estimated population frequency</th>
<th>Frequency affected individuals</th>
<th>Lambda</th>
<th>LOD Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marker 1</td>
<td>176</td>
<td>63.8%</td>
<td>89.3%</td>
<td>0.70</td>
<td>1.5</td>
</tr>
<tr>
<td>Marker 2</td>
<td>195</td>
<td>56.0%</td>
<td>85.1%</td>
<td>0.66</td>
<td>1.6</td>
</tr>
<tr>
<td>Marker 3</td>
<td>171</td>
<td>39.2%</td>
<td>75.1%</td>
<td>0.59</td>
<td>1.3</td>
</tr>
<tr>
<td>Marker 4b</td>
<td>142</td>
<td>4.6%</td>
<td>25.3%</td>
<td>0.22</td>
<td>1.2</td>
</tr>
<tr>
<td>Marker 5</td>
<td>140</td>
<td>23.0%</td>
<td>52.9%</td>
<td>0.39</td>
<td>1.3</td>
</tr>
<tr>
<td>Marker 6</td>
<td>202</td>
<td>48.3%</td>
<td>77.3%</td>
<td>0.56</td>
<td>1.1</td>
</tr>
<tr>
<td>Marker 7</td>
<td>132</td>
<td>13.0%</td>
<td>41.8%</td>
<td>0.33</td>
<td>1.6</td>
</tr>
<tr>
<td>Marker 8b</td>
<td>86</td>
<td>26.0%</td>
<td>59.7%</td>
<td>0.45</td>
<td>2.3</td>
</tr>
<tr>
<td>Marker 9</td>
<td>290</td>
<td>24.0%</td>
<td>47.3%</td>
<td>0.31</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Number of alleles for each marker is shown in parentheses. Population frequency was estimated on the whole dataset with correction for dependency due to family relationships. Lambda represents the proportion of disease chromosomes with the ancestral allele.
common ancestor. Therefore, genome regions where marker allele frequencies differ between affected individuals and the background population likely harbor disease loci. When such deviation is based on a substantially increased frequency of one or a few alleles in affected individuals, the region is almost certainly inherited IBD with the disease gene from a common ancestor. In our genome scan, we used a modification of the method described by Terwilliger to evaluate statistical evidence for excess sharing of founder alleles in our patient population. We followed established procedures to directly estimate allele frequencies from the family members in the study. We found evidence for association with 9 markers (LOD-score > 1.0, table 2). The strongest evidence for a DM2 locus was obtained for marker 8b (LOD-score 2.3).

To verify the findings of our initial genome scan, we performed a quantitative analysis of glucose levels in an independent set of first degree relatives. For 84 of the first-degree relatives fasting serum glucose concentrations were ascertained. As diabetes is characterized by high blood glucose concentrations, we investigated the correlation between the ancestral alleles identified in the initial genomic screen and fasting glucose values in these subjects. Again, the strongest evidence for a DM2 locus was found for marker 8b. Mean glucose values were on average 1.1 mmol/l higher in first degree relatives carrying the 86-bp allele of marker 8b, which was associated with DM2 in the initial screen. A linear relation was observed between the number of alleles and glucose concentration, suggesting a co-dominant effect of this ancestral allele. Of the other loci identified in the initial genome scan, also the ancestral (142-bp) allele of marker 4b showed significant evidence for association with fasting glucose concentrations (table 3). However, due to the relative low frequency of the ancestral allele no homozygous subjects were available to test for a linear correlation.

To explore these two regions in more detail, flanking markers and haplotypes in the two associated regions were investigated. For all markers, the risk for DM2 associated with the ancestral allele (as identified in the initial genome scan) and a possible association with fasting glucose levels was tested.
Table 3: Relation of ancestral alleles identified in the initial screen with fasting glucose concentration in first degree relatives

<table>
<thead>
<tr>
<th>Marker(s)</th>
<th>Associated allele (bp)</th>
<th>Mean fasting glucose concentration (SD)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>non-carriers / heterozygous carriers / homozygous carriers</td>
<td></td>
</tr>
<tr>
<td>Marker 1</td>
<td>176</td>
<td>5.3 (0.6) / 8 / 6.0 (2.2) / 38 / 5.7 (1.1) / 36</td>
<td>0.22</td>
</tr>
<tr>
<td>Marker 2</td>
<td>195</td>
<td>6.2 (2.3) / 6 / 5.8 (1.1) / 39 / 5.8 (2.1) / 35</td>
<td>0.16</td>
</tr>
<tr>
<td>Marker 3</td>
<td>171</td>
<td>5.8 (1.5) / 16 / 5.9 (2.6) / 20 / 6.2 (1.5) / 11</td>
<td>0.20</td>
</tr>
<tr>
<td>Marker 4b</td>
<td>142</td>
<td>5.7 (1.2) / 72 / 6.8 (3.5) / 10 / -</td>
<td>0.01</td>
</tr>
<tr>
<td>Marker 5</td>
<td>140</td>
<td>5.8 (1.9) / 40 / 5.8 (1.4) / 41 / 5.5 (0.05) / 3</td>
<td>0.84</td>
</tr>
<tr>
<td>Marker 6</td>
<td>202</td>
<td>6.2 (1.6) / 16 / 5.6 (1.9) / 39 / 5.8 (1.4) / 28</td>
<td>0.76</td>
</tr>
<tr>
<td>Marker 7</td>
<td>132</td>
<td>5.6 (1.6) / 53 / 6.2 (1.8) / 27 / 5.3 (0.1) / 4</td>
<td>0.38</td>
</tr>
<tr>
<td>Marker 8b</td>
<td>86</td>
<td>5.4 (0.6) / 45 / 6.2 (2.4) / 31 / 6.5 (2.6) / 5</td>
<td>0.04 / 0.01*</td>
</tr>
<tr>
<td>Marker 9</td>
<td>240</td>
<td>5.7 (1.1) / 43 / 6.0 (2.2) / 35 / 5.1 (0.9) / 5</td>
<td>0.55</td>
</tr>
</tbody>
</table>

Mean glucose values are given (standard deviation) with number of subjects. P-values are based on ANOVA for overall difference between genotypes. * p-value for linear trend based on regression analysis.
Although the flanking markers of marker 4b did not exceed a lod score of 1 in the initial genome scan, the ancestral alleles of markers 4a, 4b, 4c, 4d, 4e and 4f were all associated with an increased risk for DM2 (table 4). Also the distribution of two-marker haplotypes in this region was different between cases and controls, however, the difference was not statistically significant. Interestingly, markers 4b, 4c and 4d and haplotypes in the region 4b to 4d were also strongly associated with fasting glucose levels in first degree relatives, supporting the presence of a true diabetes susceptibility locus in this region. Also in the region of marker 8b, sharing of additional markers by affected individuals was observed, indicating the presence of another susceptibility locus, inherited IBD from a common ancestor. The ancestral alleles of the adjacent markers 8c, 8d, 8e, 8g and 8h and two-marker haplotypes in the region from marker 8a to 8i were associated with a highly increased risk for DM2 (table 5). In addition, higher fasting glucose values were observed in first degree relatives carrying the ancestral alleles of marker 8a, 8d, 8f and 8h and haplotypes in the region from marker 8d to 8h (table 5).

Although several genetic factors have been identified for some specific forms of diabetes, including mutations in the HNF-1α, HNF-1β, HNF-4α and IPF-1 genes involved in Maturity-Onset Diabetes of the Young (MODY) 20-24, the genetic defects that are responsible for the majority of diabetes with adult onset are still unknown. Linkage studies for loci influencing the risk for DM2 have been conducted in a number of populations 5-12,25-29. In 1996, Hanis et al. reported significant evidence for linkage of DM2 to the distal arm of chromosome 2 7. By using a linkage-disequilibrium approach, similar to the approach used in our study, Horikawa et al. recently demonstrated that susceptibility at this locus is confined to certain combinations of polymorphisms in the gene encoding calpain-10 30. Following the initial study by Hanis et al., several other studies have identified additional susceptibility regions throughout the genome 5-12,25-29. However, none of the significant findings of these studies and only a few of the suggestive findings have been replicated in other studies. Although this may suggest that some of the initial linkage findings have been false positives, it may also reflect the genetic heterogeneity of DM2 in diverse ethnic groups.
### Table 4: Association of markers flanking marker 4b with type 2 diabetes mellitus and fasting glucose levels

<table>
<thead>
<tr>
<th>Marker (allele in bp)</th>
<th>Cases</th>
<th>Controls</th>
<th>Heterozygous</th>
<th>Homozygous</th>
<th>p-value*</th>
<th>Heterozygous</th>
<th>Homozygous</th>
<th>Non-carriers</th>
<th>Heterozygous</th>
<th>Homozygous</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marker 4a (162)</td>
<td>36.7 / 22</td>
<td>22.4 / 11</td>
<td>0.007</td>
<td>0</td>
<td>2.2 (0.9 - 5.3)</td>
<td>-</td>
<td>5.9 (1.9) / 57</td>
<td>5.4 (0.9) / 30</td>
<td>5.5 (1.4) / 3</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>Marker 4b (142)</td>
<td>25.5 / 14</td>
<td>12.8 / 6</td>
<td>0.04</td>
<td>0</td>
<td>2.4 (0.8 - 6.8)</td>
<td>-</td>
<td>5.7 (1.2) / 72</td>
<td>6.8 (3.5) / 10</td>
<td>-</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Marker 4c (108)</td>
<td>41.4 / 24</td>
<td>41.3 / 19</td>
<td>20.7 / 12</td>
<td>8.7 / 4</td>
<td>0.05</td>
<td>1.3 (0.6 - 3.1)</td>
<td>3.1 (0.9 - 11.1)</td>
<td>5.6 (1.3) / 38</td>
<td>5.5 (1.1) / 33</td>
<td>6.7 (3.1) / 13</td>
<td>0.02</td>
</tr>
<tr>
<td>Marker 4d (171)</td>
<td>47.9 / 23</td>
<td>34.2 / 13</td>
<td>12.5 / 6</td>
<td>2.6 / 1</td>
<td>0.01</td>
<td>2.2 (0.9 - 5.5)</td>
<td>7.8 (0.9 - 68.4)</td>
<td>5.6 (1.3) / 35</td>
<td>5.5 (0.9) / 35</td>
<td>6.6 (3.3) / 11</td>
<td>0.05</td>
</tr>
<tr>
<td>Marker 4e (160)</td>
<td>34.1 / 15</td>
<td>32.1 / 17</td>
<td>18.9 / 10</td>
<td>4.5 / 2</td>
<td>0.03</td>
<td>1.2 (0.5 - 2.8)</td>
<td>5.2 (1.0 - 26.0)</td>
<td>5.9 (2.2) / 43</td>
<td>5.6 (0.8) / 33</td>
<td>5.7 (0.7) / 6</td>
<td>0.36</td>
</tr>
<tr>
<td>Marker 4f (98)</td>
<td>45.1 / 23</td>
<td>32.6 / 14</td>
<td>11.8 / 6</td>
<td>2.3 / 1</td>
<td>0.006</td>
<td>2.1 (0.9 - 4.9)</td>
<td>7.6 (0.9 - 68.0)</td>
<td>5.7 (1.9) / 43</td>
<td>5.8 (1.4) / 42</td>
<td>5.7 (0.8) / 6</td>
<td>0.49</td>
</tr>
</tbody>
</table>

### Haplotype

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Cases</th>
<th>Controls</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>4a-4b</td>
<td>8.2 / 5</td>
<td>2.0 / 1</td>
<td>0.09</td>
</tr>
<tr>
<td>4b-4c</td>
<td>8.3 / 5</td>
<td>7.7 / 4</td>
<td>0.45</td>
</tr>
<tr>
<td>4c-4d</td>
<td>14.3 / 8</td>
<td>17.4 / 8</td>
<td>0.45</td>
</tr>
<tr>
<td>4d-4e</td>
<td>8.8 / 5</td>
<td>8.3 / 4</td>
<td>0.35</td>
</tr>
<tr>
<td>4e-4f</td>
<td>16.1 / 9</td>
<td>8.5 / 4</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Genotype frequencies are presented as percentages / numbers of subjects. Risks are estimated as odds ratios (95% confidence interval). Glucose-values are given as mean (SD) / number of subjects. * p-value for trend.
<table>
<thead>
<tr>
<th>Marker (allele in bp)</th>
<th>Frequency ancestral allele or haplotype (%) / n</th>
<th>Risk for diabetes (95% CI)</th>
<th>Glucose values in first-degree relatives (SD) / n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Frequency homozygous</td>
<td>Risk heterozygous</td>
<td>Risk homozygous</td>
</tr>
<tr>
<td>Marker 8a (270)</td>
<td>58.1 / 29 Homozygous</td>
<td>1.2 (0.5 - 2.8)</td>
<td>-</td>
</tr>
<tr>
<td>Marker 8b (66)</td>
<td>56.5 / 28 Homozygous</td>
<td>3.3 (1.4 - 7.4)</td>
<td>23.2 (2.7 - 195.8</td>
</tr>
<tr>
<td>Marker 8c (146)</td>
<td>47.8 / 24 Homozygous</td>
<td>1.8 (0.8 - 3.8)</td>
<td>3.4 (1.1 - 10.5</td>
</tr>
<tr>
<td>Marker 8d (122)</td>
<td>38.1 / 19 Homozygous</td>
<td>1.2 (0.5 - 3.0)</td>
<td>4.2 (1.3 - 13.6</td>
</tr>
<tr>
<td>Marker 8e (186)</td>
<td>40.4 / 20 Homozygous</td>
<td>1.0 (0.4 - 2.4)</td>
<td>3.6 (1.1 - 12.1</td>
</tr>
<tr>
<td>Marker 8f (254)</td>
<td>37.7 / 19 Homozygous</td>
<td>1.9 (0.8 - 4.8)</td>
<td>2.7 (0.5 - 14.9</td>
</tr>
<tr>
<td>Marker 8g (208)</td>
<td>30.4 / 15 Homozygous</td>
<td>0.3 (0.2 - 1.1)</td>
<td>2.0 (0.5 - 8.5</td>
</tr>
<tr>
<td>Marker 8h (159)</td>
<td>42.6 / 21 Homozygous</td>
<td>2.9 (1.2 - 6.8)</td>
<td>4.9 (0.5 - 46.1</td>
</tr>
<tr>
<td>Marker 8i (203)</td>
<td>19.2 / 9 Homozygous</td>
<td>1.2 (0.4 - 3.5)</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Frequency homozygous</th>
<th>Risk heterozygous</th>
<th>Risk homozygous</th>
<th>Non-carriers</th>
<th>Heterozygous</th>
<th>Homozygous</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>8a - 8b</td>
<td>21.3 / 13 Homozygous</td>
<td>3.3 (1.0 - 10.7)</td>
<td>-</td>
<td>5.7 (1.6 / 65</td>
<td>6.0 (2.2 / 7</td>
<td>-</td>
<td>0.30</td>
</tr>
<tr>
<td>8b - 8c</td>
<td>41.9 / 26 Homozygous</td>
<td>4.0 (1.6 - 9.9)</td>
<td>-</td>
<td>5.7 (1.7 / 60</td>
<td>6.0 (1.6 / 12</td>
<td>-</td>
<td>0.30</td>
</tr>
<tr>
<td>8c - 8d</td>
<td>26.7 / 16 Homozygous</td>
<td>1.5 (0.6 - 3.8)</td>
<td>-</td>
<td>5.8 (1.9 / 43</td>
<td>5.6 (1.4 / 16</td>
<td>5.8 (0.5 / 2</td>
<td>0.36</td>
</tr>
<tr>
<td>8d - 8e</td>
<td>22.8 / 13 Homozygous</td>
<td>1.0 (0.4 - 2.8)</td>
<td>-</td>
<td>5.6 (1.1 / 47</td>
<td>6.9 (2.9 / 15</td>
<td>6.0 (0.7 / 4</td>
<td>0.07</td>
</tr>
<tr>
<td>8e - 8f</td>
<td>26.7 / 16 Homozygous</td>
<td>3.0 (1.0 - 9.1)</td>
<td>2.9 (0.3 - 28.6</td>
<td>5.7 (0.9 / 54</td>
<td>6.2 (1.5 / 15</td>
<td>16.2 (-/-) / 1</td>
<td>0.0001</td>
</tr>
<tr>
<td>8f - 8g</td>
<td>25.5 / 14 Homozygous</td>
<td>1.4 (0.5 - 3.5)</td>
<td>0.9 (0.1 - 6.5</td>
<td>5.5 (0.6 / 48</td>
<td>6.2 (1.5 / 17</td>
<td>10.4 (8.2 / 2</td>
<td>0.0001</td>
</tr>
<tr>
<td>8g - 8h</td>
<td>28.6 / 18 Homozygous</td>
<td>1.6 (0.7 - 3.7)</td>
<td>-</td>
<td>5.7 (1.7 / 46</td>
<td>5.9 (1.5 / 24</td>
<td>7.8 (1.6 / 2</td>
<td>0.01</td>
</tr>
<tr>
<td>8h - 8i</td>
<td>15.6 / 10 Homozygous</td>
<td>10.2 (1.3 - 78.8</td>
<td>-</td>
<td>5.9 (1.7 / 61</td>
<td>5.9 (1.2 / 12</td>
<td>-</td>
<td>0.45</td>
</tr>
</tbody>
</table>

Genotype frequencies are presented as percentages / numbers of subjects. Risks are estimated as odds ratios (95% confidence interval). Glucose values are given as mean (SD) / number of subjects. * p-value for trend.
In our study we aimed to overcome these problems by screening patients with DM2 derived from a homogeneous, recently isolated population. We reasoned that, because of the recent isolation, this population is likely to show more resemblance with the general Caucasian population than other frequently studied older isolates, such as Finland. Further, we build in a verification of the findings of our genome scan, by using information on fasting glucose levels in first-degree relatives. Glucose levels in first degree relatives offer a unique opportunity for replication. First, the disease associated haplotype is expected to be prevalent in those subjects, as first degree relatives share 50% of their DNA. Second, the association between the haplotype and glucose levels in relatives is independent of the association between the marker and DM2 in probands. Thus, the replication of our results in this independent sample of first-degree relatives makes it unlikely that our results represent a false positive finding after a search over many markers. Therefore, our findings strongly suggest the localization of two DM2 loci in the regions identified by our genome scan. Whereas several other loci have been implicated in type 2 diabetes, including a region on chromosome 2 that holds the calpain-10 gene \textsuperscript{20-30}, we could not detect significant association with any of these regions in the GRIP population.

**Research design and methods**

*Participants*

Patients with DM2 were recruited with help from the local healthcare centers and the Diabetes Service Breda, which is a regional clinical and laboratory service for the western part of the province of North Brabant. Since 1990 the Diabetes Service Breda has collected clinical and biochemical data on more than 8000 patients with DM2. All patients undergo clinical and laboratory evaluations for their diabetes at regular 3 months intervals. These evaluations include laboratory tests of glucose metabolism (fasting glucose level, HbA1c), blood lipids (HDL/LDL/Triglycerides and total cholesterol), renal function (microalbuminuria) and clinical measurements such as body weight and blood pressure. Medical records covering the last 20 years are available for all patients at the local healthcare centers. We
recruited 160 patients diagnosed with type 2 diabetes living in the isolated village. The overall participation rate was 80% (n = 128). All participants completed a questionnaire on family and medical history, underwent anthropometric and blood pressure measurements and gave blood samples for DNA-extraction and fasting serum. We applied the American Diabetes Association criteria for the diagnosis of diabetes to confirm the diagnosis\textsuperscript{13}. Individuals who were pregnant at time of diagnosis or individuals who were insulin-dependent within 1 year following diagnosis were assigned a diagnosis of "unknown". For each patient, at least 2 first-degree relatives were asked to participate in the study to be able to reconstruct haplotypes. In addition, serum samples were collected for 84 first-degree relatives to determine fasting glucose concentration.

Collection of genealogical information
In order to determine which of the subjects were descended primarily from the original founders of the isolated village, a genealogical search was completed for each patient, using church and municipal records of births, marriages and deaths. Genealogical lineage's for each patient were traced back 15 generations.

Genotyping
The genome screen was performed using 770 markers covering the whole genome, with an average spacing of 4.6 cM. No gap was greater than 18.7 cM and no more than 21 gaps (2.7%) were greater than 10 cM.

Statistical analysis
A stepwise procedure was used in the analyses. First, a whole genome scan was performed to test for linkage-disequilibrium of single markers with diabetes. The test used is based on a modification of the method described by Terwilliger and assumes that some marker allele will be over-represented on chromosomes that carry the disease mutation, when many of these chromosomes descend from a single ancestor \textsuperscript{16}. The proportion of disease chromosomes with this ancestral allele is represented by the parameter $\lambda$. It is not a priory known which marker allele will be the over-represented allele, and therefore the test considers each of
the marker alleles separately as potential founder alleles. Consequently, a total likelihood is obtained for a given value of \( \lambda \) by computing the likelihood on the data for each potential founder allele, and summing those likelihood's, weighted for the population frequency of the respective founder allele. While the procedure was originally applied to genotype data in samples of affected and unaffected individuals, it can also be used to calculate likelihoods for pedigree data \(^{17,18}\). In the estimation procedure the recombination fraction was fixed at 0.01 and the disease gene frequency was kept constant at 2.5%. Unaffected individuals or subjects without clinical information were assigned to one of the age-based liability classes, which are derived from the age of onset distribution of patients with type 2 diabetes from the Rotterdam Study, a population based cohort study in the Netherlands \(^{31}\). In this study, a LOD-score > 1.0 (corresponding to \( P < 0.016 \)) was used as a threshold to select markers for further analysis.

In the second phase, to confirm the findings of the genome scan, all markers that exceeded a LOD-score of 1.0 were tested for association with fasting glucose concentration in 84 first degree relatives. Serum glucose values were logarithmically transformed for the analyses. ANOVA was used to test for differences in mean glucose levels between carriers and non-carriers of the ancestral alleles that were identified in the initial genomic screen. A linear regression model was used to test for a possible linear association of the founder allele with glucose levels.

In the third phase, a case-control analysis was performed to test for association of flanking markers and haplotypes with type 2 diabetes and fasting glucose levels. For each marker, the analysis was restricted to the ancestral allele that had been identified in the initial genome scan. Haplotypes based on these ancestral alleles were constructed around the markers that exceeded a lod score of 1 in the initial genome scan and were associated with fasting glucose levels in first degree relatives. Haplotypes were constructed for all genotyped individuals and for deceased individuals whose genotypes could be reconstructed. For the case-control analysis one patient was selected per family and haplotypes of this patient and first degree relatives were constructed. The non-transmitted alleles or
haplotypes of the parents and the alleles or haplotypes of the partner were used as controls. A relative risk for each ancestral allele and for each haplotype was estimated as an odds-ratio with a 95% confidence interval. A p-value for trend was calculated to indicate a possible linear relation between the number of ancestral alleles or haplotypes and the risk for diabetes. To confirm the findings of the case-control analysis, ANOVA was used to test for differences in mean glucose levels between carriers and non-carriers of ancestral alleles and haplotypes in first-degree relatives. Fasting glucose values are given plus standard deviation, adjusted for differences in age and gender. Again, a p-value for trend was calculated to indicate a possible linear relation between the number of ancestral alleles or haplotypes and the mean fasting glucose concentration.

Acknowledgements

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References

CHAPTER 3

CANDIDATE GENE STUDIES
CHAPTER 3.1

A POLYMORPHISM IN THE GENE FOR INSULIN-LIKE GROWTH FACTOR-I: FUNCTIONAL PROPERTIES AND RISK FOR TYPE 2 DIABETES MELLITUS AND MYOCARDIAL INFARCTION

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Erasmus Medical Center Rotterdam, The Netherlands, Department of Epidemiology & Biostatistics (*), Center for Biomedical Genetics, Department of Clinical Genetics (†), Department of Internal Medicine (‡).
Summary

Evidence is accumulating that low levels of insulin-like growth factor-I (IGF-I) play a role in the pathogenesis of diabetes mellitus and cardiovascular diseases. We examined the role of a genetic polymorphism in the promoter region of the IGF-I gene in relation to circulating IGF-I levels and growth measured as body height, and we studied the relationship of this polymorphism with type 2 diabetes mellitus and myocardial infarction. The relation between the IGF-I polymorphism and body height was assessed in population-based sample of 900 subjects from the Rotterdam Study. Within each genotype stratum, 50 subjects were randomly selected to study the relation of this polymorphism with serum IGF-I levels. To assess the risk of type 2 diabetes mellitus, we studied 220 patients and 596 normoglycaemic controls. For myocardial infarction, 477 patients with evidence for myocardial infarction at ECG and 808 controls were studied. A 192-bp allele was present in 88% of the population, suggesting that this is the wildtype allele from which all other alleles originated. Body height was on average 2.7 cm lower (95% CI for difference -4.6 to -0.8 cm, \( p = 0.004 \)) and serum IGF-I concentrations were 18% lower (95% CI for difference -6.0 to -1.3 mmol/L, \( p = 0.003 \)) in subjects who did not carry the 192-bp allele. In non-carriers of the 192-bp allele, an increased relative risk for diabetes (1.7 [95% CI 1.1-2.7]) and for myocardial infarction (1.7 [95% CI 1.1-2.5]) was found. In patients with type 2 diabetes mellitus the relative risk for myocardial infarction in subjects without the 192-bp allele was 3.4 (95% CI 1.1-11.3). Our study suggests that a genetically determined exposure to relatively low IGF-I levels is associated with an increased risk for type 2 diabetes mellitus and myocardial infarction.
Introduction

Insulin-like growth factor-I (IGF-I) is a peptide which stimulates bone growth, cell differentiation and metabolism. The structural and functional homologies with insulin\(^1\) as well as the hypoglycemic insulin-like effects observed after the administration of recombinant IGF-I suggest that this peptide is involved in the regulation of glucose homeostasis\(^2\). In patients with diabetes mellitus low serum levels of IGF-I are frequently observed\(^3,4\). Evidence is accumulating that these low IGF-I levels may play a role in development of the vascular complications of diabetes\(^5,6\). IGF-I may also play a role in the regulation of cardiovascular function\(^7,8\) and in the development of myocardial infarction in subjects without diabetes, although different studies have yielded conflicting results regarding the direction of the association between circulating IGF-I levels and cardiovascular disease\(^9,14\).

Studies on the role of IGF-I in the development of disease have been hampered by the fact that circulating IGF-I levels do not necessarily reflect the local production of IGF-I in specific tissues, such as the myocardium or pancreatic beta cells. A genetic polymorphism in the IGF-I gene promoter region has been identified, which may influence the IGF-I production\(^15,16\). This may open the opportunity to characterize on a genetic basis subjects that are chronically exposed to low IGF-I levels throughout the body. Until now, studies of this polymorphism in relation to IGF-I levels and pathology have been limited to patients with osteoporosis and related disorders\(^16,18\).

We examined the role of a known genetic polymorphism in the promoter region of the IGF-I gene in relation to circulating IGF-I levels and growth measured as body height, and we studied the relationship of this polymorphism with type 2 diabetes and myocardial infarction.

Research design and methods

Subjects.

This study is part of the Rotterdam Study, a single-center prospective follow-up study in which all residents aged 55 years and over of the Rotterdam suburb Ommoord were invited to take part. The study was approved by the Medical Ethics
Committee of Erasmus Medical Center Rotterdam and written informed consent was obtained from all participants. The aim of the study was to investigate determinants of chronic and disabling cardiovascular, neurodegenerative, locomoter and ophthalmologic diseases. The design of the study has been described previously 19. The baseline examination of the Rotterdam Study, on which this report is based, was conducted between 1990 and 1993. A total of 7,983 participants (response rate 78%) were examined. In the present study, we only included subjects aged between 55 and 75 years at the baseline examination.

We followed a five-phase approach to study the relation of a known cytosine-adenine repeat in the promoter region of the human IGF-I gene 15 with growth, IGF-I serum levels, risk of diabetes and of cardiovascular disease. In phase 1, we selected a population based sample of 900 participants to assess genotype and allele frequencies of the polymorphism and their relationship with body height. The 900 subjects were randomly drawn from all participants in the age groups 55-65 years and 65-75 years and frequency matched for the age distribution of the myocardial infarction cases (described later). The number of subjects was based on power calculations to detect a difference in body-height of at least 1.5 cm with a power of 80% and a significance level of 0.05. In phase 2, 50 subjects were randomly drawn from each genotype stratum in the population based sample. In these subjects the relation between the promoter polymorphism and serum IGF-I concentration was studied. The selection of these 50 subjects per genotype was based on power calculations to detect a difference of at least 15% in serum IGF-I concentration between genotypes with a power of 80% and a significance level of 0.05. In phase 3, the association of the polymorphism with type 2 diabetes mellitus was assessed in a case-control study. Because of practical and financial reasons only a proportion of the Rotterdam Study (n = 1110) underwent a fasting oral glucose tolerance test. From this group we selected our cases and controls. Included were patients treated for diabetes and newly diagnosed patients based on a fasting glucose level of 7.0 mmol/l or above and/or a 2 hours post-load glucose measurement of 11.1 mmol/l or above following a fasting 75 gram oral glucose tolerance test 20. The 220 patients identified were compared to 596 normoglycaemic
controls. Normoglycemia was defined as fasting glucose below 6.1 mmol/l and 2 hours post-load glucose sample below 7.8 mmol/l.

In the fourth and fifth phase the association of the polymorphism with myocardial infarction was assessed. In phase 4, 477 participants with ECG confirmed myocardial infarction at the baseline examination were compared with 808 controls without evidence for myocardial infarction on ECG. These controls were selected from the age-frequency matched population of 900 subjects of phase 1. In all participants, a resting standard 12-lead electrocardiogram was made with an ACTA Gnosis IV (EsaciteBiomedica) 21,22. For the diagnosis of myocardial infarction an automated diagnostic classification system, the Modular ECG Analysis System (MEANS) 21,22, was used. Infarctions detected without evidence of symptoms (silent myocardial infarctions) were verified by an experienced cardiologist 21,22. Finally, in phase 5 the relative risk of myocardial infarction among the 220 subjects with type 2 diabetes was studied in relation with the IGF-1 promoter polymorphism using the clinical criteria described above.

Measurements
At the baseline examination, information concerning health status, drug use and smoking behavior was obtained with a computerized questionnaire. Height and weight were measured and body mass index (BMI in kg/m²) was calculated. Body fat distribution was assessed by the ratio of waist and hip circumferences (WHR). Blood pressure was measured in sitting position at the right upper arm with a random-zero sphygmonanometer, and the average of two measurements obtained at one occasion was used. Hypertension was defined as a diastolic blood pressure of 95 mmHg or higher and/or a systolic blood pressure of 160 or higher and/or the use of antihypertensive drugs.

Blood sampling and storage have been described elsewhere 23. Serum was separated by centrifugation and quickly frozen in liquid nitrogen. Baseline measurements were performed on non-fasting blood samples. Total serum cholesterol and HDL-cholesterol were determined with an automated enzymatic procedure 24. Glucose levels were measured by the glucose hexokinase method in
fasting serum and post-load serum samples. Total IGF-I was determined by a commercially available radioimmunoassay (Medgenix Diagnostics, with intra-assay and inter-assay variation of 6.1% and 9.9%) in non-fasting serum.

Polymerase chain reaction (PCR) was performed using oligonucleotide primers designed to amplify the polymorphic cytosine-adenine repeat 1 Kb upstream of the human IGF-I gene. The reaction was carried out in a final volume of 10 μl containing 50 ng of genomic DNA obtained from peripheral white blood cells, 0.5 mM forward primer (5'-ACCACTCTGGAGAAGGTA-3'), 0.5 mM reverse primer (5'-GCTCGCCAGCCTGTTAT-3'), 0.25 mM dNTP, 2.2 mM MgCl₂, 0.01% W1 (Gibco BRL) and 0.4 U Taq DNA polymerase (Gibco BRL). PCR was performed in 384 well plates (94°C 10 min; 35 PCR cycles of 30 seconds at 94°C, 30 seconds at 55°C and 30 seconds at 72°C; 72°C 10 min; 4°C C hold). Forward primers were labelled with FAM, HEX or NED to determine the size of PCR products by auto sequencer (ABI 377, 6.25% longranger gel, filter set D, peak-height between 100 and 2000, each lane containing 3 samples). The size of the PCR products was determined in comparison with internal ROX 500-size standard (Perkin Elmer).

Statistical analysis

Hardy-Weinberg equilibrium of the IGF-I promoter polymorphism genotypes was tested with the GENEPOP-package (Raymond M. & Rousset F, 1995. GENEPOP version 1.2). Body height and serum IGF-I were compared between genotypes using analyses of variance. Body height was adjusted for the possible confounders age and sex. Participants using diabetes or hormonal medication were excluded from the analyses of serum IGF-I, because this type of medication is known to influence IGF-I concentrations. Further, serum IGF-I was adjusted for the possible confounders age, sex and BMI. Serum IGF-I values were logarithmically transformed for the analyses. Both for body height and IGF-I, the non-transformed data and standard errors are presented.

A multiple logistic regression model was used to study the association of the IGF-I promoter polymorphism genotypes with diabetes and myocardial infarction. Each
patient group was compared to the specific control group selected. Relative risks were estimated as odds ratios and presented with a 95% confidence interval (95% CI). Both crude relative risks and relative risks after adjustment for the possible confounders age, sex, body height, BMI, WHR, total cholesterol, HDL-cholesterol and hypertension are presented in the table. All analyses were performed using the SPSS for Windows software package, version 7.5.2.

Table 1: Baseline characteristics of the study populations

<table>
<thead>
<tr>
<th></th>
<th>Population sample</th>
<th>Diabetes study</th>
<th>Myocardial infarction study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cases</td>
<td>Controls</td>
<td>Cases</td>
</tr>
<tr>
<td>Number of subjects</td>
<td>900</td>
<td>220</td>
<td>596</td>
</tr>
<tr>
<td>Age in years</td>
<td>65.9 (5.6)</td>
<td>68.2 (5.6) †</td>
<td>66.2 (5.4)</td>
</tr>
<tr>
<td>Men</td>
<td>39.9%</td>
<td>56.4%*</td>
<td>47.3%</td>
</tr>
<tr>
<td>Current smoking</td>
<td>25.3%</td>
<td>20.1%</td>
<td>24.1%</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>26.6 (3.8)</td>
<td>27.6 (3.5) *</td>
<td>25.9 (3.2)</td>
</tr>
<tr>
<td>Waist to hip ratio</td>
<td>0.90 (0.09)</td>
<td>0.94 (0.09) †</td>
<td>0.89 (0.09)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>6.8 (1.2)</td>
<td>6.4 (1.2)</td>
<td>6.6 (1.1)</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.4 (0.3)</td>
<td>1.2 (0.3) †</td>
<td>1.4 (0.3)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>38.6%</td>
<td>55.7% †</td>
<td>31.7%</td>
</tr>
</tbody>
</table>

Values are means (with standard deviation) or percentages. *p = 0.05, †p = 0.005

Results

Table 1 presents the characteristics of the participants. In both the myocardial infarction study and diabetes study, cases were slightly older and were more frequently male. Body mass index was significantly increased in patients with diabetes, while the waist to hip ratio was significantly increased both in patients with diabetes and patients with myocardial infarction. Low HDL-cholesterol levels and hypertension were more frequently seen among both case groups. In the population based sample of 900 subjects, 10 different alleles were identified in the promoter polymorphism of the IGF-I gene (table 2). Genotype and allele
distributions were in Hardy-Weinberg equilibrium (p=0.76). When considering genotypes, 88.4% of the subject from the population based sample was homozygous or heterozygous for a 192-base pair (bp) allele, suggesting that this is the wildtype allele from which all other alleles originated. The frequency of the other 9 alleles was low and these alleles were therefore pooled in the analyses. This resulted in 3 possible genotypes: subjects homozygous for the 192-bp allele (46.7%), subjects heterozygous for the 192-bp allele (41.7%) and non-carriers of the 192-bp allele (11.6%).

**Table 2: Allele distribution of the IGF-I promoter polymorphism**

<table>
<thead>
<tr>
<th>Allele (# base pairs)</th>
<th>Male (n=720)</th>
<th>Female (n=1080)</th>
<th>Total (n=1800)</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>1 (0.1%)</td>
<td>-</td>
<td>1 (0.1%)</td>
</tr>
<tr>
<td>198</td>
<td>9 (1.3%)</td>
<td>16 (1.5%)</td>
<td>25 (1.4%)</td>
</tr>
<tr>
<td>196</td>
<td>44 (6.1%)</td>
<td>80 (7.4%)</td>
<td>124 (6.9%)</td>
</tr>
<tr>
<td>194</td>
<td>118 (16.4%)</td>
<td>202 (18.7%)</td>
<td>320 (17.8%)</td>
</tr>
<tr>
<td>192</td>
<td>505 (70.1%)</td>
<td>712 (65.9%)</td>
<td>1217 (67.6%)</td>
</tr>
<tr>
<td>190</td>
<td>26 (3.6%)</td>
<td>44 (4.1%)</td>
<td>70 (3.9%)</td>
</tr>
<tr>
<td>188</td>
<td>15 (2.1%)</td>
<td>21 (1.9%)</td>
<td>36 (2.0%)</td>
</tr>
<tr>
<td>186</td>
<td>2 (0.3%)</td>
<td>3 (0.3%)</td>
<td>5 (0.3%)</td>
</tr>
<tr>
<td>184</td>
<td>-</td>
<td>2 (0.2%)</td>
<td>2 (0.1%)</td>
</tr>
<tr>
<td>182</td>
<td>1 (0.1%)</td>
<td>-</td>
<td>1 (0.1%)</td>
</tr>
</tbody>
</table>

Allele distribution based on a population-based sample of 900 participants (1800 alleles: 2 alleles per person).

In the population based sample of 900 subjects, body height increased with the number of 192-bp alleles present (p for trend=0.01, figure 1A). Mean body height was significantly lower in non-carriers of the 192-bp allele (165.4 cm) compared with subjects homozygous for the 192-bp allele (168.1 cm [95% CI for difference -4.6 to -0.8 cm, p=0.004]). Also mean serum IGF-I increased with the number of 192-bp alleles carried (p for trend=0.003, figure 1B). In non-carriers of the 192-bp allele, mean serum total IGF-I concentration was 18% lower (16.7 mmol/l) compared with those homozygous (20.5 mmol/l [95% CI for difference -6.0 to -1.3...
mmol/L, p=0.003). The findings on body height and IGF-I levels could not be attributed to a specific allele of the IGF-I promoter polymorphism.

**Figure 1**: Body height and serum IGF-I level in relation to the IGF-I promoter polymorphism

Figure 1A shows body height as mean with standard error. Figure 1B shows mean serum IGF-I concentration with standard error (mmol/L).

**Table 3**: Relation between IGF-I promoter genotype and previously identified correlates of diabetes or myocardial infarction.

<table>
<thead>
<tr>
<th></th>
<th>Homozygous 192-bp allele</th>
<th>Heterozygous 192-bp allele</th>
<th>No 192-bp allele</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>66.4 (5.4)</td>
<td>65.4 (5.6)</td>
<td>66.1 (6.0)</td>
<td>0.11</td>
</tr>
<tr>
<td>Men</td>
<td>179 (42.4%)</td>
<td>147 (39.2%)</td>
<td>34 (32.7%)</td>
<td>0.18</td>
</tr>
<tr>
<td>Current smoking</td>
<td>98 (23.4%)</td>
<td>108 (29.0%)</td>
<td>21 (20.6%)</td>
<td>0.30</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>26.6 (3.9)</td>
<td>26.8 (3.8)</td>
<td>26.5 (3.0)</td>
<td>0.71</td>
</tr>
<tr>
<td>Waist to hip ratio</td>
<td>0.90 (0.09)</td>
<td>0.91 (0.09)</td>
<td>0.90 (0.1)</td>
<td>0.52</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>6.8 (1.3)</td>
<td>6.8 (1.2)</td>
<td>6.7 (0.9)</td>
<td>0.93</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.3 (0.4)</td>
<td>1.4 (0.4)</td>
<td>1.4 (0.3)</td>
<td>0.27</td>
</tr>
<tr>
<td>Hypertension</td>
<td>170 (40.5%)</td>
<td>140 (37.3%)</td>
<td>37 (35.6%)</td>
<td>0.53</td>
</tr>
</tbody>
</table>

Measurements based on population sample of 900 subjects. Values are means (with standard deviation) or percentages.
Table 3 demonstrates that the IGF-I genotype is not associated with previously identified correlates of myocardial infarction or diabetes. Table 4 shows that fewer subjects with diabetes or myocardial infarction were homozygous for the 192-bp allele compared with the control groups. Compared to those homozygous for the 192-bp allele, non-carriers of the 192-bp allele had a relative risk of 1.7 for diabetes (95% CI 1.1-2.7) as well as for myocardial (95% CI 1.1-2.5). For subjects heterozygous for the 192-bp allele, the relative risk was 1.4 for diabetes (95% CI 1.0-1.9) and 1.2 for myocardial infarction (95% CI 0.9-1.5). Although the IGF-I polymorphism was strongly associated with body height, no statistically significant association between body height and diabetes or myocardial infarction was found. On average however, diabetes patients were 0.6 cm shorter (p = 0.23) and myocardial infarction cases were 0.4 cm shorter (p = 0.29) than controls. Correction for these small differences in body height in our analysis did not change the risk estimates for diabetes or myocardial infarction associated with the IGF-I genotype. Within the group of type 2 diabetes patients, 34 subjects with myocardial infarction were identified. The prevalence of myocardial infarction was 25% in subjects without the 192-allele (figure 2). The relative risk for myocardial infarction in non-carriers of the 192-bp allele was 3.4 (95% CI 1.1-11.3) while the relative risk for those heterozygous was 2.9 (95% CI 1.1-7.7).

<table>
<thead>
<tr>
<th></th>
<th>Homozygous 192-bp allele</th>
<th>Heterozygous 192-bp allele</th>
<th>No 192-bp allele</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diabetes</strong></td>
<td>83 (37.7%)</td>
<td>102 (46.4%)</td>
<td>35 (15.9%)</td>
</tr>
<tr>
<td><strong>Controls</strong></td>
<td>277 (46.5%)</td>
<td>248 (41.6%)</td>
<td>71 (11.9%)</td>
</tr>
<tr>
<td>Crude relative risk</td>
<td>reference</td>
<td>1.4 (1.0-1.9)</td>
<td>1.6 (1.0-2.6)</td>
</tr>
<tr>
<td>Adjusted relative risk*</td>
<td>reference</td>
<td>1.4 (1.0-1.9)</td>
<td>1.7 (1.1-2.7)</td>
</tr>
<tr>
<td><strong>Myocardial infarction</strong></td>
<td>205 (43.0%)</td>
<td>204 (42.8%)</td>
<td>68 (14.3%)</td>
</tr>
<tr>
<td><strong>Controls</strong></td>
<td>383 (47.4%)</td>
<td>340 (42.1%)</td>
<td>85 (10.5%)</td>
</tr>
<tr>
<td>Crude relative risk</td>
<td>reference</td>
<td>1.1 (0.9-1.4)</td>
<td>1.5 (1.0-2.1)</td>
</tr>
<tr>
<td>Adjusted relative risk *</td>
<td>reference</td>
<td>1.2 (0.9-1.5)</td>
<td>1.7 (1.1-2.5)</td>
</tr>
</tbody>
</table>

Number of subjects for each category are given (% of cases or controls). Risks are given with a 95% confidence interval. *Adjustment for the possible confounders age, sex, WHR, BMI, total cholesterol, HDL-cholesterol and hypertension
Discussion

In this population-based study we found that the absence of the wildtype (192-bp) allele of a genetic polymorphism in the regulatory region of the insulin-like growth factor-I gene is significantly associated with low body height and low serum levels of IGF-I. The main finding of the study is that the absence of this allele is also significantly associated with an increased risk of type 2 diabetes mellitus and myocardial infarction. Particularly, in subjects with type 2 diabetes mellitus the relative risk for myocardial infarction is strongly increased in subjects not carrying the 192-bp allele.

The polymorphism under study is a cytosine-adenosine (CA) repeat 1 kilobase upstream from the transcription site of the IGF-I gene. Given the population-based approach of our study, we cannot distinguish whether this polymorphism itself is involved in regulation of IGF-I expression or merely flags
another polymorphism in the promoter region functionally involved in IGF-I expression. However, the association with low body height and low IGF-I serum level, suggests that the absence of the 192-bp allele characterizes subjects chronically exposed to low IGF-I levels throughout the body. Using this polymorphism, the difficulty in measuring local IGF-I production in relevant tissues, such as the myocardium or the pancreatic beta cell, can be circumvented. Further, this genetic approach overcomes the problem that cross-sectional studies cannot distinguish whether changes in IGF-I levels are a cause or rather a consequence of disease.

In our population-based sample from the Rotterdam Study, the 192-bp allele was present in 88% of subjects. This finding is in agreement with other reports in Caucasian populations. In contrast with a previous report by Rosen et al., subjects homozygous for the 192-bp allele had significantly higher serum levels of IGF-I compared with subjects not carrying this allele. However, the study of Rosen et al was based on a relatively small, highly selected study population including patients with chronic heart disease, patients with idiopathic osteoporosis, participants of a calcium intervention trial and participants of a study on body mass. The inclusion of patients with putative IGF-I related pathology might explain for a large part the discrepancy with the findings in our population-based study. Our observation of high IGF-I concentrations in those homozygous for the 192-bp allele are supported by the fact that these subjects were also significantly taller by nearly 3 cm compared with subjects not possessing the 192-bp allele.

This is the first study of the role of the IGF-I promoter polymorphism in the pathogenesis of diabetes and cardiovascular disease. The increased relative risk for type 2 diabetes and myocardial infarction in non-carriers of the 192-bp allele, suggests that a lifetime exposure to moderate alterations in IGF-I expression may also be biologically relevant in terms of disease risk. These observations are in accordance with earlier reports of low-normal circulating IGF-I levels in patients with cardiovascular disease and diabetes. Given the association with both myocardial infarction and diabetes, this IGF-I promoter
polymorphism may explain to some extent the clustering of these diseases. The polymorphism is particularly a strong predictor of myocardial infarction in subjects with type 2 diabetes mellitus. Provided that these findings can be replicated by others, this may open an opportunity to identify type 2 diabetes patients at high risk for myocardial infarction, who may benefit from specific therapy influencing the IGF-I metabolism. Further studies are needed to lead to a better understanding of the specific pathogenic pathways involved.

Our population-based study suggests that a genetic polymorphism in the promoter region of the IGF-I gene is associated with IGF-I expression. The absence of a 192-bp allele of this polymorphism, as observed in almost 12% of the general population, is associated with lower body height, low IGF-I levels and an increased risk for type 2 diabetes mellitus and myocardial infarction. The increased risk for these disorders is most likely a result of a genetically determined, lifelong exposure to relatively low IGF-I concentrations in those without the 192-bp allele.

Acknowledgements
This work was supported by the Netherlands Organization for Scientific Research (NWO) and the Municipality of Rotterdam. N Vaessen is supported by a grant of the Dutch Diabetes Foundation. We would like to thank I Meulenbelt and P E Slagboom for the collaboration in IGF-I research, R P Stolk and D E Grobbbee for help in design and data collection of the diabetes study and J Vergeer and B de Graaf for their help in genotyping.
References


POPULATION-BASED EVIDENCE FOR GENETICALLY PROGRAMMED FAILURE OF THE BETA-CELL IN TYPE 2 DIABETES MELLITUS

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Erasmus Medical Center Rotterdam, The Netherlands. * Genetic Epidemiology Unit, Department of Epidemiology & Biostatistics, Department of Clinical Genetics, ‡ Department of Internal Medicine. §University Medical Center Utrecht, The Netherlands. Julius Center for Patient Oriented Research.
Summary
It has been put forward that insulin resistance and genetically programmed pancreatic beta-cell dysfunction interact in susceptible persons to cause type 2 diabetes mellitus. Recent studies indicate that insulin-like growth factor-I (IGF-I) is an important regulator of pancreatic beta-cell growth and maturation. In this study we examined whether a genetically determined low expression of IGF-I has an effect on pancreatic beta-cell function and, if so, whether this genetic factor influences the risk for diabetes in persons with insulin resistance. We studied a polymorphism in the regulatory region of the IGF-I gene which is known to be associated with IGF-I expression and body height. The relation between the IGF-I polymorphism, beta-cell function and diabetes mellitus was assessed in a population-based sample of 220 persons with type 2 diabetes, 253 persons with impaired glucose tolerance and 596 persons with normal glucose tolerance. Persons with the lowest expression of IGF-I showed a significant reduction in beta-cell function (P for trend = 0.02). In persons with insulin resistance, genetically determined low IGF-I expression was associated with a 7.5 fold increased risk of diabetes (95 percent confidence interval of odds ratio, 3.4 to 16.2). In those with obesity, genetically determined low IGF-I expression was associated with a 6.2 fold increased risk of diabetes (95 percent confidence interval of odds ratio, 2.8 to 13.5). Our findings show that the increased risk for diabetes mellitus in persons with genetically determined low IGF-I expression is most likely the result of an inadequate compensatory beta-cell response to the increasing insulin needs of persons with insulin resistance and obesity. The IGF-I promoter region may in part determine the genetically programmed pancreatic beta-cell dysfunction that leads to diabetes in persons with insulin resistance or obesity.
Introduction

Type 2 diabetes mellitus is the most common metabolic disorder in the Western world, affecting more than 120 million people worldwide.\textsuperscript{1} It has been hypothesized that insulin resistance and genetically programmed pancreatic beta-cell dysfunction may interact in susceptible persons to cause diabetes.\textsuperscript{2} Insulin resistance, i.e., a decreased response of the liver and peripheral tissues to insulin, is present in approximately 20-25 percent of the Western population and often occurs in combination with obesity.\textsuperscript{3} Despite the fact that insulin resistance is one of the earliest features of type 2 diabetes mellitus, only 20 percent of persons with insulin resistance will ultimately develop diabetes mellitus.\textsuperscript{4} This is largely determined by the ability of the beta-cells to compensate insulin secretion in order to maintain normal glucose levels. The functional pancreatic beta-cell mass is the major factor determining the amount of insulin that can be secreted. Although the functional pancreatic beta-cell mass is partly determined at birth, beta-cell mass may also increase or decrease during life to meet changes in insulin demands.\textsuperscript{4}

Insulin-like growth factor-I (IGF-I) is a hormone that regulates proliferation and differentiation of numerous cell types. IGF-I has been shown to be an important regulator of the pancreatic beta-cell mass.\textsuperscript{5-8} Recent studies indicate that IGF-I is involved in pancreatic islet development during embryogenesis and also promotes compensatory beta-cell proliferation and survival in situations of increasing insulin demands.\textsuperscript{5,7-10} Patients with diabetes mellitus are characterized by low serum levels of IGF-I.\textsuperscript{11,12} We recently demonstrated that the absence of the wildtype allele of a polymorphism in the promoter region of the IGF-I gene is associated with low circulating IGF-I concentrations, low body height and an increased risk for type 2 diabetes mellitus.\textsuperscript{13}

In this study we tested the hypothesis that a genetically determined reduction in IGF-I expression results in a reduced pancreatic beta-cell function. This genetically determined beta-cell dysfunction may be an important factor in the development of diabetes mellitus, in particular in persons with increasing insulin demands, such as persons with insulin resistance or obesity. We therefore specifically investigated the effect of diminished IGF-I expression in the
pathogenesis of type 2 diabetes mellitus in persons with insulin resistance and obesity.

**Research design and methods**

*Participants*

This study is part of the Rotterdam Study, a single-center prospective follow-up study in which all residents aged 55 years and over of the Rotterdam suburb Ommoord were invited to take part. The study was approved by the Medical Ethics Committee of Erasmus Medical Center Rotterdam and written informed consent was obtained from all participants. The aim of the study was to investigate determinants of chronic and disabling cardiovascular, neurodegenerative, locomotor and ophthalmologic diseases. The design of the study has been described previously. The baseline examination of the Rotterdam Study was conducted between 1990 and 1993. A total of 7,983 participants (response rate 78 percent) were examined. In the present study, we only included persons aged between 55 and 75 years at the baseline examination.

Of the 7,983 participants of the Rotterdam Study, 1110 persons underwent a fasting 75 gram oral glucose tolerance test. For this study, we selected all persons with normal glucose tolerance (n = 596), impaired glucose tolerance (n = 253) and diabetes mellitus (n = 220) from whom DNA was available. Normal glucose tolerance was defined as fasting glucose below 6.1 mmol/l and 2 hours post-load glucose below 7.8 mmol/l. Impaired glucose tolerance was defined as fasting glucose between 6.1 and 7.0 mmol/l and/or a 2 hours post-load glucose between 7.8 and 11.1 mmol/l. A diagnosis of diabetes mellitus was made if patients were treated for diabetes or had a fasting glucose level of 7.0 mmol/l or above and/or a 2 hours post-load glucose of 11.1 mmol/l or above.

*Measurements*

Information concerning health status and drug use was obtained with a computerized questionnaire. Height and weight were measured and body mass index (BMI in kg/m²) was calculated. Obesity was defined as BMI higher than
27 kg/m². Body fat distribution was assessed by the ratio of waist and hip circumferences (WHR). Blood sampling and storage have been described elsewhere. Serum was separated by centrifugation and quickly frozen in liquid nitrogen. Glucose and insulin levels were measured in fasting serum and post-load serum samples. Persons that were treated for diabetes did not underwent a glucose tolerance test. Therefore, post-load glucose and insulin measurements were only available for persons not using anti-diabetic medication. Glucose was measured by the glucose hexokinase method. Insulin was measured by a commercially available assay (IRMA, Medgenix Diagnostics, intra-assay and inter-assay variation of 3-6 percent and 5-12 percent).

Beta-cell function was assessed as the insulogenic index 30⁻¹⁸

\[
\frac{(\text{ins}30' - \text{ins}0')}{(\text{glu}30' - \text{glu}0')}
\]

In patients with overt diabetes mellitus, it is not possible to distinguish defects in insulin secretion that are pathogenetically involved in the development of diabetes mellitus from defects that are secondary to hyperglycemia. Therefore, analysis of beta-cell function was restricted to persons whose plasma glucose concentrations were within the non-diabetic range.

As indicator of insulin sensitivity we used the Matsuda index:¹⁹

\[
\frac{10,000}{\sqrt{\text{FPG} \times \text{FPI}} \times (\text{mean OGTT glucose} \times \text{mean OGTT insulin})}
\]

**Genotyping of the IGF-I promoter polymorphism**

Polymerase chain reaction (PCR) was performed using oligonucleotide primers designed to amplify the polymorphic cytosine-adenine repeat 1 Kb upstream of the human IGF-I gene. The reaction was carried out in a final volume of 10 µl containing 50 ng of genomic DNA obtained from peripheral white blood cells, 0.5 mM forward primer (5' -ACCACCTGGAACAGGCGTA-3'), 0.5 mM reverse primer (5' -GCTAGCCAGCTTGTGTTATT-3'), 0.25 mM dNTP, 2.2 mM MgCl₂, 0.01 percent W1 (Gibco BRL) and 0.4 U Taq DNA polymerase (Gibco BRL). PCR was performed in 384 well plates (94°C 10 min; 35 PCR cycles of 30 seconds at 94°C, 30
seconds at 55°C and 30 seconds at 72°C; 72°C 10 min; 4°C hold). Forward primers were labeled with FAM, HEX or NED to determine the size of PCR products by auto sequencer (ABI 377, 6.25 percent longranger gel, filter set D, peak-height between 100 and 2000, each lane containing 3 samples). The size of the PCR products was determined in comparison with internal ROX 500-size standard (Perkin Elmer).

Statistical analysis

Hardy-Weinberg equilibrium of the IGF-I promoter polymorphism genotypes was tested with the GENEPOP-package (Raymond M. & Rousset F, 1995. GENEPOP version 1.2). Mean age, BMI, WHR, fasting glucose and insulin, insulin sensitivity and beta-cell function were compared between groups using analyses of variance. A multiple logistic regression model was used to study the association of the IGF-I promoter polymorphism genotypes with diabetes mellitus. For all continuous measurements, mean values ± SD are presented, adjusted for age and sex. Relative risks were estimated as odds ratios and presented with a 95 percent confidence interval. For all analysis, P-values are given for the difference between genotypes and a P-value for trend is given to indicate a possible linear relation between the number of 192-bp alleles and the outcome variable. To study the role of genetically determined low IGF-I expression in persons with insulin resistance, a stratified analysis was performed. In this analysis the risk for diabetes mellitus was assessed in persons with low insulin sensitivity (Matsuda index below the 50th percentile) versus high insulin sensitivity (Matsuda index higher or equal to the 50th percentile). From this analysis we excluded persons using anti-diabetic medication, because insulin sensitivity measurements were not available for these subjects. Similar, a stratified analysis was conducted, based on presence or absence of obesity. In this analysis, all persons with diabetes were included. All analyses were performed using the SPSS for Windows software package, version 7.5.2.
CHAPTER 3.2

Results

The characteristics of the study population are shown in table 1. In our study diabetes patients were more frequently male than persons with normal glucose tolerance \((p < 0.05)\). Mean age, BMI, WHR, fasting plasma glucose and insulin concentrations were significantly higher in persons with impaired glucose tolerance and/or diabetes mellitus \((p < 0.005)\). In the total study population, 10 different alleles were identified in the promoter polymorphism of the IGF-I gene. Genotype and allele distributions were in Hardy-Weinberg equilibrium \((p=0.76)\). Based on the presence of the 192-bp allele, 3 possible genotypes were identified: persons homozygous for the 192-bp allele \((44.5\%\) percent), persons heterozygous for the 192-bp allele \((43.3\%\) percent) and non-carriers of the 192-bp allele \((12.2\%\) percent).

Table 1: Characteristics of the Study Population

<table>
<thead>
<tr>
<th>Variable</th>
<th>Normal Glucose Tolerance</th>
<th>Impaired Glucose Tolerance</th>
<th>Diabetes Mellitus</th>
</tr>
</thead>
<tbody>
<tr>
<td>n = 596</td>
<td>n = 253</td>
<td>(n = 220)</td>
<td></td>
</tr>
<tr>
<td>Men (%)</td>
<td>46.0</td>
<td>51.0 (\dagger)</td>
<td>56.4 (\dagger)</td>
</tr>
<tr>
<td>Age - yr</td>
<td>65.9 ± 5.4</td>
<td>66.6 ± 5.5 (\dagger)</td>
<td>67.9 ± 5.5 (\dagger)</td>
</tr>
<tr>
<td>Body Mass Index - kg/m(^2)</td>
<td>25.9 ± 3.2</td>
<td>27.3 ± 3.8 (\dagger)</td>
<td>27.6 ± 3.5 (\dagger)</td>
</tr>
<tr>
<td>Waist to Hip Ratio</td>
<td>0.89 ± 0.089</td>
<td>0.93 ± 0.084 (\dagger)</td>
<td>0.94 ± 0.089 (\dagger)</td>
</tr>
<tr>
<td>Fasting glucose - mmol/l</td>
<td>5.5 ± 0.4</td>
<td>6.2 ± 0.5 (\dagger)</td>
<td>8.5 ± 2.5 (\dagger)</td>
</tr>
<tr>
<td>Fasting insulin - mU/l</td>
<td>11.6 ± 5.6</td>
<td>15.5 ± 8.8 (\dagger)</td>
<td>19.2 ± 20.5 (\dagger)</td>
</tr>
</tbody>
</table>

Data are expressed as percentage or mean ± SD. Mean Body Mass Index, Waist to Hip Ratio, fasting glucose concentration and fasting insulin concentration are corrected for differences in age and gender between the groups. Overall difference between groups \(\dagger p < 0.05\) versus persons with normal glucose tolerance. \(\dagger p < 0.005\) versus persons with normal glucose tolerance.

No significant difference in mean BMI, WHR, fasting glucose levels or insulin sensitivity was observed between the 3 IGF-I promoter genotypes. However, as shown in figure 1, fasting insulin levels and beta-cell function differed strongly between genotypes. Fasting insulin levels increased with the number of 192-bp
alleles present (P for trend = 0.04). Insulin levels were 14 percent higher in persons homozygous for the 192-bp allele (13.0 ± 7.0 mU/L, p = 0.05) compared with persons without the 192-bp allele (11.4 ± 6.4 mU/L). Also the beta-cell function strongly increased with the number of 192-bp alleles present (p for trend = 0.02). The beta-cell function was 38 percent higher in non-diabetic persons homozygous for the 192-bp allele (32.5 ± 36.2, p = 0.05) compared with non-diabetic persons without the 192-bp allele (23.5 ± 15.6).

**Figure 1:** Fasting Insulin Concentrations and Beta cell Function, Stratified by IGF1 Genotype.

Beta-cell function was assessed as the insulinogenic index 30'. *White bars*, persons homozygous for the 192-bp allele; *hatched bars*, persons heterozygous for the 192-bp allele; *grey bars*, non-carriers of the 192-bp allele. Group results are given as means ± SEM. *Insulin levels significantly increased with the number of 192-bp alleles present (P for trend = 0.04). **Beta-cell function significantly increased with the number of 192-bp alleles present (P for trend = 0.02).*

As published earlier, the odds ratio for diabetes mellitus was 1.4 for persons heterozygous for the 192-bp allele (95 percent confidence interval, 1.0 to 1.9; P = 0.03) and 1.7 for persons without the 192-bp allele (95 percent confidence interval,
1.1 to 2.7; P = 0.02) compared with persons homozygous for the 192-bp allele (P for trend = 0.007). In figure 2, the risk for diabetes mellitus is shown, stratified by the degree of insulin sensitivity. In persons with high insulin sensitivity the

**Figure 2: Risk for Type 2 Diabetes Mellitus, Stratified by IGF-1 Genotype and Insulin Sensitivity.**

Relative Risks are expressed as odds ratios. Genotypes are based on the presence or absence of the 192-bp allele. Hatched bars, insulin sensitive persons (Matsuda index equal or above 50th percentile); Grey bars, insulin resistant persons (Matsuda index below 50th percentile).

* Significantly different from insulin sensitive persons, homozygous for the 192-bp allele (P<0.005). ** Significantly different from insulin sensitive persons, homozygous for the 192-bp allele (P<0.0005).

odds ratios for heterozygous carriers and non-carriers of the 192-bp allele were not significantly increased. Low insulin sensitivity was associated with 3.4 fold increased risk (95 percent confidence interval of odds ratio, 1.8 to 6.4; P = 0.0001) for diabetes mellitus. In persons with low insulin sensitivity, the risk for diabetes mellitus showed a strong relation with the number of 192-bp alleles present (P for trend = 0.005). The odds ratio in those heterozygous for the 192-bp allele was 5.2 (95 percent confidence interval, 2.8 to 9.7; P < 0.0001), while the combination of
Low insulin sensitivity and the absence of the 192-bp allele was associated with a 7.5 times increased risk for diabetes mellitus (95 percent confidence interval of odds ratio, 3.4 to 16.2; P < 0.0001).

**Figure 3:** Risk for Type 2 Diabetes Mellitus, Stratified by IGF-I Genotype and Obesity.

Relative Risks are expressed as odds ratios. Genotypes are based on the presence or absence of the 192-bp allele. Hatched bars, non-obese persons (BMI equal or below 27 Kg/m²); Grey bars, obese persons (BMI above 27 Kg/m²). * Significantly different from non-obese persons, homozygous for the 192-bp allele (P<0.0005).

In figure 3, the risk is shown for persons stratified by the presence or absence of obesity. Obesity itself was associated with a 2.6 times increased risk of diabetes mellitus (95 percent confidence interval of odds ratio, 1.5 to 4.7; P = 0.0003). Also in persons with obesity the risk for diabetes mellitus was related with the number of 192-bp alleles (P for trend = 0.01). The odds ratio for diabetes mellitus was 2.9 in persons heterozygous for the 192-bp allele (95 percent confidence interval, 1.6 to 5.2; P = 0.0003), while the odds ratio in persons without the 192-bp allele was 6.2 (95 percent confidence interval, 2.8 to 13.5; P < 0.0001).
Discussion

In this population-based study we found that a genetically determined low expression of IGF-I was significantly associated with a strong decrease in beta-cell function. Particular in persons with insulin resistance and/or obesity, genetically determined low IGF-I expression was associated with a highly increased risk for type 2 diabetes mellitus. To our knowledge, this is the first study using a genetic approach to investigate the role of low IGF-I expression in pancreatic beta-cell function. We previously demonstrated that the absence of the wildtype (192-bp) allele of a cytosine-adenosine (CA) repeat 1 kilobase upstream from the transcription start site of the IGF-I gene is associated with low circulating IGF-I concentrations and reduced body height.\textsuperscript{13} By using this polymorphism as a marker of lifetime IGF-I expression, difficulties in measuring local IGF-I expression in vivo in relevant tissues, such as the pancreatic beta-cell, can be circumvented. Further, because the genetic make-up will not change during life, this approach overcomes the problem of cross-sectional studies in which one cannot distinguish whether changes in IGF-I concentrations are a cause or rather a consequence of disease. In the current study, the 192-bp allele was present in 88 percent of persons. This finding is in agreement with our previous findings in the Rotterdam Study and with other reports in Caucasian populations.\textsuperscript{13,21,22} We estimated the beta-cell function and insulin sensitivity from fasting and OGTT measurements using established validated techniques.\textsuperscript{18,19} Although estimates derived from these techniques correlate highly with more invasive and time-consuming gold-standard methods, i.e., the hyperglycemic and euglycemic clamp, some measurement error may have occurred. Since all clinical measurements were performed without knowledge of the genotype, such misclassification is most likely not related to the genotype and will therefore result in an underestimation of the true effect.

Experimental data have demonstrated that IGF-I is an important regulator of the beta-cell mass, possibly by regulating differentiation, proliferation and survival of pancreatic beta-cells.\textsuperscript{7,9} We observed a strong decrease in beta-cell function and low insulin levels in persons with genetically determined low IGF-I
expression. This suggests that diminished IGF-I expression may result in a physiologically relevant shortage of functional beta-cells in humans. An adequate functional beta-cell mass is an important factor in the maintenance of normal glucose homeostasis, particularly in subjects with insulin resistance or obesity. In these individuals glucose uptake by peripheral tissues is diminished, resulting in mild hyperglycemia. This hyperglycemia is an important trigger for IGF-I mediated beta-cell replication and growth. The strongly increased risk for diabetes that was observed in obese and insulin resistant persons with genetically determined low IGF-I expression in our study, suggests that diminished IGF-I expression may result in inadequate expansion of the beta-cell mass. This in turn will predispose to the development of diabetes mellitus in situations of increasing insulin needs. Earlier, Polonsky et al. put forward that a genetically determined beta-cell dysfunction may be an important factor in the disturbance of normal glucose homeostasis in persons with insulin resistance or obesity. Our data suggest that genetically determined low expression of IGF-I may in part explain this genetically programmed failure of the beta-cell.

In conclusion, our population-based study supports the hypothesis that insulin resistance and genetically programmed pancreatic beta-cell dysfunction may interact in susceptible persons to cause diabetes. Our data suggest that genetically determined low expression of IGF-I results in a diminished functional pancreatic beta-cell mass, thereby increasing the susceptibility for diabetes mellitus, particularly when insulin demands are high. In view of that, genetically determined low IGF-I expression may be an important physiological difference between persons with insulin resistance or obesity who become diabetic and those who do not.

Acknowledgements

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References


CHAPTER 3.3

GENETIC EVIDENCE FOR A CARDIOPROTECTIVE EFFECT OF INSULIN-LIKE GROWTH FACTOR-I

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* Genetic Epidemiology Unit, Department of Epidemiology & Biostatistics, Department of Clinical Genetics. †Department of Internal Medicine. Erasmus Medical Center Rotterdam, The Netherlands.
Summary

Background. We recently demonstrated that genetically determined low expression of insulin-like growth factor-I (IGF-I) is associated with an increased risk of myocardial infarction (MI). In the present study, we examined the potential mechanisms underlying this increased risk for MI. First, we investigated whether low IGF-I expression is involved in the pathogenesis of atherosclerosis. Second, we investigated whether IGF-I has direct cardioprotective properties.

Methods and results. In a case-control study of 477 individuals with prevalent MI, 137 individuals with newly diagnosed MI and 780 controls we found that genetically determined low IGF-I expression was associated with a 1.7 fold increased risk of prevalent MI and a 1.9 fold increased risk for incident MI. Using a population-based sample of 900 subjects from the Rotterdam Study we found no evidence for an increased frequency of atherosclerosis in subjects with low IGF-I expression. However, in those subjects that had already developed generalized atherosclerosis or suffered from angina pectoris, IGF-I expression proved to be a strong modifier of the risk to develop MI. The combination of angina pectoris and low IGF-I expression was associated with a 9.7 times increased risk of prevalent MI and a 10.2 times increased risk for incident MI, while the combination of atherosclerosis and low IGF-I expression was associated with a 3.7 times increased risk for prevalent MI and a 5.2 times increased risk for incident MI.

Conclusion. These findings suggest that IGF-I has important cardioprotective effects by reducing myocardial injury in subjects exposed to cardiac ischemia.
Introduction

Evidence has accumulated that a relative shortage of the hormone insulin-like growth factor-I (IGF-I) may predispose to the development of myocardial infarction (MI). However, the mechanism by which IGF-I is involved in the pathogenesis of MI is not known. Several experimental studies have indicated that IGF-I has important cardioprotective effects by reducing myocardial apoptosis and injury in response to ischemia. On the other hand IGF-I has also been suggested to play a role in the pathogenesis of atherosclerosis, the leading determinant of cardiac ischemia. Until now, studies on the role of IGF-I in the pathogenesis of cardiovascular disease in humans have been inconclusive. One explanation may be that most studies have focussed on circulating IGF-I levels, which may not truly reflect local production of IGF-I in the myocardium or the vessel wall. Furthermore, these studies have been hampered by the fact that IGF-I expression may change merely as a result of the ongoing disease process. We recently demonstrated that the absence of the wildtype (192-bp) allele of a genetic polymorphism in the promoter region of the IGF-I gene is associated with low circulating IGF-I levels as well as a reduced body height. This polymorphism can be used to characterize subjects with genetically determined low expression of IGF-I. Absence of the 192-bp allele of this polymorphism was found to be associated with an increased prevalence of MI.

The aim of the present study is to examine the potential mechanisms underlying the increased risk for MI in subjects genetically exposed to low IGF-I levels. First, we examined the role of genetically determined low IGF-I expression in the pathogenesis of atherosclerosis in order to assess whether IGF-I is involved in the development of coronary atherosclerotic lesions. Second, the effect of IGF-I expression was studied in the late stage of disease, i.e. in the development of MI in individuals that have already developed angina pectoris or generalized atherosclerosis.
Methods

Study Population

This study is part of the Rotterdam Study, a single-center prospective follow-up study in which all residents aged 55 years and over of the Rotterdam suburb Ommoord were invited to take part. The study was approved by the Medical Ethics Committee of Erasmus Medical Center Rotterdam and written informed consent was obtained from all participants. The design of the study has been described previously. The baseline examination of the Rotterdam Study was conducted between 1990 and 1993. A total of 7,983 participants (response rate 78 percent) were examined. Currently, follow-up on the occurrence of MI is complete for 7,976 subjects with a mean follow-up time of 5.2 years.

The present study is based on the following samples from the Rotterdam Study: (1) A population based sample of 900 subjects, randomly drawn from all participants aged between 55 and 75 years at the baseline examination. (2) A case-control sample, consisting of 477 individuals with prevalent MI, 137 individuals with incident MI and 780 controls without evidence for a prevalent or incident MI.

Measurements

At the baseline examination, information concerning health status, drug use and smoking behavior was obtained with a computerized questionnaire. Height and weight were measured and body mass index (BMI in kg/m²) was calculated. Hypertension was defined as a diastolic blood pressure of 95 mmHg or higher and/or a systolic blood pressure of 160 or higher and/or the use of antihypertensive drugs. Diabetes mellitus was defined as the use of blood glucose lowering medication. The definition of angina pectoris was based on a Dutch version of the Rose questionnaire.

The presence of atherosclerosis was evaluated at different sites. Atherosclerosis of the abdominal aorta was assessed using a lateral X-ray of the lumbar spine (T12-S1) on which the presence of calcified deposits was determined. The severity of atherosclerosis was graded from 0 (no calcified plaques) to 5 (aorta outlined with calcified plaques) according to the length of the
affected area.\textsuperscript{15,16} The intima-media thickness of the carotid artery and the presence of atherosclerotic plaques in the carotid artery were assessed with ultrasound.\textsuperscript{17,18} For each subjects mean intima-media thickness ((left + right)/2) was taken as measure for wall thickness of the distal common carotid artery.\textsuperscript{17,18} The common and internal carotid artery and the carotid bifurcation were both on-line and off-line (from tapes) evaluated for the presence of atherosclerotic lesions.\textsuperscript{17,18} A total carotid plaque score was defined by summation of the presence of plaques at the left and right side at three locations (score ranging from 0 to 6).\textsuperscript{17,18} The presence of atherosclerosis in the arteries of the lower extremities was evaluated by measuring the systolic blood pressure level of the posterior tibial artery at both the left and right side.\textsuperscript{19} The ratio of the systolic blood pressure at the ankle to the systolic blood pressure at the arm was calculated for each leg. The lowest ankle-arm index in either leg was used in the analysis.\textsuperscript{19}

Blood sampling and storage have been described elsewhere.\textsuperscript{13} Total serum cholesterol and HDL-cholesterol were determined with an automated enzymatic procedure.\textsuperscript{20} Genotypes of the IGF-I gene promoter polymorphism were determined as described earlier.\textsuperscript{12}

\textit{Definition of prevalent and incident myocardial infarction}

A MI before baseline was diagnosed on the basis of ECG-findings or self-report.\textsuperscript{21,22} At the baseline examination, a resting standard 12-lead electrocardiogram was made with an ACTA Gnosis IV (EsaoteBiomedica)\textsuperscript{21,23} in all participants of the Rotterdam Study. For the diagnosis of MI an automated diagnostic classification system, the Modular ECG Analysis System (MEANS)\textsuperscript{21,22}, was used. Infarctions detected without evidence of symptoms (silent myocardial infarctions) were verified by an experienced cardiologist.\textsuperscript{21,23} The occurrence of incident MI during follow-up was reported by general practitioners. All reported information was verified by research physicians. All MIs during follow-up were diagnosed using the definition of the 10\textsuperscript{th} revision of the International Classification of Diseases (ICD-10).\textsuperscript{24}
In this study, prevalent MI was defined as evidence for MI before the baseline examination. Incident MI was defined as the first occurrence of MI after the baseline examination. By definition individuals with prevalent MI were excluded from this group.

Data Analysis
The relationship between the IGF-I promoter polymorphism, atherosclerosis and angina pectoris was studied using logistic regression and analysis of variance. Mean intima-media thickness and ankle-brachial index were logarithmically transformed for the analyses. The non-transformed data and standard deviation (SD) are presented. A multiple logistic regression model was used to study the association of the IGF-I promoter polymorphism genotypes with MI. Relative risks were estimated as odds ratios and presented with a 95 percent confidence interval. To examine if the IGF-I genotype influenced the risk for MI in subjects who are frequently exposed to periods of cardiac ischemia, a stratified analysis was performed. First, the risk for prevalent and incident MI was calculated for subjects with or without angina pectoris at baseline. Similar, a stratified analysis was conducted, based on the presence or absence of atherosclerosis. For this analysis atherosclerosis was defined as the presence of moderate to severe atherosclerosis at either the abdominal aorta (aorta atherosclerosis grade ≥ 3), and/or the carotid artery (plaque score ≥ 3) and/or the lower extremities (ankle-arm index below the lowest quartile). In all analyses, subjects homozygous for the 192-bp allele and without angina pectoris or atherosclerosis were used as the reference category. All analyses were performed using the SPSS for Windows software package, version 7.5.2. Hardy-Weinberg equilibrium of the IGF-I promoter polymorphism genotypes was tested with the GENEPOP-package (Raymond M. & Rousset F, 1995. GENEPOP version 1.2).

Results
Table 1 presents the characteristics of the participants. Patients were older, were more frequently male, had lower HDL-cholesterol levels and were more frequently hypertensive than control subjects. Total cholesterol was significantly increased in
incident cases at baseline. In the total study population, 10 different alleles were identified for the IGF-I promoter polymorphism. Genotypes in the population sample and in the cases en controls were in Hardy-Weinberg equilibrium. Based on the presence of the wildtype (192-bp) allele, 3 possible genotypes were identified: subjects homozygous for the 192-bp allele (associated with normal IGF-I expression), subjects heterozygous for the 192-bp allele (associated with intermediate IGF-I expression) and non-carriers of the 192-bp allele (associated with low IGF-I expression).

Table 1: General characteristics of the study population

<table>
<thead>
<tr>
<th></th>
<th>Population sample</th>
<th>Myocardial infarction study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prevalent cases</td>
<td>Incident cases</td>
</tr>
<tr>
<td>Number of Subjects</td>
<td>900</td>
<td>477</td>
</tr>
<tr>
<td>Age - yr</td>
<td>65.9 ± 5.6</td>
<td>66.5 ± 5.2 *</td>
</tr>
<tr>
<td>Men - no (%)</td>
<td>359 (39.9%)</td>
<td>330 (692) †</td>
</tr>
<tr>
<td>Body Mass Index - kg/m2</td>
<td>26.6 ± 3.8</td>
<td>26.6 ± 3.8</td>
</tr>
<tr>
<td>Current Smoking - no. (%)</td>
<td>228 (25.3)</td>
<td>126 (26.5)</td>
</tr>
<tr>
<td>Total cholesterol - mmol/l</td>
<td>6.8 ± 1.2</td>
<td>6.6 ± 1.2</td>
</tr>
<tr>
<td>HDL cholesterol - mmol/l</td>
<td>1.4 ± 0.3</td>
<td>1.2 ± 0.3 †</td>
</tr>
<tr>
<td>Hypertension - no. (%)</td>
<td>347 (38.6)</td>
<td>282 (59.1) †</td>
</tr>
<tr>
<td>Diabetes - no. (%)</td>
<td>45 (5.0%)</td>
<td>21 (6.7%)</td>
</tr>
</tbody>
</table>

Values are means ± standard deviation.
* Significantly different from the control population; p < 0.05.
† Significantly different from the control population; p < 0.005.

The prevalence of atherosclerosis and angina pectoris in the population sample of 900 subjects is given in table 2. The number of calcifications in the abdominal aorta (p = 0.44), the number of plaques in the common carotid artery (p = 0.64), the mean intima-media thickness of the common carotid artery (p = 0.12) and the mean ankle-arm index (p = 0.16) were not significantly different between the genotype groups. In addition, no significant association was found between the 192-bp allele and angina pectoris (p = 0.68).
Table 2: Prevalence of atherosclerosis and angina pectoris in the population sample of 900 subjects

<table>
<thead>
<tr>
<th>Presence of calcifications in aorta - no. (%)</th>
<th>Homozygous carriers</th>
<th>Heterozygous carriers</th>
<th>Non carriers</th>
</tr>
</thead>
<tbody>
<tr>
<td>No calcifications</td>
<td>164 (42.5)</td>
<td>158 (47.0)</td>
<td>47 (48.2)</td>
</tr>
<tr>
<td>Calcification score 1-2</td>
<td>154 (39.9)</td>
<td>113 (33.6)</td>
<td>35 (36.1)</td>
</tr>
<tr>
<td>Calcification score 3 or higher</td>
<td>68 (17.6)</td>
<td>65 (19.3)</td>
<td>15 (15.5)</td>
</tr>
<tr>
<td>Presence of plaques in the a.carotis - no. (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No plaques</td>
<td>158 (44.5)</td>
<td>155 (48.6)</td>
<td>40 (45.5)</td>
</tr>
<tr>
<td>Plaque score 1-2</td>
<td>122 (34.4)</td>
<td>111 (34.8)</td>
<td>32 (36.4)</td>
</tr>
<tr>
<td>Plaque score 3 or higher</td>
<td>75 (21.1)</td>
<td>53 (16.6)</td>
<td>16 (18.2)</td>
</tr>
<tr>
<td>Mean intima-media thickness a.carotis (mm)</td>
<td>0.77 ± 0.15</td>
<td>0.77 ± 0.13</td>
<td>0.73 ± 0.13</td>
</tr>
<tr>
<td>Mean ankle-arm index</td>
<td>1.1 ± 0.2</td>
<td>1.1 ± 0.2</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>Angina pectoris - no. (%)</td>
<td>23 (5.5)</td>
<td>17 (4.6)</td>
<td>5 (4.9)</td>
</tr>
</tbody>
</table>

Values are means ± standard deviation or number of persons (%).

As demonstrated previously, subjects heterozygous for the 192-bp allele had a relative risk of 1.2 for prevalent MI (95% CI 0.9-1.5)\textsuperscript{13}, while non-carriers of the 192-bp allele had a relative risk of 1.7 (95% CI 1.1-2.5), compared to subjects homozygous for the 192-bp allele. The genotype distributions of incident MI cases and controls are shown in Table 3. The risk for incident MI increased significantly with the number of 192-bp alleles present (p for trend = 0.04). The relative risk for MI in those heterozygous for the 192-bp allele was 1.3 (95% CI 0.9 - 1.9), while the absence of the 192-bp allele was associated with a 1.9 times increased risk for MI (95% CI 1.0 - 3.4).

Table 3: Relative risk of incident myocardial infarction based on the presence of the 192-bp allele

<table>
<thead>
<tr>
<th></th>
<th>Homozygous carriers</th>
<th>Heterozygous carriers</th>
<th>Non carriers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myocardial infarction</td>
<td>57 (41.6%)</td>
<td>59 (43.1%)</td>
<td>21 (15.3%)</td>
</tr>
<tr>
<td>Controls</td>
<td>369 (47.3%)</td>
<td>330 (42.3%)</td>
<td>81 (10.4%)</td>
</tr>
<tr>
<td>Relative risk *</td>
<td>reference</td>
<td>1.3 (0.9-1.9)</td>
<td>1.9 (1.0-3.4)</td>
</tr>
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</table>

Number of subjects for each category are given (% of cases or controls). Risks are given with a 95% confidence interval. * p for trend = 0.04.
Figure 1: Risk for prevalent and incident myocardial infarction, stratified by IGF-1 genotype and the presence of angina pectoris at the baseline examination.

Findings are based on 477 individuals that had evidence for a prevalent myocardial infarction at the baseline examination (prevalent cases), 137 individuals that developed their first myocardial infarction after the baseline examination (incident cases) and 780 subjects without evidence for a prevalent or incident myocardial infarction (controls). Relative Risks are expressed as odds ratios. Genotypes are based on the presence or absence of the 192-bp allele. Hatched bars, no angina pectoris at the baseline examination; Grey bars, angina pectoris at the baseline examination. Individuals without angina pectoris and homozygous for the 192-bp allele were used as the reference group.

* p < 0.05, † p < 0.005, ‡ p < 0.0005 (Significantly different from the reference group)
In figure 1 the results of the case-control analyses are shown, stratified by the presence or absence of angina pectoris at the baseline examination. In subjects without evidence for angina pectoris at the baseline examination, the relative risk of MI for heterozygous carriers was not significantly increased. Non-carriers of the 192-bp allele had a significant increased risk for prevalent MI (OR 1.5; 95% CI 1.0 - 2.1) and a, non-significantly, increased risk for incident MI (OR 1.5; 95% CI 0.9-2.8). In subjects with evidence for angina pectoris at the baseline examination, both the risk for prevalent and incident MI showed a strong relation with the number of 192-bp alleles present (p for trend = 0.05 and 0.002 respectively). The risks for prevalent and incident MI in those heterozygous for the 192-bp allele were 3.8 (95% CI 2.0 - 7.1) and 2.5 (95% CI 1.0 - 6.8) respectively. The combination of angina pectoris and the absence of the 192-bp allele was associated with a 9.7 times increased risk for prevalent MI (95% CI 21 - 44.7) and a 10.2 times increased risk for incident MI (95% CI 1.7 - 62.4).

In figure 2 the risks are shown for subjects stratified by the presence or absence of atherosclerosis. The relative risks for heterozygous carriers and non-carriers of the 192-bp allele were not significantly increased in subjects without evidence for atherosclerosis at the baseline examination. In subjects with atherosclerosis at the baseline examination the risk for prevalent and incident MI was significantly related with the number of 192-bp alleles (p for trend = 0.01 and 0.006 respectively). The relative risks for prevalent and incident MI were 1.6 (95% CI 1.2 -2.3) and 2.7 (95% CI 1.5 - 4.9) respectively in subjects heterozygous for the 192-bp allele. In subjects without the 192-bp allele the risk estimated in prevalent cases was 3.7 (95% CI 2.2 - 6.3) and in incident cases 5.2 (95% CI 2.3 - 11.8).

Discussion

In our prospective population-based study, we confirmed the previously described association between a polymorphism in the regulatory region of the gene for insulin-like growth factor-I and MI in a series of newly diagnosed patients. There was no evidence that this increased risk for MI is the result of an increased frequency of generalized atherosclerosis in subjects with low IGF-I expression.
Figure 2: Risk for prevalent and incident myocardial infarction, stratified by IGF-I genotype and the presence of atherosclerosis at the baseline examination.

Findings are based on 477 individuals that had evidence for a prevalent myocardial infarction at the baseline examination (prevalent cases), 137 individuals that developed their first myocardial infarction after the baseline examination (incident cases) and 780 subjects without evidence for a prevalent or incident myocardial infarction (controls). Relative Risks are expressed as odds ratios. Genotypes are based on the presence or absence of the 192-bp allele. Hatched bars: No atherosclerosis at the baseline examination; Grey bars, moderate or severe atherosclerosis at the baseline examination. Individuals without atherosclerosis and homozygous for the 192-bp allele were used as the reference group. * p < 0.05, † p < 0.005, ‡ p < 0.0005 (Significantly different from the reference group)
However, genetically determined exposure to low IGF-I levels particularly is a strong risk factor for MI in subjects with prevalent angina pectoris or generalized atherosclerosis.

This is the first population based study addressing the mechanism through which genetically determined low IGF-I expression influences the risk for MI. We previously demonstrated that the mean serum IGF-I concentration is 18% lower in subjects not carrying the wildtype (192-bp) allele of a cytosine-adenosine (CA) repeat 1 kilobase upstream from the transcription site of the IGF-I gene.\textsuperscript{12} In addition, we demonstrated that the absence of this 192-bp allele is associated with reduced body height and an increased risk for prevalent MI and diabetes.\textsuperscript{12} By using this polymorphism as a marker of lifetime IGF-I expression, difficulties in measuring local IGF-I expression in vivo in relevant tissues such as the blood vessels and the myocardium can possibly be circumvented.

It has been suggested that IGF-I is involved in the pathogenesis of atherosclerosis.\textsuperscript{5,11} We found no significant association between the IGF-I polymorphism and measurements of generalized atherosclerosis, i.e. aortic calcifications, atherosclerotic plaques in the carotid artery, increased intima-media thickness of the common carotid artery, or a decreased ankle-arm index. These measurements have all been shown to be associated with generalized atherosclerosis and their presence has been found to be a predictor of coronary heart disease and cardiovascular mortality.\textsuperscript{15,17,19} Although we cannot exclude that IGF-I is implicated in the pathogenesis of atherosclerosis, our findings do not support the hypothesis that the increased risk for MI in subjects with low IGF-I expression is the result of an increased frequency of generalized atherosclerosis.

Recently, it has been demonstrated that mice over-expressing IGF-I display less myocyte death following coronary ligation.\textsuperscript{6} Furthermore, IGF-I administered to rats reduces myocardial apoptosis and injury in response to ischemia.\textsuperscript{8} These studies suggest that IGF-I has important cardioprotective effects by reducing myocardial injury in response to ischemia. Our observations are compatible with the view that also in humans IGF-I has direct cardioprotective properties. Both in our cross-sectional study as well as in our follow-up study, we observed that the
CHAPTER 3.3

risk for MI in subjects with genetically determined low expression of IGF-I was most pronounced in subjects with prevalent angina pectoris or generalized atherosclerosis. This suggests that the lack of a cardioprotective effect in those with low expression of IGF-I results in an increased risk for MI, particularly in subjects with atherosclerosis or angina pectoris, i.e., in subjects likely to exhibit periods of cardiac ischemia. This is in agreement with our previous observation that genetically determined low IGF-I expression increases the risk of MI in subjects with type 2 diabetes mellitus, a disease that is characterized by a high prevalence of atherosclerosis and ischemic heart disease. We postulate that the increased risk for MI in diabetic patients with low IGF-I expression may for a large part be explained by the interaction of low IGF-I availability and cardiac ischemia.

In conclusion, our population-based study confirms the observation that genetically determined low expression of IGF-I increases the risk for myocardial infarction. We found no evidence for a role of altered IGF-I expression in the pathogenesis of atherosclerosis. However, our findings suggest that IGF-I has direct cardioprotective effects, possibly by reducing myocardial injury in response to ischemia. The findings of our study argue for further clinical studies assessing the possibility of IGF-I treatment in the primary and secondary prevention of myocardial infarction.

Acknowledgements

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References


GENETIC VARIATION IN THE GENE FOR INSULIN-LIKE GROWTH FACTOR-I IS ASSOCIATED WITH LOW BIRTHWEIGHT AND POSTNATAL REALIGNMENT OF WEIGHT

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Summary
The finding that low birth weight is a strong predictor of diabetes and cardiovascular disease in later life has led to an ongoing debate on the role of nature and nurture in this relation. On the one hand, Barker has argued that poor nutrition in utero, leading to low birth weight, programs an infant to the development of diabetes and cardiovascular disease. On the other hand, Hatterley and others have put forward that fetal genetic factors may underlie this relationship. Insulin-like growth factor-I (IGF-I) and insulin are essential growth factors during fetal life. We recently showed that the absence of the wildtype allele of a common polymorphism in the promoter region of the IGF-I gene is associated with low IGF-I expression, reduced adult body height, reduced insulin secretion and an increased risk of type 2 diabetes mellitus and myocardial infarction. We hypothesized that, if this polymorphism influences fetal IGF-I and insulin secretion, it may also be involved in the regulation of intrauterine growth and may thus explain part of the relationship between low birth weight and late-onset disease. In 463 elderly individuals, absence of the wildtype allele was associated with a 213 gram lower birthweight (p = 0.04). In addition, the IGF-I genotype was strongly associated with a postnatal realignment of weight; non-carriers of the wildtype allele who started with a low birthweight showed a relative gain in weight during life (p = 0.001). We propose that these differences in birthweight reflect genetically determined differences in fetal IGF-I and insulin secretion. Our data support the hypothesis that the association between low birthweight, diabetes and cardiovascular disease may follow from pleiotropic effects of common variations in genes influencing both fetal growth and susceptibility to late onset disease.
Insulin-like growth factor-I (IGF-I) is an important determinant of fetal growth. IGF-I is also involved in the growth and development of the insulin producing beta-cells in the pancreas. In addition to IGF-I, insulin secreted by the fetal pancreas in response to maternal glucose is a key growth factor for the fetus. A relative shortage of IGF-I has been implicated in the pathogenesis of type 2 diabetes and cardiovascular disease. We recently showed that the absence of the wildtype (192-bp) allele of a common polymorphism in the promoter region of the IGF-I gene results in low circulating IGF-I concentrations, reduced body height in adulthood, reduced insulin secreting capacity and an increased risk of type 2 diabetes and myocardial infarction. If this common polymorphism influences fetal IGF-I and insulin secretion, it may also be involved in the regulation of birth weight and may thus explain part of the relationship between low birth weight and type 2 diabetes mellitus and cardiovascular disease later in life. We analyzed the relation between birth weight and the IGF-I promoter polymorphism genotype in 463 participants of the Rotterdam Study, a single-center prospective follow-up study in the elderly.

Figure 1 shows the effect of the IGF-I genotype on birth weight. Absence of the 192-bp allele, which is associated with diminished IGF-I expression, resulted in a 213 gram decrease in birthweight compared with individuals homozygous for this allele (95% CI for the difference: -413 gram to -12 gram, p = 0.04). The difference in birthweight between genotypes was even more pronounced in individuals with a history of diabetes in the mother. As expected, the overall birthweight was higher in individuals with maternal diabetes (3431 ± 806 gram) as compared to those born to a mother who did not develop diabetes later in life (3234 ± 696 gram, p = 0.03). Within the group of individuals with a history of diabetes in the mother, absence of the 192-bp allele was associated with a 599 gram lower birthweight compared to individuals that were homozygous for this allele (95% CI for the difference: -1184 gram to -15 gram, p = 0.03) (see figure 1). To investigate whether variation in birth weight resulting from potential altered fetal IGF-I and insulin secretion has an effect on adult weight, we studied the relation of this IGF-I polymorphism with current weight, body mass index.
Figure 1: Mean birth weight (SEM) of 463 elderly individuals (A) and a subgroup of individuals with a history of diabetes in the mother (B), stratified by the presence of the wildtype (192-bp) allele of a polymorphism in the promoter region of the IGF-I gene.

195 individuals were homozygous for the 192-bp allele (42.1%), 204 individuals were heterozygous for the 192-bp allele (44.1%) and 64 individuals did not carry the 192-bp allele (13.8%). Mean birthweight was significantly lower in individuals that did not carry the 192-bp allele both in the whole study population (p = 0.04) as in the subgroup of individuals with a history of diabetes in the mother (p = 0.03).

and obesity. No relation was observed between the genotype and weight or obesity at adulthood. However, the IGF-I genotype was strongly associated with a postnatal realignment of weight, defined as a shift from the lower end of the weight distribution at birth to the mean weight of the overall population at old age (see figure 2). Of all individuals homozygous for the 192-bp allele 19 individuals (10%) showed a shift towards the mean weight of the general population during life. In individuals without the 192-bp allele this was true for 17 individuals (27%; p = 0.001).

In our population the absence of the wildtype allele of a common polymorphism in the promoter region of the IGF-I gene results in low IGF-I expression and an increased risk for type 2 diabetes mellitus and myocardial
Figure 3: Frequency of postnatal realignment of weight stratified by the presence of the wildtype (192-bp) allele.

Postnatal realignment of weight is expressed as % of individuals that shifted from the lower end of the weight distribution at birth to the mean weight of the overall population at old age. The frequency in individuals without the 192-bp allele was significantly different from the other genotypes (p = 0.001).

Infarction at older age. We now demonstrate that the absence of the wildtype allele also reduces fetal growth. Although IGF-I itself is a strong regulator of fetal growth, IGF-I is also involved in the regulation of growth and development of the insulin producing pancreatic beta-cells. IGF-I and insulin are structurally related molecules, both of which are essential for optimal embryonic and fetal development. A fundamental role of IGF-I in the regulation of birth size has been conclusively shown using targeted gene deletion technology. Deletion of the IGF-I gene in mice results in homozygotes that have a birth weight about 60% that of normal. The central role of insulin in the regulation of fetal growth is clearly demonstrated by the intra-uterine growth retardation in homozygous monogenetic disorders that severely reduce insulin secretion or action.
Therefore the diminished fetal growth may be a result of a reduction in fetal IGF-I secretion as well as a reduction in fetal insulin secretion. Both fetal IGF-I and insulin production are stimulated by high maternal glucose levels. This can explain the increased weight of infants born to mothers with poorly controlled diabetes. Our observation that the difference in birthweight between genotypes was more pronounced in individuals with a history of maternal diabetes suggests that maternal hyperglycemia is only able to adequately stimulate IGF-I and/or insulin secretion in individuals that carry the wildtype allele of the IGF-I polymorphism.

Absence of the wildtype allele was associated with a strong postnatal realignment of weight. The same pattern has also been observed in individuals with a mutation in the glucokinase gene, a disorder that is characterized by disturbances in glucose sensing and insulin secretion of the pancreatic beta-cells. These observations suggest that although genetically determined expression of IGF-I and insulin are important determinants of growth during the fetal period, they do not play a major role in the regulation of body weight in postnatal life. Most likely, outside the uterine environment, other genetic factors as well as environmental factors like diet and physical activity are likely to have a more important impact on weight regulation.

Many researchers have regarded intrauterine starvation as the driving force behind both low birthweight and later development of type 2 diabetes mellitus and cardiovascular disease. Our data support the alternative hypothesis that the association between low birthweight, diabetes and cardiovascular disease may be a result of common genetic variation influencing both fetal growth and susceptibility to late onset disease.

**Research design and methods**

**Participants**

This study is embedded in the Rotterdam Study, a single-center prospective follow-up study in which all residents aged 55 years and over of the Rotterdam suburb Ommoord were invited to take part. The study was approved by the Medical Ethics
Committee of Erasmus Medical Center Rotterdam. The design of the study has been described previously. Of the 7,983 participants of the Rotterdam Study, 1,110 persons participated in a study focusing on the pathogenesis of diabetes. Data on birth weight and DNA were available for 463 of these persons, including 93 persons with type 2 diabetes mellitus.

**Measurements**

Information concerning health status, birthweight and a family history of diabetes mellitus was obtained with a computerized questionnaire. Height and weight were measured and body mass index (BMI in kg/m²) was calculated. Obesity was defined as BMI higher than 27 kg/m². All individuals were categorized according to quartiles of the birthweight distribution and the adult weight distribution. A relative weight gain during life was defined as a shift within the weight distribution to a higher quartile.

**Genotyping of the IGF-1 promoter polymorphism**

Polymerase chain reaction (PCR) was performed using oligonucleotide primers designed to amplify the polymorphic cytosine-adenine repeat 1 Kb upstream of the human IGF-1 gene. The reaction was carried out in a final volume of 10 μl containing 50 ng of genomic DNA obtained from peripheral white blood cells, 0.5 nM forward primer (5'-ACCACCTCTGGGAAGGTTA-3'), 0.5 nM reverse primer (5'-CTAGCCAGCTGTGTTATT-3'), 0.25 mM dNTP, 2.2 mM MgCl₂, 0.01 percent W1 (Gibco BRL) and 0.4 U Taq DNA polymerase (Gibco BRL). PCR was performed in 384 well plates (94°C 10 min; 35 PCR cycles of 30 seconds at 94°C, 30 seconds on 55°C and 30 seconds on 72°C; 72°C 10 min; 4°C hold). Forward primers were labeled with FAM, HEX or NED to determine the size of PCR products by auto sequencer (ABI 377, 6.25 percent longranger gel, filter set D, peak-height between 100 and 2000, each lane containing 3 samples). The size of the PCR products was determined in comparison with internal ROX 500-size standard (Perkin Elmer).
Functional properties of the IGF-I promoter polymorphism.
Based on the presence of the wildtype (192-bp) allele, 3 possible genotypes were identified: individuals homozygous for the 192-bp allele (42.1%), individuals heterozygous for the 192-bp allele (44.1%) and non-carriers of the 192-bp allele (13.8%). Functional analysis of this polymorphism has been published earlier\(^7\). We have shown that body height and circulating IGF-I levels increase with the number of 192-bp alleles present. Mean body height was significantly lower in non-carriers of the 192-bp allele (165.4 cm) compared with subjects homozygous for the 192-bp allele (168.1 cm). In addition, mean serum total IGF-I concentration was 18% lower (16.7 mmol/l) in non-carriers of the 192-bp allele compared with those homozygous for the wildtype allele (20.5 mmol/l).

Statistical analysis
In the current study, mean birth weight, current weight and BMI were compared between the 3 genotypes using one-way analysis of variance (ANOVA). A possible association between the polymorphism and obesity or weight gain during life was investigated using a logistic regression model. All analyses were performed using the SPSS for Windows software package, version 7.5.2.
CHAPTER 3.4

References


CHAPTER 4

GENERAL DISCUSSION
Introduction

The etiology of the common forms of diabetes mellitus and its vascular complications are still unclear. Genetic studies may provide the means to disentangle the etiology of these diseases through identification of genes and hence proteins involved. The aim of the studies described in this thesis was to identify genes that play a role in the etiology of type 1 and type 2 diabetes mellitus and to elucidate the role of genetic variation in the gene for insulin-like growth factor-I in the pathogenesis of diabetic vascular complications.

In recent years, remarkable progress has been made in unraveling the etiology of several monogenetic diseases, in which there is a clear-cut relation between the genetic factor and the occurrence of disease. Diabetes mellitus and diabetic vascular complications, however, show a complex etiology. In these diseases the risk associated with a mutation most likely depends on the interaction with other genetic and environmental risk factors. Given the lack of success of family studies in the identification of genetic risk factors involved in complex diseases, we have focussed on the possibility of identifying relevant genes using population-based patient series. In this thesis, two different strategies are followed to identify new genes, the genome-wide screen and the candidate gene approach. To maximize the statistical power of the study, the genome-wide screen was conducted in the GRIP population, a genetically isolated population in the Southwest of the Netherlands. The candidate gene studies were performed as part of the Rotterdam study, a single-center prospective follow-up study in which all residents aged 55 years and over of the Rotterdam suburb Ommoord were invited to take part. We chose the gene for insulin-like growth factor-I (IGF-I) as a candidate gene, because low circulating levels of this hormone have been associated with an increased risk for both diabetes mellitus and cardiovascular disease.

Each chapter in this thesis includes a discussion on the merits and limitations of the study described in that chapter. In the first part of this chapter an integrated overview is given of the findings as well as the limitations. The second part of this chapter will provide a more general view on the opportunities for population-based studies of complex genetic disorders.
CHAPTER 4.1

MAIN FINDINGS AND CLINICAL RELEVANCE
Genomic screens

Chapter 2 describes the results of two genome-wide screens in the GRIP population, a recently genetically isolated population in the Netherlands. This population has been founded by approximately 150 people in the middle of the 18th century and has now expanded up to 20,000 inhabitants. We hypothesized that, given the genetic heterogeneity of diabetes, studies in this genetically homogeneous population may be more successful in mapping susceptibility loci than studies in complex outbred populations.

In chapter 2.1 we focussed on the etiology of type 1 diabetes mellitus. We identified 43 patients with type 1 diabetes mellitus that could be traced back to a common ancestor within 15 generations. The HLA DR3/DR4 genotype, a well-known genetic risk factor for type 1 diabetes mellitus, is found in 35% of the patients in the general population. In the patients from this isolated population both carriers of the DR3 and/or DR4 allele or the DR3/DR4 genotype were less frequent, suggesting that genetic drift has reduced the contribution of HLA to the benefit of other genetic factors implicated in type 1 diabetes mellitus. To identify these genes, we performed a genome-wide scan by using a combined linkage and association based approach. In the initial analysis, a p value < 0.016 (corresponding to a LOD-score > 1.0) was used as a threshold to select a genomic region for further analysis. Of the 391 markers tested, one marker in the HLA region and two markers on chromosome 8q and 17q exceeded this threshold. In an additional case-control analysis we demonstrated that flanking markers in the 8q and 17q region were also associated with an increased risk for type 1 diabetes. Further, the risk for diabetes increased with the number of ancestral alleles present for each of these markers. This strongly supports the presence of a type 1 diabetes susceptibility gene in these regions. The findings on chromosome 8q, which are further supported by the presence of a conserved ancestral haplotype in this region, confirm previous findings of a type 1 diabetes mellitus susceptibility locus on chromosome 8q. Our study further suggests the presence of a new susceptibility locus on chromosome 17q. Evidence for this locus is less strong compared to that of the 8q locus because no conserved ancestral
haplotype could be found. Therefore, this finding still needs to be confirmed in other studies to exclude that this represents a false positive finding after a search over many markers. Apart from the HLA-region and the locus on chromosome 8q, no significant association was found with other susceptibility regions that have been identified in previous genomic screens 4-12.

In chapter 2.2 we focussed on the etiology of type 2 diabetes mellitus. We identified 79 families with at least one patient with type 2 diabetes mellitus that could be traced back to a common ancestor within 13 generations. We performed a genome-wide scan using a more dense map consisting of 770 markers with an average spacing of 4.6 cM. In the initial analysis, nine markers yielded a lod score over 1.0 and the strongest evidence for a susceptibility locus was observed for marker 8b. In contrast to the type 1 diabetes screen, we were able to confirm the findings of our genome scan on type 2 diabetes, by using information on fasting glucose levels in an independent set of first-degree relatives. Of the nine markers identified in the initial screen, marker 4b and marker 8b were strongly associated with fasting glucose levels. Although we cannot conclude that the results of all other markers were false positive findings, further research on these loci is needed. The findings for marker 4b and 8b were further confirmed by the observation that affected individuals shared additional markers in these regions and by the observation that flanking markers and haplotypes in both regions were significantly associated with fasting glucose concentration in first-degree relatives. There have been three previous reports of linkage to a locus on chromosome ##q and recently a locus locus on **p was reported. Apart from confirming our finding, this also suggests that findings in the GRIP population can be extrapolated to other populations. Whereas several other loci have been implicated in type 2 diabetes, including a region on chromosome 2 that holds the calpain-10 gene 13-27, we could not detect significant association with any of these regions in the GRIP population. Our failure to replicate the role of calpain-10 in type 2 diabetes may be ascribed to the fact that a genetic isolate is not suitable for replication studies.

Even though the findings in our genome scans are very promising, the
progression of the association with the genomic regions to the eventual identification of causative mutations within a gene requires additional research. If obvious candidate genes are located within the identified regions, variants within these regions can be tested directly for association with diabetes or glucose levels. These studies are ongoing in the GRIP type 1 and type 2 diabetes studies. Usually several hundreds of genes are located within the identified regions and finding the right candidate can be a hard task if the protein encoded by these genes is not known or is not obviously involved in the disease. To facilitate the identification of candidate genes, the genomic regions that have been identified should be narrowed down sufficiently. Haplotype analysis in additional patients from the GRIP area or segregation analysis in multigenerational families will help us further to narrow down the genomic regions that have been identified in our genome scans. Once we have identified association with a specific variant, a causal relation with diabetes will have to be established. The ultimate proof of causality will have to come from in vitro and in vivo models of the disease phenotype, using the mutant genes uncovered by positional cloning.

**Candidate gene studies**

In chapter 3, a candidate gene approach is used to study the pathogenesis of type 2 diabetes mellitus and cardiovascular complications in the Rotterdam Study; a large population based cohort study. We focused on the gene for insulin-like growth factor-1 (IGF-I) because evidence is accumulating that this hormone plays a role in the regulation of glucose homeostasis and in the development of diabetic vascular complications \(^ {28,29}\). In *chapter 3.1* we started out with a study on the functional properties of a genetic polymorphism in the promoter region of the IGF-I gene and examined its relationship with type 2 diabetes mellitus and myocardial infarction. We identified a 192-bp allele in 88% of the population, suggesting that this is the wildtype allele from which all other alleles originated. Individuals that did not carry the 192-bp allele had a significantly lower body height, lower IGF-1 levels and an increased risk for type 2 diabetes mellitus and myocardial infarction. Particular in subjects with diabetes, absence of the 192-bp
allele was associated with a highly increased risk for myocardial infarction. These findings suggest that the increased risk for these disorders is a result of a genetically
determined, lifelong exposure to relatively low IGF-I concentrations.

In chapter 3.2, we examined the mechanism underlying the increased risk for
diabetes in subjects with genetically determined low IGF-I expression. We found
that a genetically determined low expression of IGF-I was significantly associated
with a significant decrease in beta-cell function. Particular in persons with insulin
resistance and/or obesity, genetically determined low IGF-I expression was
associated with a highly increased risk for type 2 diabetes mellitus. Experimental
data have demonstrated that IGF-I is an important regulator of the beta cell mass,
possibly by regulating differentiation, proliferation and survival of pancreatic
beta cells. Our data suggest that genetically determined low expression of IGF-I
results in a physiologically relevant shortage of functional beta cells, thereby
increasing the susceptibility for diabetes mellitus, particularly when insulin
demands are high. We therefore propose that genetically determined low IGF-I
expression may be an important physiological difference between individuals with
insulin resistance or obesity who become diabetic and those who do not.

In chapter 3.3, we focused on the relation between the IGF-I polymorphism
and myocardial infarction. Using an independent sample of incident cases from the
Rotterdam Study, we were able to confirm the increased risk for myocardial
infarction in subjects with genetically determined low IGF-I expression. This
observation makes it unlikely that the initial association with prevalent myocardial
infarction was a result of selective survival in an elderly population. There was no
evidence that the increased risk for myocardial infarction was the result of an
increased frequency of generalized atherosclerosis. However, genetically
determined low expression of IGF-I was particularly a strong risk factor for incident
myocardial infarction in subjects with prevalent angina pectoris or generalized
atherosclerosis. This work supports the findings of several experimental studies that
have indicated that IGF-I has important cardioprotective effects by reducing
myocardial apoptosis and injury in response to ischemia. Our observations
are compatible with the view that also in humans IGF-I has direct cardioprotective
properties. The lack of such a protective effect in those with genetically determined low expression of IGF-I increases the risk for myocardial infarction, particular in subjects with atherosclerosis or angina pectoris, i.e. in subjects likely to exhibit periods of cardiac ischemia. This is in agreement with our previous observation that genetically determined low IGF-I expression increases the risk of myocardial infarction in subjects with type 2 diabetes mellitus, a disease that is characterized by a high prevalence of atherosclerosis and ischemic heart disease\textsuperscript{36}. The increased risk for myocardial infarction in diabetic patients with low IGF-I expression may for a large part be explained by the interaction of low IGF-I availability and coronary atherosclerosis.

Because IGF-I is an important determinant of fetal growth\textsuperscript{37,38}, we also studied the relation between the IGF-I polymorphism and birth weight (chapter 3.4). There is substantial evidence that low birth weight is a strong predictor of diabetes and cardiovascular disease in later life. Barker et al. hypothesized that intrauterine starvation is the driving force behind both low birth weight and later development of disease\textsuperscript{39,40}. On the other hand, Hattersley and others have put forward that fetal genetic factors may underlie this relationship \textsuperscript{41,42}. We hypothesized that, if the IGF-I polymorphism influences fetal IGF-I and insulin secretion, it may also be involved in the regulation of intrauterine growth and may thus explain part of the relationship between low birth weight and late-onset disease. We observed that absence of the 192 bp allele of the IGF-I polymorphism was associated with a lower birth weight and a postnatal realignment of weight. Preliminary findings in another Dutch cohort of children with intra-uterine growth retardation confirm this observation (personal communication N. Arends), however, preliminary findings of a study in the UK do not (personal communication A. Hattersley). This suggests that the polymorphism studied in this thesis is in linkage-disequilibrium with the causal variant in the Dutch population but not in the UK population. Nevertheless, our findings support the hypothesis that the associations between low birth weight, diabetes and cardiovascular disease may follow from pleiotropic effects of common variations in genes influencing both fetal growth and susceptibility to late onset disease. For the IGF-I gene, it is likely that the growth promoting effects of IGF-I are
responsible for the variation in birth weight, adult height and beta cell growth and differentiation, while the anti-apoptotic effects of IGF-I mainly influence survival of beta cells and myocardial cells during life (see figure).

**Figure 1: Schematic representation of the pleiotropic effects of IGF-I**

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<tr>
<th>Protection against myocardial infarction following ischemia</th>
<th>Protection against diabetes in case of increasing insulin demand</th>
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<td>Survival of beta cells</td>
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<td>Promotion of cell growth and differentiation</td>
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**Clinical implications of the findings in this thesis**

The findings of our genome scans do not have clinical implications yet. Nevertheless, they are an important first step in the identification of genes involved in the pathogenesis of diabetes. The identification of these genes might ultimately lead to new insights in the metabolic pathways leading to diabetes and might also provide novel therapeutic targets. Conversely, the observation that genetically determined exposure to relatively low IGF-I concentrations is a risk factor for the development of diabetes or myocardial infarction may have an impact on future clinical practice.

It has been argued that genetic testing will not have a major effect on the way in which common diseases are identified or prevented. The probability
that the disease will develop in a person with a certain genotype (the positive predictive value) is a function of the frequency of both the genotype, the relative risk of the disease, and the risk of disease in a given population. From the data in chapter 3, it can be estimated that the probability that type 2 diabetes mellitus or a myocardial infarction will develop in a person without the 192-bp allele of the IGF-I polymorphism is only 16%. We therefore agree that genetic screening for variation in the IGF-I gene in the general population will not be very helpful as a diagnostic or predictive tool. However, in specific subgroups predictive testing may be helpful. For example, in individuals with obesity the probability that type 2 diabetes mellitus will develop in a person without the 192-bp allele is as high as 75%, while for individuals without the 192-bp allele that suffer from angina pectoris the probability to develop a myocardial infarction is 78%.

These findings provide a rationale for clinical studies assessing the role of IGF-I treatment in the primary and secondary prevention of diabetes and myocardial infarction in individuals with low IGF-I expression. Several randomized, double blind, placebo controlled trials have already shown that recombinant IGF-I (rIGF-I) may have an important role in the treatment of diabetes 44-46. Although these studies have shown that rIGF-I can be administered without causing severe side effects, most studies have only focused on the short-term effect of rIGF-I treatment. Recent studies suggest that exposure to high levels of IGF-I may increase the risk of cancer 47,48. Our preliminary findings on the association between the IGF-I promoter polymorphism and cancer do not confirm this hypothesis (personal communication R.Osborne). Nevertheless, although the results of our candidate gene studies are very promising, further basic and clinical studies are required to gain insight into the specific role of IGF-I in cancer before rIGF-I therapy is proposed as a preventive strategy for individuals at high risk for diabetes or myocardial infarction.
CHAPTER 4.2

FUTURE OPPORTUNITIES FOR POPULATION-BASED STUDIES OF COMPLEX GENETIC DISORDER
In recent years, remarkable progress has been made in unraveling the etiology of several genetic disorders, including Huntington's disease and cystic fibrosis. Progress has been most notable for monogenetic disorders, in which there is a clear-cut relation between the genetic factor and the occurrence of disease. At present, genetic research focuses on chronic disorders with a complex etiology, such as diabetes mellitus, cardiovascular disease and cancer. In these diseases the risk associated with a mutation may depend for a large part on interaction with other genetic or environmental risk factors. Given the lack of success of family studies in the identification of genetic factors involved in these complex diseases, there is growing interest in the possibility of identifying relevant genes using population-based patient series. These expectations are fuelled largely by the developments of the human genome project 49. These developments will bring genetic research closer to epidemiological practice. Here we discuss the opportunities for epidemiological research in the identification of genetic risk factors involved in complex genetic disorders.

Finding new genes involved in the pathogenesis of a disease is typically a fishing expedition. In epidemiology, case-control studies provide an efficient tool for such an expedition. Two different strategies can be followed to identify new genes, the candidate gene approach and the genome-wide screen. The approach most intuitive for epidemiologists is to target candidate genes, genes that are expected to be involved in the disease. A role of these genes in the pathogenesis may be suspected based on the function of the gene product, the protein, or homology of the gene or protein to a gene or protein that is known to be involved in the disease. To determine whether a candidate gene is involved in the pathogenesis of a disease, DNA variations in the gene can be studied. The rationale of this approach is that if a variation is associated with an increased risk of disease, this particular variation should at least be present more often in patients than in unaffected relatives or unrelated controls. In case of a protective effect, a reduced frequency in patients is expected.

Candidate gene studies may be a powerful tool to unravel the pathogenesis of a disease. This approach was successfully used in chapter 3 of this thesis. The
finding that absence of the wildtype allele of a polymorphism in the promoter region of the gene for insulin-like growth factor-I (IGF-I) is associated both with low circulating IGF-1 levels and an increased risk for type 2 diabetes mellitus and myocardial infarction strongly points at a causal role of diminished IGF-I expression in the pathogenesis of these diseases.

A major drawback of the candidate gene approach is that a detailed *a priori* knowledge of the pathogenesis of the disease is required: proteins or genes involved in the disease should be known. For the role of IGF-I in the pathogenesis of diabetes there was a clear case. For a large number of common disorders knowledge of proteins involved in the etiology is limited or absent. Further, there may be yet unknown proteins involved in a disorder, which will never be detected in a candidate gene study. For instance, before cloning of the genes involved in Maturity-Onset Diabetes of the Young (MODY), a dominant inherited subtype of diabetes mellitus, the proteins HNF-1α, HNF-1β, HNF-4α and IPF-1 and their functions were unknown. To find such novel disease genes requires a search through the entire genome, a method that has been utilized in chapter 2 of this thesis.

Hypothetically, a search for disease genes over the full genome involves testing up to 3 billion base-pairs. Testing each of these base-pairs for an alteration is not feasible. Searching the full genome is only feasible in practice if we use the basic principle that patients who inherit a disease gene from a common ancestor not only receive the disease mutation, but also adjacent parts of the chromosome. This principle is referred to as linkage-disequilibrium. Genome mapping starts by genotyping a set of polymorphic markers which are more or less equally distributed across the genome. Whenever a sizeable proportion of patients have inherited a causal mutation from a common ancestor, specific alleles of marker(s) physically in the vicinity of this mutation should at least be present more often in cases than in unaffected relatives or unrelated controls. Using this principle, disease genes may be localized by screening the genome of patients by using only a comparatively few markers.
The probability of detecting linkage disequilibrium is largely determined by the age of the mutation, which determines how large a segment of DNA is shared. Owing to further recombination, the piece of DNA shared by patients who inherited disease from a common ancestor and therefore the extent of linkage-disequilibrium around a disease locus becomes smaller and smaller over generations. Furthermore, the chances of success in identifying the gene are affected by the number of persons that have inherited a mutation from a common ancestor. A major problem encountered in a population-based study is that there is only a small probability that a substantial number of patients with a common complex disorder have inherited a disease gene from a common ancestor. Patients with a common trait, randomly derived from the general population, are usually distantly related, if at all. Any two individuals who inherited a gene from a common ancestor hence only share a small amount of DNA. Therefore the marker and disease locus must be close to localize the gene in a genome screen. Kruglyak estimated that in the general population linkage-disequilibrium is unlikely to extend beyond an average distance of 3 kilobases, which implies that approximately 500,000 markers will be required for a whole genome screen. At present, testing this large number of markers is not technically feasible. Nevertheless, as demonstrated in chapter 2 of this thesis, successful genome screens can be conducted in genetically isolated populations. Isolated populations are typically populations that have arisen from a small number of ancestors. As a consequence, these populations show only a limited amount of genetic variation. If one considers genes with a high number of mutations present at a low frequency (<1/500) in the general population, e.g., the BRCA-1 mutations that have been involved in familial breast cancer, one expects only a limited number of different mutations in a population that was founded by only 150 individuals. Indeed, one finds in the Ashkenazi Jewish population predominantly three founder mutations in the BRCA-1 gene and the BRCA-2 gene. The studies described in this thesis demonstrate that also the allele frequency of more frequent polymorphisms may change. We have shown that the frequencies of the HLA-DR3 and DR4 allele were much lower in type 1 diabetes patients from the
GRIP population than the frequencies which are found in most Caucasian type 1 diabetes patients, suggesting that other genes play a more important role in this population. In addition to these specific founder effects, genetic drift may reduce genetic variability in isolated populations. As a result of the reduced genetic variability in genetically isolated populations, there is a higher probability that patients carry a common mutation inherited from a common ancestor. Furthermore, one can easily select patients from these populations who are closely related, thus increasing the probability of finding linkage disequilibrium in a large area of DNA surrounding a disease-related mutation. Recent studies have suggested that linkage disequilibrium is not larger in genetically isolated populations compared to the general population. We and others have shown that this is not true for recent isolates. Service et al. found linkage disequilibrium over a distance up to 7 cM in Costa Rica, while preliminary findings in the GRIP population suggest that linkage disequilibrium extents over a region of at least 15 cM (personal communication, J. Hoving-Duistermaat). It has been argued that, because many isolated populations are atypical to start with, findings will be difficult to generalize to other groups. The fact that within the GRIP population we confirmed a type 1 diabetes locus on chromosome 8 that has previously been identified in patients from the UK and the US and two type 2 diabetes loci that have been previously identified, suggests that this will not be a major problem for complex diseases.

**Pitfalls in population-based research**

Population-based studies in the past have been widely criticized because of the repeated failure to replicate results. There may be different reasons for this. It has been suggested that population admixture may be an important source of confounding. Whenever a study population comprises different subgroups with respect to genetic make-up, confounding may occur if these differences in genetic background are not controlled in the design or analysis of a study. For example, in a study of diabetes mellitus in Pima Indians, a spurious association was found that could be fully explained by the difference in degree of Caucasian ancestry.
between patients and controls. The Pima example, however, may represent an extreme situation. Wacholder et al demonstrated that the risk ratio is expected to be biased by less than 10 percent in US studies except under extreme conditions. This result suggests that there will only be a relatively small effect from population stratification in a well-designed case-control study of genetic factors.

What are alternative explanations for the general failure of replication of population-based studies? In genetic studies, association between DNA variation and disease is often measured without a prior hypothesis about which variant is associated to the risk of disease. For instance, one may measure the association between disease and a DNA variant that may take forms A, B, C and D. There are 10 possible combinations on the two chromosomes (AA, AB, AC, AD, BB, BC, BD, CC, CD, DD). In the stage of gene identification, there is no prior hypothesis about which of the four variants (and their 10 possible combinations) is associated with the disease. This number of possibilities opens the door for false positive findings, and lack of replicability.

There may also be biological explanations why studies are difficult to reproduce. The first one may be the fact that if we rely on linkage disequilibrium between a marker and the disease-related mutation in a candidate gene study or genome screen, we have to acknowledge that the extent of linkage disequilibrium and even the DNA variant may differ across populations. Second, there may be factors in populations modifying the risk of disease. We tend to think of effects of genes as fixed effects, but they are not. As Khaw has pointed out, the variation in a trait explained by a certain mutation may depend on the variability of other genetic or environmental causes. For instance, in a population with a homogeneous diet, the impact of genetics on blood pressure may be relatively larger than that in a population in which dietary habits are diverse.

Present opportunities
At present, the most obvious approach for genetic studies in epidemiology is the candidate gene approach. Although we are confronted with a bad track record of population-based studies, there is ample reason for renewed optimism. There is
no doubt that genetic research will benefit from incorporating standard epidemiological methods in study design. It is often assumed that DNA variations are stable over time and similar within populations. Many studies have used prevalent patient series without specifying the study population or the duration of disease of the patients leaving ample room for selection and survival bias. When reviewing the method section of studies, control selection is rarely described at all. Although no major difference in allele frequencies may be anticipated within ethnic groups, differences in DNA variations have been shown between groups of Caucasian, African and Asian origin. To prevent bias due to population admixture, sampling of patients and controls from a single study base and evaluating the presence of population admixture are both necessary.

A major improvement in the validity of candidate gene studies may be achieved by adding functional studies in the research program. In the past, anonymous DNA variants were used, without knowledge of function. As pointed out earlier, this has increased the probability of false positive findings. If possible, candidate gene studies should be accompanied by functional assessment of the effect of the allele associated with the disease. This work may demand molecular studies in vitro (transfection of the variant into cells) or studies in transgenic animals (incorporation of the human mutated gene into an animal). For a number of disorders functional studies are possible in humans. Studies on variations in promoter regions may be easily accompanied by studies of various functional outcomes. For instance, in chapter 3 of this thesis, we could show a relation between a polymorphism in the promoter region of the insulin-like growth factor-1 gene (IGF-I) and serum levels of IGF-I as well as body height.

In a candidate gene study, one of the key problems is heterogeneity. In assessing the evidence for a role of a gene in disease, one approach is to focus on familial patients. Another approach to reduce the complexity of a disorder may be to focus on a single etiologic pathway. At the gene level a large number of genes may be involved in a complex disease such as myocardial infarction, e.g. genes that regulate lipid metabolism, blood pressure, hemostasis, glucose metabolism...
and obesity. Thus, in a genome scan, in which the DNA of patients with myocardial infarction is screened, one can anticipate only a weak association between a particular gene and a disease. Large datasets will be necessary to measure these small effects. An alternative approach may be to study levels of proteins in relation to the genes involved in specific etiologic pathways. For instance a polymorphism in the APOE gene is strongly related to serum levels of ApoE and cholesterol but only weakly to atherosclerosis and myocardial infarction. By comparison of subjects with extremely low ApoE levels versus subjects with high ApoE levels one is targeting a stronger association, which can be detected more easily and reliably. After the unraveling of the genes related to an intermediate protein, the relation with myocardial infarction can be studied subsequently in a candidate gene approach. Last but not least, it will be crucial to evaluate the evidence for a causal role of a gene in the etiology of a disorder in the context of all previous studies. Meta-analyses of studies will increase precision and will enable to detect potential false positive or negative findings in a single study. Such studies are crucial to evaluate whether the variation itself is related to the disease, or, whether another variation closely linked to the one studied is involved in the causal pathway. In the first case, one expects an association with the same allele in all populations, while in the second case, different alleles may be associated to the disease in different population. In particular, studies of populations distantly related, e.g. Caucasians, Asians, and Africans, are valuable.

Future developments

After the identification and cataloging of all human genes as a spin-off of the human genome project, further studies on the functional variations within them may provide an opportunity to define candidate genes more accurately. New developments in technology will provide added insights regarding gene function on the cellular level, improving our ability to predict phenotypic effects of genes.

An approach feasible in the post-genome era will be genome scans targeting only genes instead of using markers randomly dispersed over the genome. The major advantage of this approach is that it more directly focuses
on etiologic relevant sequences and that linkage disequilibrium is only necessary at a relatively small distance, i.e., within a gene. A technical development that is important for the feasibility of such large-scale genetic epidemiologic research is the introduction of microarrays, which include information on presence or absence of a variation in a gene. These approaches will create the opportunity to screen rapidly for DNA mutations or variations in large series of affected individuals. In this respect, identification of single-nucleotide polymorphism maps (SNPs) throughout the human genome is crucial. Major progress is to be anticipated in this field within the next decade.

The major question for genetic-epidemiologists to prepare for future genetic studies is which samples are most valuable for genome searches. There is ongoing debate on the most "intelligible" approach. On the one hand scientists and industry are focusing on isolated populations, such as Iceland. The advantage of this approach is that only a limited number of patients and, in the case of a recently isolated population with strong linkage disequilibrium, a limited number of genetic markers will be sufficient for a genome scan. The recent successes of genomic screens in genetically isolated populations, including the ones described in this thesis, support this approach. Others advocate studying large numbers of cases and controls from outbred populations, because findings in these heterogeneous populations will be representative of the general population. The aim of these case-control studies is to study all (candidate) genes in large populations. In the United Kingdom, the Medical Research Counsel (MRC) and the Wellcome Trust contemplate studies of about 500,000 middle-aged subjects. As is shown in this thesis, population based studies in the general population, such as the Rotterdam Study, are very suitable for targeted candidate gene studies. Given the technical developments, also genome screens in the general population may become feasible.

Here the focus was on the identification of new genes involved in the etiology and pathogenesis of diabetes and vascular pathology. A second important task will be to accurately assess the risks of these diseases associated with the identified genetic variations. Both for diabetes and cardiovascular
disease, non-genetic factors such as smoking, diet and viral infections contribute to the occurrence of disease. Combinations of predisposing alleles, environmental factors, and behavior may lead to the same pathogenic effect. Therefore, it will be necessary to study gene-associated risks in interaction with other genetic and environmental risk factors. Given the need of information on a broad spectrum of exposures, it will be efficient to implement these studies in ongoing epidemiological follow-up studies, such as the Rotterdam Study.
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CHAPTER 5

SUMMARY & SAMENVATTING
Diabetes mellitus is the most common metabolic disorder in the Western world, affecting more than 120 million people worldwide. The etiology of the most frequent forms, type 1 and type 2 diabetes mellitus, and the etiology of diabetic vascular complications are still unclear. Substantial evidence exists that susceptibility to these diseases has a genetic basis. This thesis focuses on the identification of specific genetic factors that may be involved in the etiology of diabetes mellitus and the development of vascular complications. Given the lack of success of family studies in the identification of genetic risk factors involved in complex diseases, we have focussed on the possibility of identifying relevant genes using population-based patient series. In this thesis, two different strategies are followed to identify new genes, the genome-wide screen and the candidate gene approach.

In chapter 1 a brief overview is given of the clinical characteristics and pathophysiology of diabetes mellitus and the current knowledge of the etiology, with a major emphasis on the role of genetic factors.

Chapter 2 describes the results of two genome-wide screens in the GRIP population, a genetically isolated population in the Southwest of the Netherlands. Due to the limited number of founders and the low immigration rate, the genetic variability of this population has been strongly reduced which makes this population very suitable for mapping of genes for complex genetic disorders. In chapter 2.1 we have focused on the etiology of type 1 diabetes mellitus (DM1). We identified 43 patients with (DM1) that could be traced back to a common ancestor within 15 generations and performed a genome-wide scan by using a combined linkage and association based approach. In addition to the well-known HLA locus, evidence for a susceptibility locus was observed with markers on chromosome 8q and 17q. The observation that flanking markers in these regions were also associated with an increased risk for DM1 and the observation that the risk for diabetes increased with the number of ancestral alleles present for each of these markers, strongly supports the presence of a true susceptibility gene in these regions. The findings on chromosome 8q, which are further supported by the presence of a conserved ancestral haplotype in this region, confirm previous
findings of a DM1 susceptibility locus on chromosome 8q. In addition, this study suggests the presence of a new locus on chromosome 17q. However, evidence for this locus is less strong and therefore this finding still needs to be confirmed in other studies. In chapter 2.2 we have focused on the etiology of type 2 diabetes mellitus (DM2). We identified 79 families with at least one patient with DM2 that could be traced back to a common ancestor within 13 generations and performed a genome-wide scan. Nine markers showed evidence for association with DM2 and the strongest evidence for a susceptibility locus was observed on chromosome **p. Of these nine markers, markers on chromosome #q and **p showed additional evidence for association with fasting glucose levels in an independent set of first-degree relatives. Both localizations were confirmed by the observation that affected individuals shared additional markers in these regions and by the observation that flanking markers and haplotypes in both regions were significantly associated with fasting glucose concentration in first-degree relatives of patients. The replication of the findings of the initial genomic scan in first degree relatives suggests that we have identified 2 susceptibility loci for DM2.

In chapter 3, a candidate gene approach is used to study the pathogenesis of type 2 diabetes mellitus and cardiovascular pathology in the Rotterdam Study; a large population based cohort study. We chose the gene for insulin-like growth factor-I (IGF-I) as a candidate gene, because low circulating levels of this hormone have been associated with an increased risk for both diabetes mellitus and cardiovascular disease. In chapter 3.1 we examined the role of a genetic polymorphism in the promoter region of the IGF-I gene in relation to circulating IGF-I levels and body height, and we studied the relationship of this polymorphism with type 2 diabetes mellitus and myocardial infarction. The relation between the IGF-I polymorphism and body height was assessed in population-based sample of 900 subjects. Within each genotype stratum, 50 subjects were randomly selected to study the relation of this polymorphism with serum IGF-I levels. To assess the risk of type 2 diabetes mellitus, we studied 220 patients and 596 normoglycaemic controls. For myocardial infarction, 477 patients with evidence for myocardial infarction at ECG and 808 controls were studied. A 192-bp allele was present in 88%
of the population, suggesting that this is the wildtype allele from which all other alleles originated. Body height was on average 2.7 cm lower and serum IGF-I concentrations were 18% lower in subjects who did not carry the 192-bp allele. In non-carriers of the 192-bp allele, an increased relative risk for diabetes and for myocardial infarction was found. In patients with type 2 diabetes mellitus the relative risk for myocardial infarction in subjects without the 192-bp allele was 3.4. This study suggests that a genetically determined exposure to relatively low IGF-I levels is associated with an increased risk for type 2 diabetes mellitus and myocardial infarction. In chapter 3.2, we examined whether genetically determined low expression of IGF-I has an effect on pancreatic beta-cell function and, if so, whether this genetic factor influences the risk for diabetes in persons with insulin resistance or obesity. The relation between the IGF-I polymorphism, beta-cell function and diabetes mellitus was assessed in a population-based sample of 220 persons with type 2 diabetes, 253 persons with impaired glucose tolerance and 596 persons with normal glucose tolerance. Persons with the lowest expression of IGF-I showed a significant reduction in beta-cell function. In persons with insulin resistance, genetically determined low IGF-I expression was associated with a 7.5 fold increased risk of diabetes. In those with obesity, genetically determined low IGF-I expression was associated with a 6.2 fold increased risk of diabetes. Our findings show that the increased risk for diabetes mellitus in persons with genetically determined low IGF-I expression is most likely the result of an inadequate compensatory beta-cell response to the increasing insulin needs of persons with insulin resistance and obesity. We conclude that the IGF-I promoter region may in part determine the genetically programmed pancreatic beta-cell dysfunction that leads to diabetes in persons with insulin resistance or obesity. In chapter 3.3, we aimed to confirm the association between the IGF-I polymorphism and myocardial infarction using prospective follow-up data. In addition, we examined the mechanism underlying this increased risk for myocardial infarction. We compared the genotype distribution of the polymorphism in the regulatory region of the IGF-I gene in 108 individuals with newly diagnosed myocardial infarction and 779 controls. Genetically determined low IGF-I expression was
CHAPTER 5

associated with a 1.8 fold increased risk of myocardial infarction. Using a population based sample of 900 subjects we found no evidence for the hypothesis that this increased risk for myocardial infarction is the result of an increased frequency of atherosclerosis in subjects with low IGF-I expression. However, in those subjects that had already developed generalized atherosclerosis or suffered from angina pectoris at baseline, IGF-I expression proved to be a strong modifier of the risk to develop myocardial infarction. The combination of angina pectoris and low IGF-I expression was associated with 12.9 times increased risk for incident myocardial infarction, while the combination of atherosclerosis and low IGF-I expression was associated with a 6.8 times increased risk. This study confirms the observation that genetically determined low expression of IGF-I increases the risk for myocardial infarction. In addition, these findings are compatible with the view that IGF-I has direct cardioprotective properties. We conclude that the lack of such a protective effect in those with genetically determined low expression of IGF-I increases the risk for myocardial infarction, particular in subjects that are likely to exhibit periods of cardiac ischemia. Finally, in Chapter 3.4, we examined the relation between the IGF-I promoter polymorphism and birthweight. We hypothesized that, if this polymorphism influences fetal IGF-I and insulin secretion, it may also be involved in the regulation of intrauterine growth and may thus explain part of the relationship between low birth weight and late-onset disease that has been described in many studies. In 463 elderly individuals, absence of the wildtype allele was associated with a 213 gram lower birth weight. In addition, the IGF-I genotype was strongly associated with a postnatal realignment of weight; non-carriers of the wildtype allele who started with a low birth weight showed a relative gain in weight during life. We propose that these differences in birth weight reflect genetically determined differences in fetal IGF-I and insulin secretion. Our data support the hypothesis that the association between low birth weight, diabetes and cardiovascular disease may follow from pleiotropic effects of common variations in genes influencing both fetal growth and susceptibility to late onset disease.

In the first part of Chapter 4 an integrated overview is given of the findings as well as the limitations of the different studies. Further, the possible clinical
implications are discussed. The last part of this chapter provides a more general view on the future opportunities for population-based studies of complex genetic disorders.
Samenvatting

Suikerziekte (diabetes mellitus) is de meest voorkomende endocrinologische aandoening in de westerse wereld. De toegenomen levensverwachting, de toenemende wereldpopulatie en de mondiale overname van een Westerse levensstijl zullen leiden tot een explosieve toename van het aantal diabetes patiënten in de komende decennia. Een beter begrip van de factoren die een rol spelen bij het ontstaan van diabetes zal uiteindelijk leiden tot een verbeterde diagnose, behandeling en mogelijk preventie van deze ziekte.

Diabetes mellitus is een zogenaamde complexe genetische aandoening. Dit wil zeggen dat zowel omgevingsfactoren als genetische factoren een rol spelen bij het ontstaan van deze ziekte. Dit proefschrift richt zich op de identificatie van specifieke genetische factoren die een rol spelen bij het ontstaan van diabetes mellitus en vasculaire complicaties. Gezien de geringe mate van succes van familiestudies in het opsporen van genetische risicofactoren voor complexe aandoeningen, hebben wij ons gericht op de mogelijkheid om relevante genen op te sporen met behulp van populatie onderzoek. In dit proefschrift worden twee strategieën gevolgd om genen op te sporen: de zogenaamde “genome wide scan” en het kandidaat-gen onderzoek.

In hoofdstuk 1 wordt een kort overzicht gegeven van de klinische verschijnselen en de pathofysiologie van diabetes mellitus. Tevens wordt kort ingegaan op de huidige kennis met betrekking tot het ontstaan van diabetes, waarbij de nadruk ligt op de rol van genetische factoren.

Hoofdstuk 2 beschrijft de resultaten van twee genome wide scans in de GRIP populatie, een genetisch geïsoleerde populatie in het Zuidwesten van Nederland. Door het beperkte aantal founders en de minimale immigratie is de genetische variabiliteit van deze populatie verminderd. Hierdoor is deze populatie uitermate geschikt voor het opsporen van genen die een rol spelen in de etiologie van complexe genetische aandoeningen. In hoofdstuk 2.1 hebben we ons gericht op de etiologie van type 1 diabetes mellitus (DM1). We hebben 43 DM1 patiënten opgespoord welke met elkaar verwant bleken te zijn binnen 15 generaties. Bij deze patiënten is een genome wide scan uitgevoerd waarbij
gebruik is gemaakt van een gecombineerde linkage en associatie methode. In dit onderzoek is het gehele DNA van de patiënten onderzocht op het voorkomen van genen die het risico op DM1 beïnvloeden. Behalve de associatie met een reeds bekend locus in de HLA-regio werden aanwijzingen gevonden voor een DM1 gen op chromosoom 8q en 17q. De bevindingen op chromosoom 8 bevestigen bevindingen uit eerdere studies in andere populaties. Het bewijs voor een DM1 gen op chromosoom 17q is minder sterk en dient derhalve nog bevestigd te worden in andere studies. In hoofdstuk 2.2 hebben we ons gericht op de etiologie van type 2 diabetes mellitus (DM2). We hebben 117 patiënten met DM2 opgespoord. Al deze patiënten bleken binnen 13 generaties van een gezamenlijke voorouder af te stammen. In de genome wide scan werd associatie gevonden met 9 genetische markers. Van deze 9 markers waren markers op chromosoom **p en ##q eveneens geassocieerd met nachtver glucose concentraties in een onafhankelijke set van eerstegraads familielieden. De aanwezigheid van een mogelijk DM2 gen in deze regio's werd uiteindelijk bevestigd door de observatie dat aangedane personen ook aanliggende markers deelden, terwijl deze chromosomale regio's ook geassocieerd waren met nachtver glucose spiegels in de eerstegraads familielieden. De replicatie van de bevindingen van de genome scan in de DM2 patiënten met de glucose spiegels in eerste graads familielieden suggereert dat we twee regio's in het genoom hebben geïdentificeerd die het risico op DM2 beïnvloeden.

In hoofdstuk 3 is een kandidaat-gen studie gebruikt om de pathogenese van type 2 diabetes mellitus en vasculaire pathologie te bestuderen in de ERGO-studie; een groot populatie onderzoek in Rotterdam. Het gen dat codeert voor insuline-achtige groefactor-I (IGF-I) werd gekozen als kandidaat-gen omdat er aanwijzingen zijn dat dit hormoon een rol speelt in de regulatie van het glucose metabolisme en bij de ontwikkeling van vasculaire complicaties. Hoofdstuk 3.1 begint met een studie waarin de functionaliteit van een variant in het IGF-I gen wordt onderzocht en waarin de relatie van deze variant met type 2 diabetes mellitus en het hartinfarct wordt bestudeerd. 88% van de onderzoeksgroep bleek een specifiek alleles, het zgn.192-bp allele, te dragen. Individuen die dit allele niet
droegen hadden een significant lagere lichaamslengte, lagere circulerende IGF-I spiegels en een verhoogd risico op type 2 diabetes mellitus en een hartinfarct. Vooral diabetes patiënten die het 192-bp allele niet droegen hadden een sterk verhoogd risico op een hartinfarct. Deze bevindingen suggereren dat het verhoogde risico op diabetes en een hartinfarct in personen zonder het 192-bp allele een gevolg is van een genetisch bepaald chronisch verlaagde productie van IGF-I.

In hoofdstuk 3.2 hebben we het mechanisme onderzocht waardoor een genetisch bepaald tekort aan IGF-I leidt tot een verhoogd risico op type 2 diabetes mellitus. We vonden dat genetisch bepaalde lage IGF-I productie gepaard ging met een verminderd vermogen om insuline te produceren. Vooral in personen met insuline resistentie of overgewicht was een genetisch verminderde IGF-I productie geassocieerd met een sterk verhoogd risico op diabetes. Experimentele studies hebben aangetoond dat IGF-I een belangrijke rol speelt bij de aanleg en groei van de insuline producerende beta-cellen in de pancreas. Onze bevindingen suggereren dat genetisch bepaalde lage IGF-I productie resulteert in een relatief tekort aan functionele beta-cellen. Hierdoor zal het risico op diabetes verhoogd zijn, vooral in situaties waarin er een verhoogde behoefte aan insuline is, zoals bij zwangerschap, overgewicht of insuline resistentie.

In hoofdstuk 3.3 is de relatie tussen de variant in het IGF-I gen en het hartinfarct nader onderzocht. In een onafhankelijke groep van personen die een eerste hartinfarct ontwikkelden tijdens de follow-up periode van de ERGO studie konden we het verhoogde risico op een hartinfarct in mensen met genetisch bepaalde lage IGF-I productie bevestigen. Het verhoogde risico op een hartinfarct kon niet verklaard worden door het vaker voorkomen van aderverkalking in personen met lage IGF-I productie. Lage IGF-I productie ging vooral gepaard met een hoog risico op een hartinfarct in personen die reeds klachten hadden van angina pectoris of die reeds leden aan uitgebreide aderverkalking. Deze bevindingen steunen de bevindingen van een aantal experimentele studies die aantonen dat IGF-I een belangrijke rol speelt bij de bescherming van hartcellen in perioden van zuurstof tekort.
Omdat IGF-I een belangrijke rol speelt bij de groei van de foetus, hebben we in hoofdstuk 3.4 de relatie tussen de variant in het IGF-I gen en geboortegewicht onderzocht. In een groot aantal studies is een relatie aangetoond tussen een laag geboortegewicht en het ontstaan van diabetes en hartvaatziekten op latere leeftijd. Er is gesuggereerd dat ondervoeding van de foetus tijdens de zwangerschap verantwoordelijk is voor het lage geboortegewicht en tevens voor het optreden van ziekte op latere leeftijd. Een alternatieve hypothese is dat genetische factoren de gevonden relatie kunnen verklaren. Wij vonden dat personen met een genetisch bepaalde verlaagde IGF-I productie een lager geboortegewicht hadden. Deze bevinding steunen de hypothese dat genetische factoren, in plaats van omgevingsfactoren, ten grondslag kunnen liggen aan de relatie tussen een laag geboortegewicht en het optreden van ziekte op latere leeftijd.

In hoofdstuk 4 wordt een geïntegreerd overzicht gegeven van de belangrijkste bevindingen. Vervolgens worden de voor en nadelen en de eventuele klinische implicaties besproken. In het laatste deel van dit hoofdstuk worden de huidige en toekomstige mogelijkheden voor populatie onderzoek van complexe genetische aandoeningen besproken in het licht van de ontwikkelingen van het humane genome project.
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LIST OF PUBLICATIONS


