VRIJE UNIVERSITEIT

Genetic influences on β-cell function

a Dutch twin-family study

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad Doctor aan
de Vrije Universiteit Amsterdam,
op gezag van de rector magnificus
prof.dr. L.M. Bouter,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de faculteit der Geneeskunde
op dinsdag 23 november 2010 om 15.45 uur
in de aula van de universiteit,
De Boelelaan 1105

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Chapter 1

General introduction

Introduction

Type 2 diabetes mellitus is a serious chronic disease, with important consequences for every- day life. Incidence and prevalence are rapidly increasing in the last decennium, not only in the developed countries but even more in the developing countries. At 1 January 2008, 670.000 inhabitants of the Netherlands had type 2 diabetes (± 4%) and another 250.000 adults were unaware that they had this disease (1). It is expected that the amount of diabetic patients in the Netherlands will increase to 1.3 million in 2025. The WHO has estimated that in the year 2000, 171 million adults had type 2 diabetes mellitus and that this will increase to 366 million in the year 2030 (2). To prevent this increase, we need more knowledge of the pathophysiology of type 2 diabetes mellitus and the phenotypes at high risk, in order to develop more effective prevention strategies and to improve treatment possibilities.

Type 2 diabetes mellitus will develop when insulin secretion is not adequate for the prevailing insulin sensitivity. As long as the insulin secretion can keep up with the decreasing insulin sensitivity, there will be no symptoms of glucose intolerance. There is a continuing debate about what comes first; the decreasing insulin sensitivity or the impaired insulin secretion. During each stage of the development of type 2 diabetes mellitus, insulin resistance and insulin secretory dysfunction are independent predictors of worsening glucose tolerance and are, therefore, both targets for the primary prevention of the disease (3). Since better tests were developed to assess the insulin secretion and more research was performed in persons with different degrees of glucose tolerance, small impairments of β-cell function can already be detected in persons without any symptom of hyperglycaemia or type 2 diabetes mellitus (4).

Insulin secretion

β-cells in the pancreas islets are responsible for the insulin secretion. Glucose is the most potent secretagogue as it produces robust insulin secretion in a few minutes after entering the β-cell and the stimulatory effect lasts as long as the plasma glucose is elevated. The β-cell insulin secretory response to glucose occurs in two phases: an acute first phase, lasting a few minutes and then declining followed by a gradually increasing second phase to a peak within 30-40 minutes. Glucose is rapidly transported into the β-cells, largely via the

GLUT1 transporter and partly via the GLUT2 transporter (5;6). Next glucose phosphorylation takes place by glucokinase, a strict glucose specific enzyme, that has demonstrated to be the key regulator of the glucose sensing in β-cells (7;8). The end product of this glucose metabolism, pyruvate, enters the mitochondria, were it follows two different routes. The first route is oxidation to acetyl-CoA, which provides a large amount of ATP. The increased cellular ATP/ADP ratio closes K_{ATP}-sensitive channels, resulting in membrane depolarization followed by Ca^{2+} influx through voltage-gate-dependent Ca^{2+} channels. This causes exocytose of insulin granules. Next to this K_{ATP}- dependent route the mitochondria provide a K_{ATP}- independent way of glucose stimulated insulin secretion by carboxylation of pyruvate to oxaloacetate by the enzyme pyruvate carboxylase. Metabolites produced by the mitochondria are exported to the cytosol and function as intracellular messengers to support insulin secretion. Among these amplifying signals are NADPH, GTP, Malonyl-CoA, Long chain acyl-CoA, Glutamate and PEP (9).

The most important physiologic non-glucose secretagogues that increase the insulin secretion are incretins such as glucose-dependent insulin releasing polypeptide (GIP) and glucagon-like peptide-1(GLP-1). Immediately after oral ingestion of nutrients GIP is mainly secreted by the K cells in the upper small intestine while GLP-1 is predominantly secreted by entero-endocrine L cells located in the distal intestine (10-12). This prompt release is probably more indirect controlled by neural and endocrine factors in the proximal gastrointestinal tract, while later incretin secretion is maintained by arrival of nutrients lower in the intestine. Binding of GIP and GLP-1 to their specific receptor at the β-cell membrane causes the activation of adenyl cyclase via the G protein and leads to an increase of intracellular cyclic adenosine monophosphate (cAMP). This evokes a cascade of intracellular events resulting in increased concentration of cytosolic Ca^{2+} which drives the exocytose of insulin granules. Incretin receptors are expressed in many other tissues including several brain areas and the heart. Besides the enhancement of insulin secretion, both incretins promote β-cells proliferation while GLP-1 also stimulates insulin biosynthesis, reduces food intake, inhibits glucagon secretion and decreases gastrointestinal secretion and motility.

Amino acids and fatty acids stimulate insulin secretion not only by enhancing the incretin production in the intestinal cells (10;13), they have also a specific effect on β-cells. Charged amino-acids like lysine and arginine cross the β-cell membrane via a transport system specific for cationic amino acids. The accumulation of the positive charged

molecules directly depolarizes the β-cell membrane leading to calcium influx and consequently increased insulin secretion (14;15). On the other hand, fatty acids play a role in the intracellular amplification pathway of insulin secretion (15) and may remodel the plasma membrane to facilitate insulin secretion (9).

Age appears to be negatively correlated with β-cell function in glucose tolerant Caucasians, even after correction for insulin sensitivity and this might be due to an impairment in proinsulin conversion to insulin (16;17).

The autonomic regulation of the β-cell function is influenced by the splanchnic nerve of the sympathetic nervous system (SNS) and the vagus nerve of the parasympathetic nervous system (PNS) (18). The splanchnic nerve releases norepinephrine from nerve terminals and epinephrine from the adrenal medulla, initiating catabolic metabolic processes including inhibition of insulin secretion. In contrast the vagus nerve mediates anabolic responses to internal stimuli from the viscera and external stimuli from the sensory components of food. Activation of the vagal efferent activity occurs at the onset of and during meal ingestion and plays an important role in the acute and further postprandial insulin responses. The well-known neurotransmitter of the vagus nerve, acetylcholine, acts on the β-cells by muscarine receptors. Consequently, a combination of reactions follows, including increment of cytosolic concentration of Ca^{2+}, independently of extracellular Ca^{2+} uptake, stimulation of the formation of arachidonic acid and activation of protein kinase C, resulting in a rapid stimulation of exocytosis and insulin secretion (19). There are three more neurotransmitters localized to islet parasympathetic nerves: vasoactive intestinal polypeptide, gastrin releasing peptide and pituitary adenylate cyclase activating polypeptide. They all stimulate insulin secretion by activating intracellular signalling mechanisms, which are partially different. Prolonged mild hyperglycaemia results in a compensatory increase in insulin secretion, which is partially mediated by an induction in vagal efferent activity (20).

Autonomic and endocrine responses to food consumption, which are evoked by sensory mechanisms before nutrients have been absorbed, are called ‘cephalic phase responses’ (19). The insulin secretion in the first 3-4 minutes of a meal intake is the result of three successive pathways: first, the afferent pathway activated by olfactory, visual, gustatory and oropharyngeal mechanical receptors, secondly the integration of these stimuli in the brain and finally the efferent pathway, mediated by the cholinergic neurons. Although the contribution of the cephalic phase to the entire postprandial insulin secretion

is only 1-3%, the cephalic phase is probably of considerable functional importance for glucose tolerance after meal intake. Inhibition of the early (15 min) response to a meal by the ganglionic antagonist , trimetophane, is accompanied by increased post prandial glucose concentrations at min 45 and 60 (19).

Increased sympathetic activity results in inhibition of glucose stimulated insulin secretion in situations of stress, including exercise and trauma (21). The neurotransmitter norepinephrine activates the α_2- adrenergic receptors in the β-cell membrane. The inhibition of insulin secretion is mediated by hyperpolarisation of the β-cells through opening of the ATP- regulated K^+ channels. This inhibits the Ca^{2+} uptake and reduces the cytosolic concentration of Ca^{2+}. Reduced formation of cyclic AMP and inhibition of the metabolic processes leading to exocytosis have also been shown as cause of reduced insulin secretion after activation of α_2- adrenergic receptors in the β-cell membrane. Although norepinephrine can also stimulate insulin secretion by activation of the β_2-adrenergic receptors on the β-cell membrane, resulting in increased formation of cAMP in the β-cells (19), increased sympathetic activity results predominantly in decreased insulin secretion (21).

Besides the parasympathetic and sympathetic nervous systems, each individual islet is also extensively innervated by a network of sensory nerves and by nerve fibres, stained for a marker of nitric oxide synthethase. However the role of these two types of nerve fibres is far from understood (19).

Insulin secretion in adult life may also be related to pre-natal circumstances. The consequences of the famine during the Dutch Hunger Winter of 1944-1945 have been extensively investigated. It appeared that foetal malnutrition especially during the first 6 months gives rise to impaired glucose tolerance in adult life based on an insulin secretion defect (22).

Insulin signalling and insulin action

The insulin molecule consists of two polypeptide chains, the A chain (21 amino acids) and the B chain (30 amino acids), linked by two disulphide bridges. The insulin cell-surface receptor is a heterotetrameric receptor, composed of two extracellular α subunits and two β subunits that contain an extracellular portion, a transmembrane domain and an intracellular part. Insulin binding to the α subunit results in phosphorylation and activation of the

tyrosine kinase in the intracellular part of the β subunits (23). This activates the insulin receptor substrate (IRS) proteins 1-4, which are the main mediators of the intracellular insulin receptor signalling events (24). The four IRS proteins are tissue specific; IRS-1 protein mediates the insulin action specifically in the skeletal muscle, while IRS-2 protein acts in the liver. The tyrosine phosphorylation of IRS-1 leads to two major signalling pathways e.g. the phosphatidylinositol-3'-kinase (P13K) pathway and the mitogen-activated protein kinase (MAPK) pathway. The P13K pathway plays a crucial role in the metabolic actions of insulin, by stimulating glycogen, lipid and protein synthesis. It also stimulates nitric oxide production a potent vasodilator and anti-atherogenic agent. In muscle and fat cells this pathway also affects the insulin regulated glucose transport (GLUT4) system, which facilitates the rapid uptake of glucose through the cell membrane (23). The activation of MAPK pathway leads to intra-nuclear processes, which influence transcription factors and DNA synthesis. This results not only in cell growth, cell proliferation and cell differentiation, but also in activation of multiple inflammation pathways (25). In short, the core business of insulin in the body is energy storage.

Insulin sensitivity is at the physiological level associated with obesity, physical inactivity and aging. Decreased insulin sensitivity is characterized by an impaired ability of insulin to inhibit hepatic glucose production and to stimulate glucose uptake by skeletal muscle. Insulin also fails to suppress lipolysis in adipose tissue. The molecular mechanisms underlying a decrease in insulin sensitivity are not all precisely known, but may be mainly based on a deregulation of one of the many steps of the insulin signalling pathway. Protein tyrosine phosphatises (PTPs), which dephosphorylate the insulin receptor or downstream substrates may be key regulators of the insulin receptor signal transduction pathway and for the most part attenuate insulin action (26). Recent studies in human skeletal muscle of insulin resistant type 2 diabetic and obese non diabetic individuals showed profound insulin resistance in the P13K pathway with normal stimulatory effect of insulin on the MAPK pathway (25). This defect in insulin signalling impairs not only glucose uptake, glucose metabolism in the muscle cells and NOS synthesis but, because of the persistent hyperinsulinaemia, at the same time activates via the MAPK pathway multiple genes coding for pro–inflammatory .mediators (TNFα, IL-1B, PKC). These pro-inflammatory mediators inhibit the intracellular insulin signalling and induce the degradation of IRS-1 by phosphorylation of the Serine residues on the IRS proteins (27).

Insulin signalling can also be inhibited by signals from other pathways, like that in lipotoxicity. Obesity is often characterised by a state of low grade chronic inflammation with increased levels of pro-inflammatory cytokines and their effects on insulin resistance by serine phosphorylation of IRS-1(25). Studies have shown that fat accumulation in muscle and hepatic cells are correlated with organ-specific insulin resistance. Increased release of free fatty acids from the adipose tissue decreases insulin mediated glucose transport in skeletal muscle and impairs suppression of glucose production by the liver(27). Adipocytes and infiltrated macrophages of visceral fat of obese and type 2 diabetic individuals secrete pro-inflammatory cytokines (TNFα, Interleukin-6), acute phase reactants (C-reactive protein) and hormones (leptin and resistin) which also induce insulin resistance. Moreover, visceral adiposity is a state with a relative deficiency of adiponectin, a potent insulin-sensitizing hormone (24).

The importance of insulin sensitivity and specially the role of the adipose tissue in the development of diabetes mellitus has recently been shown by the results of the CANOE (Canadian Normoglycemia Outcomes Evaluation) trial (28). A low dose combination therapy of rosiglitazone (a PPARγ agonist that increases insulin sensitivity among others by its action on adipose tissue and fatty acids in the muscle) with metformin (a biguanide that reduces hepatic glucose production and increases the peripheral insulin sensitivity) appeared to be highly effective in the prevention of type 2 diabetes in patients with impaired glucose tolerance. The low dose combination therapy did not only results in a smaller decline of insulin sensitivity but also in a reduction in inflammation and improvement in hepatic function.

Insulin sensitivity declines slowly during aging, but this may be due to age–related changes in body composition, rather than a consequence of aging itself (29). Increased insulin resistance in elderly was found to be associated with fat accumulation in muscle and liver cells that may be a result of age-associated decrease in ATP production by the mitochondria (30). However, a recent study of Karakelides (31) showed that an age related decrease in muscle mitochondrial function was neither related to adiposity nor insulin sensitivity.

Genetic and environmental factors

The importance of genetic influences is sustained by twin studies, and a strong familial aggregation. In 1981 Barnett (32) showed a nearly complete concordance rate for type 2 diabetes mellitus in identical twins while in the few discordant pairs the unaffected twin already showed metabolic abnormalities. His conclusion that genetic factors are predominant in the aetiology of type 2 diabetes mellitus has been confirmed by many twin (33-52) and family (53-63) studies in the following decades. A positive family history immediately increases the chance to get the disease. The risk is six times higher when two first degree relatives have type 2 diabetes mellitus and at least two times when one first degree relative is affected (64;65). Further evidence for a genetic role is the wide variation in prevalence among different ethnic groups (66;67).

At the end of the 20th century twin and family studies also started to estimate the heritability of individual differences in glucose and insulin levels. Most of these studies were performed with only fasting glucose and insulin levels, but a few studies have also addressed heritability of the responses to glucose challenge tests like the Oral Glucose Tolerance tests, the intravenous glucose tolerance test (mainly for assessment of β-cell function) and the euglycaemic-hyperinsulinaemic clamp test (for insulin sensitivity only). Table 1.1 and 1.2 give an overview of twin and family studies, performed from 1996 till 2010, that assessed the heritability of insulin sensitivity and insulin response in many different ways. Table 1.3 summarizes the results from studies, performed in the same period, that estimated heritability for clinical indicators of (pre)diabetic state.

A further step towards a better understanding of the genetic variation involved in type 2 diabetes mellitus was the identification of the actual genetic variants. In the last decade studies came out that tested the association of variants in candidate genes with measures of glucose metabolism and/or the risk of type 2 diabetes mellitus. But increasingly the candidate gene approach has given way to the genome wide association (GWAS) approach. Large collaborative consortia across many different research groups like MAGIC (the Meta-Analyses of Glucose an Insulin related Traits Consortium) made it possible to combine the data of tens of thousands of subjects to identify new genetic variants that affect glucose metabolism and/or the risk of type 2 diabetes mellitus. So far, GWA studies have uncovered 26 confirmed gene variants that are associated with a higher risk for the development of type 2 diabetes mellitus (68;69) and at least fifteen of these

Table 1.1: Heritability estimates of insulin resistance and insulin sensitivity from studies between 1996 and 2010

Test	reference no	formula	Heritability %	covariate
Insulin RESISTANCE				
Fasting blood levels	(38;53;63)	HOMA-IR= (fasting insulin mU/l x fasting glucose mmol/l)/22.5	22 – 42 - 48	A, S
	(50;56)	HOMA-IR= (fasting insulin mU/l x fasting glucose mmol/l)/22.5	23 - 48	A, S, BMI
	(61;71)	HOMA-IR= (fasting insulin mU/l x fasting glucose mmol/l)/22.5	8 - 38	A, S, E
	(37)	Log (HOMA-IR)	8	
	(54)	Log (HOMA-IR)	12	
	(54)	Log (HOMA-IR)	16	A, S, BMI
	(37)	HOMA-R'=[(log fasting insulin)-c] x fasting glucose	59	
	(36)	HOMA2-IR computer model	58	A, BMI, W
Insulin SENSITIVITY				
Fasting blood levels	(57)	ISI 0= Insulin Sensitivity Index 0 =	37	A, S
			28	A, S, BMI
	(60)	HOMA-%S	46	A, S, BMI
	(46)	Basal glucose uptake in μmol/l $kgFFM^{-1}$ min^{-1}	Young 27	S
			Old 67	S
Oral glucose tolerance test (OGTT)	(57)	ISI 120 = 10^4 / Insulin 120 minute x glucose 120 minute	34	A, S
			32	A, S, BMI
FSIVGTT+insulin	(54)	Log(S1+1)	29	
			33	A, S, BMI
FSIVGTT+ tolbutamide	(55)	Si = insulin sensitivity index	38	
	(62)	Si = insulin sensitivity index	28	A, S, BMI
FSIVGTT	(59)	Si MINMOD analysis computer program	44	A, S, BMI
Euglycaemic hyperinsulinaemic Clamp	(61)	M (lbm)= glucose disposal in mg $kglbm^{-1}$ min^{-1}	24	A, S, E
	(39;46)	Insulin stimulated glucose uptake in μmol/l $kgFFM^{-1}.min^{-1}$.	37 - 55	S

FSIVGTT = frequently sampled intravenous glucose tolerance test; HOMA-IR = Homeostasis Model Assessment of insulin resistance; HOMA-%S = Homeostasis Model Assessment of insulin sensitivity, computer model; ISI = insulin sensitivity index; c = a constant derived from regression analysis of Ln(Ins0) *vs* Glucose 0; S_1 = insulin sensitivity index, based on minimal model of Bergman, ref (54); A = age; S = sex; BMI = Body mass index; E = ethnicity.

Table 1.2: Heritability estimates of insulin levels c.q.insulin response with respect to glucose level as surrogate measure of β-cell function

Test	1st Author	Reference no	Formula	Heritability %	covariates
Fasting blood levels	Katoh	(38)	HOMA- β = (20xIns0(mU/l)/Gluc0(mmol/l-3.5)	38	A, S
	Souren	(50)	HOMA- β	62	A, S, BMI
	Falci	(36)	HOMA2-%B	63	A, BMI
	Jenkins	(37)	HOMA- β'= (Ln(Ins0) – c)/Gluc0	68	women
	idem		Log (HOMA- β)	28	women
	Mills	(60)	HOMA%-B (computer model)	78	A, S, BMI
OGTT	Hanson	(57)	CIR_{120}=Ins $_{120}$/G_{120}x(G_{120}-70mgr/dl)	24	A, S, BMI
OGTT insulin secretion	Lehtovirta	(40)	OGTT β index computer model	53	
FSIVGTT+ tolbutamide	Elbein	(55)	Acute Insulin Response (2-10)	38	
	Hong	(59)	Acute Insulin Response (2-10)	46	A, S, BMI
	Lehtovirta	(39)	Acute Insulin Response (1-10)	55	S
			Acute Insulin Response (1-10), adjusted for isgu	41	S
	Lehtovirta	(39)	Acute Insulin Response (10-60)	58	S
	Lehtovirta	(40)	Readily Releasable Insulin	76	S

HOMA-β = Homeostasis Model Assessment of β-cell activity; A = age; S = sex; BMI = Body mass index; c = a constant derived from regression analysis of Ln(Ins0) *vs* Gluc0; HOMA2-%B = software (http://www.dtu.ox.ac.uk/homa) was used. OGTT = oral glucose tolerance test; OGTT β index: β-cell ability to increase insulin secretion in response to glucose; FSIVGTT = frequently sampled intra venous glucose tolerance test; Readily Releasable Insulin = the first peaking phase of insulin secretion during IVGTT, computer model. isgu = Insulin stimulated glucose uptake

Table 1.3: Range of Heritability estimates for clinical indicators of (pre)diabetic state in 27 twin and family studies

Test	Fasting glucose	OGTT Glu 120	Fasting insulin	OGTT Ins 120	HbA1c	D.I.	BMI
Heritabilities in % in twin studies	12 - 75	35 - 62	14 - 54	28 - 51	62	75 - 84	50 - 90
Reference no	(33;37-39;41-44;47;48;50)	(33;38;41;44;45;47)	(33;34;38;39;41-45;47;48;50;52)	(41)	(49)	(46)	(33;34;38;43-45;50;72)
Heritabilities in % family studies	7 - 77	17	8 - 51	35	55 - 60	23 - 67	20 - 80
Reference no	(53;56;58;60;61;63)	(53)	(53;54;57;58;61)	(57)	(60;63)	(55;59)	(44;53;58;61;63;72)

OGTT Glu 120 = Glucose concentration at 120 minutes during Oral Glucose Tolerance Test; OGTT Ins 120 = Insulin concentration at 120 minutes during Oral Glucose Tolerance Test; D.I.= Disposition index (Insulin secretion x insulin sensitivity); BMI = Body mass index.

genes affect β-cell function. A number of genetic loci have also been revealed for glucose and insulin metabolism as reviewed by Ingelsson (70). Nearly all these loci derive from studies that performed glucose and insulin measurements in the fasting state or during an OGTT.

Outline of the thesis

Despite impressive progress still much of the pathophysiology of type 2 diabetes mellitus is unknown. In part this reflects a poor understanding of the causes of interindividual differences in insulin production, even in healthy individuals. The twin-family study presented in this thesis focuses on the function of the healthy β-cell. Its aim is to reveal the genetic and environmental contribution to individual variation in different aspects of β-cell function and to associate the heritable aspects of β-cell function with candidate genotypes arising from ongoing GWA studies.

Chapter 2 details the design of the study, including the recruitment of the participants and a description of the tests of the β-cell function performed. In Chapter 3 we estimate the heritability of the main diagnostic parameters used in type 2 diabetes mellitus, fasting glucose and HbA1c, with special attention to a possible overlap in the genetic influences on these parameters. In chapter 4 the heritability of classical and mathematical model derived β-cell function parameters is estimated during a highly naturalistic challenge, the mixed meal test. This test includes the influence of incretins on the insulin secretion. In chapter 5 we present the use of the extended hyperglycaemic clamp to assess the heritability of insulin secretion after different intravenous secretagogues. A euglycaemic-hyperinsulinaemic clamp was performed in the same subjects to estimate the heritability of insulin sensitivity. Associations between selected genotypic variants from recent GWA studies and β-cell function are described in the last two chapters. Chapter 6 shows the association between eight type 2 diabetes mellitus related gene variants and the insulin response, stimulated by the three different secretagogues during hyperglycaemic clamps. To increase the power of this investigation, four different clamp studies were combined. In Chapter 7 we show that variation in several type 2 diabetes mellitus risk genes is associated with different aspects of β-cell function, assessed with the extended hyperglycaemic clamp tests.

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Chapter 2

Study Design and data collection

Study design and data collection

This study on the heritability of the betacel function started with a pilot study in 20 healthy male students (10 lean and 10 obese). We performed the designed meal and clamp tests twice in each participant of this group to assess the feasibility and reproducibility of these tests. Our conclusion was that the tests were well feasible and reproducible but the GLP-1 doses had to be reduced.

The actual twin/family study took place from September 2004 till the end of October 2007. It is a collaborative effort between the Diabetes Centre of the VU University Medical Centre and the Netherlands Twin Register (NTR), kept by the department of Biological Psychology at the VU University in Amsterdam (1). When the study was designed the adult NTR comprised more than 12,000 twins and 3,000 siblings. These subjects have indicated their willingness to be approached for participation for scientific research.

Participants

Between September 2004 and the end of 2006 154 families were selected from the NTR on the basis of the presence of a same-sex twin pair and one ore more same-sex siblings. The twins and their siblings were invited by mail to take part in our study. By personal referral, we came in contact with another 7 families, who then became member of the NTR and were also enrolled in the study. Additional inclusion criteria for participation in this study were: no known diabetes mellitus, Caucasian origin, good general health, minimum age for all participants 20 years. The maximum age for twins was 45 years; the same-sex sibling could have a maximal difference of five years with the twin. Of each family, a minimum of two persons (a twin pair or one of the twin pair and a same-sex sibling) had to participate. They all had to sign an informed consent. Exclusion criteria for participation were: metabolic disorders as diabetes mellitus, uncontrolled thyroid and/or adrenal disease; use of the following drugs: antiviral, corticosteroids, antihypertensive or other drugs that affect insulin secretion and/or insulin sensitivity; serious heart-, pulmonary-, interfering malignant- or haematological diseases; renal disease/impairment (creatinine >150 μmol/l) or hepatic disease (enzyme values > 3 x upper limit of normal). Women who were pregnant or intended to become pregnant within the study period were excluded as well as women in

the first six months after child birth. Participants should not have a serious mental impairment i.e. preventing to understand the study protocol.

The zygosity of the invited twin pairs was 108 MZ (45 male) and 46 DZ (21 male). Of the 154 families, 72 had one or more siblings, in total 92 sibs (50% male). Altogether 400 persons were approached. A few weeks after the families received the information about the study from the NTR, we tried to contact them by phone. After we gave more details about the study, 211 subjects from about half of the families decided not to participate, mostly because the lack of time, sometimes because of fear for needles or pregnancy (see table 2.1). A total of 77 twin families were successfully included in the study. The zygosity of all participating twins in this sample was determined by DNA polymorphisms and the twin pairs consisted of 51 MZ pairs (22 male) and 21 DZ pairs (7 male). In 5 other families only one of the twins of the pair participated (1MZM, 2DZM, 1MZF en 1DZF). An additional forty siblings (from 31 of the twin families) took part in the study (21 male). The mean age of the participating MZF twins was 2 years higher than that of the originally invited MZF twins and the mean age of the participating sibs was 5 years higher than that of the invited sibs. There was no significant difference in sex distribution between the participating subjects and the originally invited subjects or, for the twins, in their zygosity.

Table 2.1 Reasons of not participating in this study

Reason	number	%
No time or no desire to participate	97	45.5
Co twin and /or sibling does not participate	33	15.6
Does not like medical examination/ scared for needles	26	12.3
Parents or spouse did not want them to participate	12	5.7
Not of Caucasian origin	10	4.7
No contact possible	8	3.8
Living abroad	6	2.8
Bad health	6	2.8
Health problems in the family	4	1.9
Pregnancy	4	1.9
Living to far away to visit hospital	2	0.95
Participant had died since last contact	1	0.45
Does not want any further contact with the NTR	1	0.45
Meal test not completed	1	0.45
Total	211	99.75%

Oral glucose tolerance test

The study consisted of three separate test days. On the first test day, an Oral Glucose Tolerance Test (OGTT) was performed to be sure they had no latent diabetes mellitus. The OGTT was performed in 190 subjects at the home of the participants after a 12 hour overnight fast. Fasting and 2hr post load (75 gram glucose solution) capillary blood glucose was determined with a glucose dehydrogenase method (HemoCue glucose 201 Ängelholm, Sweden). During this test the subject also completed two questionnaires concerning general health, medical history, use of alcohol, drugs and medicines, sport activities, social economic state, family diseases and food habits. The fasting capillary blood glucose had to be less than 6.1 mmol/l and the 2hours post load blood glucose less than 11.0 mmol/l. Nobody had to be excluded because of the presence of diabetes mellitus.

Mixed meal test

On the second test day 190 participants came to the clinical research unit of the VU University Medical Centre after an overnight fast beginning at 20.00 hours the evening before, but one sibling discontinued the test before the meal was consumed. The meal test protocol is presented in table 2.2. A physical examination was first performed, including weight to the nearest 0.1 kg (in underwear, balance scale Seca, Nieuwegein, The Netherlands) and standing height to the nearest 0.1 cm (barefooted, mean of two measurements, LOG Harpenden fixed Stadiometer, Holtain Limited Crymych, Dyfed, Great Britain). BMI was calculated as weight (in kg) divided by the square of height (in m). Waist circumference was measured twice to the nearest 1 mm with a tape measure at the level midway between the lowest rib margin and the iliac crest, and hip circumference was measured twice with a tape measure at the widest level over the greater trochanters. Next the subject was placed in bed for physical examination, while the non-dominant hand was resting in a heating box (50 degrees Celsius) to warm for arterialised blood sampling. Subsequently we attached the Vrije Universiteit Ambulatory Monitoring System (VU-AMS) to the body of the participant (4 electrodes on the chest, 2 electrodes on the back, and attaching the device around the waist of the participant using a belt). The VU-AMS is a device to record at the same time electrocardiogram, impedance cardiogram, the thorax impedance and changes in impedance (2;3). Blood pressure and pulse measurements were the first time performed by hand (Speidel and Keller maxi stabile 3, Welch Allyn, Delft,

Table 2.2. Test protocol of the mixed meal test

Time	Study time	Procedure	Blood sampling							Pulse /blood pressure
			G	I	P	A	Gn	TG		
8.00	-30	Anthropometric measurements and physical examination								
8.10	-20	VU-AMS attached								x x
8.15	-15	i.v. cannula placed	x	x	x	x	x	x	F	
8.20	-10		x							x x
8.28	-2		x	x	x	x	x	x	D	
8.30	0	Meal started								
8.35	5									x x
8.40	10	Meal finished	x	x			x		D	
8.50	20		x	x	x		x			
9.00	30		x	x	x	x	x			
9.10	40		x	x			x			
9.20	50		x	x			x			
9.30	60		x	x		x	x	x		
10.00	90		x	x			x			
10.30	120		x	x	x	x	x	x		
11.00	150		x	x			x			
11.30	180		x	x		x	x	x		
12.00	210		x	x			x	x		
12.30	240		x	x	x	x	x	x	C	x x
12.35	245	i.v.cannula removed								
12.40	250	VU-AMS detached								

First pulse and blood pressure measurement by hand, following pulse and blood pressure measurements by automatic blood pressure meter, always performed in duple. The participants were confined to bed from the start of the physical examination to the detachment of the AMS device. G = glucose; I = insulin, C peptide and incretins; P = proinsulin; A = diabetes related hormones; Gn = glucagon; TG = triglyceride; D = DNA; F = haematology, liver and kidney functions, HbA1c, fat spectrum and DNA; C = CRP and TSH

The Netherlands) and later during the test with an automatic blood pressure meter (Dinamap procare 100, KP medical B.V., Houten, The Netherlands). All pulse and blood pressure measurements were performed in duple.

A cannula was retrogradely placed in a heated dorsal hand vein for blood sampling of fasting haematological, biochemical and hormonal values. Participants then received a precisely weighed meal. For men the meal consisted of 110 g brown bread, 20 g margarine, 25 g fat-rich cheese, 30 g jam, 19 g honey cake and 200 cc semi-skimmed milk (721 kcal,

89 g carbohydrates [50% energy], 30 g fat [37% energy] and 24.4 g protein [13% energy]). Women consumed 79% of that meal with the same proportions of nutrients and energy (570 kcal, 71 g carbohydrates, 23 g fat and 19.4 g protein). This difference in meal consumption between the sexes was not a confounding factor because all analyses were performed with same-sex pairs.

Before and during the meal and 240 minutes afterwards the venflon cannula was kept patent by flushing 2 cc of Saline (0.9 % NaCl) after every withdrawal. In total 14 times blood was sampled for blood glucose, insulin and C peptide. When taking blood, the first two ml were discarded to prevent dilution of the test sample with Saline. At some moments extra blood was sampled for proinsulin, incretins, other diabetes related hormones, CRP, TG and TSH. Four hours after the beginning of the meal, final blood sampling, and pulse and blood pressure measurement were performed. After the intra venous cannula was removed and the VU-AMS device was detached the participant could be mobilised.

Shortly after the first sampling the blood for haematological and biochemical analyses was transported to the clinical laboratory at the VU University Medical Centre for immediate assessment. Blood glucoses were assessed at bedside using a glucose oxidase method (YSI 2300 Stat plus, Yellow Springs, OH, USA). An EDTA sample was frozen at -80^0 Celsius and stored for future DNA extraction. Blood for incretins and glucagon were kept cool at 4 degrees Celsius. All blood was centrifuged (10 minutes, 3000 rounds, at 4^0 Celsius) and the serum was divided in micro tubes and stored at minus 80^0Celsius for later assessments.

Clamp tests

The third test day consisted of two parts, starting at 8.00 a.m. in the clinic after a 12 hour fast. One hundred and thirty persons were willing to participate in this demanding test day. First a euglycaemic-hyperinsulinaemic clamp was performed for an optimal assessment of the insulin sensitivity (4). After weight measurement (balance scale Seca, Schinkel, Nieuwegein, The Netherlands) the participant was confined to bed and the VU-AMS device was attached as on test day 2. Blood pressure and pulse measurements were again performed in duple at fixed intervals with an automatic blood pressure meter (Dinamap procare 100, KP medical B.V., Houten, The Netherlands).

One cannula was retrogradely placed in a heated dorsal hand vein to obtain arterialized blood. A second cannula was inserted into the antecubital vein of the arm for infusion of 0.9% saline, glucose 20% and insulin. After baseline samples for blood glucose, insulin and C-peptide levels were taken twice, a primed-continuous (first 4minutes 160 mU m^{-2} min^{-1}, min 4 to 7, 80 mU m^{-2} min^{-1}, min 7 to 120, 40 mU m^{-2} min^{-1}) insulin infusion (Velosuline/Actrapid, Novo Nordisk, Bagsvaer, Denmark in NaCl 0.9% with 2% albumin) was given for 120 minutes. Glucose 20% was infused at a variable rate to maintain the blood glucose at 0.3mmol/l below the fasting level and within the range of 4.5 – 5.5 mmol/l. Blood glucose was monitored at 5 minutes interval while blood samples for hormonal levels were obtained at 60, 90, 105 and 120 minutes.

One hour after the completion of the euglycaemic-hyperinsulinaemic clamp, the hyperglycaemic clamp was performed at 10 mmol/l according to Fritsche et al. (5) to assess the insulin secretion after different secretagogues. The blood glucose level was frequently monitored (at least every 5 minutes) and the infusion rate of glucose 20% accordingly adjusted. Blood samples for measurement of insulin and C-peptide were drawn at fixed time points: at t= -5 and t= -2 before the start of the hyperglycaemic clamp, after a bolus of each secretagogue every minute during 10 minutes and in between at 5 to 30 minutes interval. At some moments blood was also sampled for proinsulin and glucagon. At t=0 the subject received an intravenous bolus of glucose over 1 minute to acutely raise glucose level to 10 mmol/l. Two hours later (t=120) GLP-1 (7-36 Amide Human, Polypeptide Laboratories, Wolfenbuettel, Germany) was given as a bolus injection (1.5 pmol kg^{-1}) over 1 minute, followed by a continuous infusion of 0.5 pmol kg^{-1} min^{-1}. At t=180 a bolus of 5 gram arginine was injected over 50 seconds on top of the GLP-1 infusion. Twenty minutes after the arginine bolus, the GLP-1 infusion was terminated and the hyperglycaemic clamp finished. The glucose infusion was gradually decreased. After the last blood sampling for hormonal values, the VU-AMS recording was detached and a meal was offered to the participant, while monitoring of the blood glucose was continued. Once the blood glucose was stable, the infusions were removed and the participant could be mobilized. The exact sampling scheme for the various blood samplings and blood pressure measurements is given in table 2.3.

Table 2.3 Test protocol of euglycaemic-hyperinsulinaemic clamp and hyperglycaemic clamp

Time	Study time	Procedure	Blood sample	Pulse and Blood pressure	infusion			
					NaCl 0.9%	Gluc 20%	ins	GLP-1
8.05		Weight measured						
8.10	-40	VU-AMS attached						
8.30	-30	2 i.v. cannula placed	I, P, Gn		x			
8.40	-20				x			
8.50	-15		I, P, Gn	x x	x			
9.00	0	Euglycaemic hyper-insulinaemic clamp			x	x	x	
10.00	60		I		x	x	x	
10.30	90		I, P		x	x	x	
10.45	105		I		x	x	x	
11.00	120	end euglycaemic-hyperinsulin.clamp	I, P	x x	x	x	stop	
		rest			x	x		
11.45	-5		I, P, Gn	x x	x	x		
12.00	0	Hyperglycaemic clamp, Glucose bolus			x	x		
12.01 - 12.10	1-10	Blood sampling every minute*	I, P, Gn		x	x		
12.15	15		I		x	x		
12.30	30		I, P, Gn		x	x		
13.00	60		I, P, Gn		x	x		
13.20	80		I		x	x		
13.40	100		I		x	x		
13.55	115		I, P, Gn	x x	x	x		
14.00	120	GLP-1 bolus			x	x		x
14.01 - 14.10	121 - 130	Blood sampling every minute*	I, P, Gn		x	x		x
14.30	150		I, P, Gn		x	x		x
14.40	160		I		x	x		x
14.50	170		I		x	x		x
14.55	175		I, P, Gn	x x				
15.00	180	Arginine bolus			x	x		x
15.01 - 15.10	181 - 190	blood sampling every minute*	I, P, Gn		x	x		x
15.20	200	End hyperglycaemic clamp	I, P, Gn	x x	x	x		stop
15.30	210		I		x	x		
15.40	220		I		x	x		
15.45	225	VU-AMS detached			x	x		

Blood sampling: glucose measurement at least every 5 minutes during the whole test.
Participants were confined to bed from the time the VU-AMS was attached till after the hyperglycaemic clamp test was finished and the blood sugar was stable. Gluc = glucose intra venous infusion; ins = insulin intra venous infusion; I = insulin and C peptide; P = proinsulin; Gn = glucagon; *: every minute glucose, insulin and C-peptide; every 3 to 5 minutes proinsulin and glucagon.

Table2.4 gives an overview of all demographic, anthropometric, haematological, metabolic, cardiovascular and questionnaire variables collected in this study. In this thesis I will focus exclusively on the variables that are relevant to beta-cell function.

Statistical Analyses

All genetic analyses were carried out in Mx (6), a structural equation modelling program specifically designed for the analysis of twin and family data. The raw data option in Mx was used for uni- and multivariate analyses. At first it was confirmed that the variances of the variables were not significant different for twins and siblings, and that the covariances between DZ twins could be equated to those between a twin and a singleton sibling. The latter allowed us to treat all sibling pairs sharing 50% of their genetic material, whether DZ twin or twin-sibling pair, in the same way.

In the univariate analyses the within-variable cross-person correlations were assessed with age and sex as covariates. The MZ and DZ/sibling correlations describe the resemblance for a variable in MZ twins and in all other pairs of first-degree relatives (DZ twins, twin-sibling, sibling-sibling). These correlations form the basis to estimate the relative contribution of genetic and environmental factors to individual differences in each variable. MZ twin pairs have all, or nearly all, genes in common, and DZ twin pairs, twin-sibling pairs and sibling-sibling pairs share on average half of their segregating genes. With the structural equation modelling technique (SEM) the total phenotypic variance in an observed variable was decomposed in sources of variance: additive genetic A, the sum of affects of multiple alleles at different loci; dominance genetic D, when there is interaction between alleles at the same locus or across loci; environmental influence C, shared by members, growing up in the same family and environmental influences E, unique to each family member (7). Because C and D are confounded and cannot be estimated simultaneously in analyses of twin, reared together, the pattern of twin correlations is first used to choose for an ACE or an ADE model.

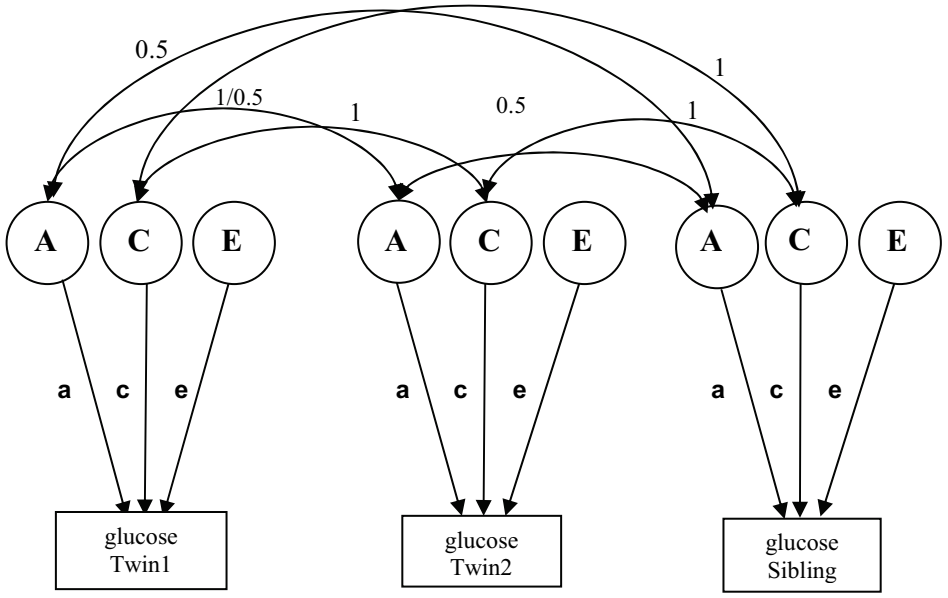

Figure 2.1: Univariate path diagram for a twin design with one additional sib
A = additive genetic, C = shared environmental E = non shared environmental influences to the trait. The factor loadings of these influences are a, c and e. In MZ twins the correlation of the additive genetic factors is 1.0, and 0.5 in DZ twins and between twins and siblings. The correlation of the shared environmental effects is 1.0 between twins and between twins and siblings. E is not correlated by definition.

If the MZ twin correlations are much higher than twice the DZ/twin-sib correlations (e.g. MZ = 0.7, DZ = 0.2) this suggests dominance and an ADE model is then the most likely model to fit the data. If the MZ twin correlations are much less than twice the DZ/twin-sib correlations (e.g. MZ =0.7, DZ = 0.55) this suggests shared environmental influences and an ACE model is then the most likely model to fit the data. Figure 2.1 depicts the ACE model for fasting glucose. The contributions of these A and C factors are all tested for significance using likelihood-ratio tests. The difference in minus two times the log-likelihood (-2LL) between two nested models (e.g. ACE and AE) has a χ^2 distribution. A corresponding p-value > 0.05 indicates that the more parsimonious model (AE) does not fit the data less well than the full model (ACE). This procedure is repeated for each variable to arrive at the most parsimonious model that fits the data. Under this model we estimated the heritability of each of relevant variables individually.

Multivariate analyses were performed to asses the phenotypic correlations between selected variables and to reveal the overlapping and separate genetic influences on these

variables. First the cross variable, within person correlations and then the cross variable cross person correlations in MZ and DZ/sibling pairs were estimated. When the cross-variable cross-person correlation is larger in MZ twin than in DZ/sibling pairs, this indicates that part of the association between the variables is explained by overlapping genetic factors. Next, a multivariate genetic ACE or ADE model was fitted to the data. The contributions of these factors were again tested for significance using likelihood-ratio tests. Under the most parsimonious model the heritability of each variable was estimated individually, and this heritability was decomposed into components that were specific to each variable and components that were overlapping two or more variables.

Table 2.4: Overview of all variables, collected in this study

Demographic variables		
Date of birth Gender Place of birth Family of origin Highest level of education Occupation Civil status		
Anthropometric Measures		Derived variables
Length (in duple) Weight (before meal and before and after clamp) Waist circumference (m in duple) Hip circumference (m in duple)		BMI, BSA Waist-to-hip ratio
Measures of the cardio-respiratory system		Derived variables
Electro cardiogram and Impedance Cardiogram during meal and clamp		Inter beat Interval (IBI) and heart rate /minute Pre ejection period (PEP) Left ventricular ejection time (LVET) Stroke volume (SV)
Respiration rate (RR)		Respiratory sinus arrhythmia (RSA)
SBP, DBP and pulse rate fasting in duple at two different test day's SBP, DBP and pulse rate in duple during meal SBP, DBP and pulse rate in duple after euglycaemic-hyperinsulinaemic clamp SBP, DBP and pulse rate in duple during hyperglycaemic clamp		
Laboratory measurements		
Haematology Biochemistry	hb, ht, ery, leuco, platelets Fasting HbA1c, ALAT, alk. phosphatise, γ-GT, Creatinine Fasting Total, HDL and LDL cholesterol, Free Fatty Acids, Triglycerides (fasting and 5 x during meal) C-reactive Protein 1 x end meal	
Hormones	Insulin) C-Peptide)	2 x fasting at 2 test day's, 12 x during meal, 4x during euglycaemic-hyperinsulinaemic clamp, 44 x during hyperglycaemic clamp
	Glucagon	2 x fasting at two test day's, 12 x during meal, 14 x during the clamp
	GIP	2 x fasting and 12 x during meal
	GLP-1	2 x fasting and 11 x during meal
	proinsulin	2 x fasting and 4 x during meal
	Adiponectine) Leptin) Ghreline) Resistin)	2 x fasting and 5 x during meal
	PYY	2 x fasting and 9 x during meal
	TSH	end meal
DNA		

Table 2.4 continued: Overview of all variables, collected in this study

Questionnaire Variables		
Health behaviour	**Health & disease**	**Food questionnaire**
Smoking	Use of medication /contraceptives	Food used the evening before the tests
Exercise	Family history of diabetes mellitus, obesitas, cardio- vascular disease or cerebro- vascular disease	use of caffeine, alcohol, fish and fibres

SBP: systolic blood pressure; DBP = diastolic blood pressure; BSA = Body Surface Area

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Chapter 3

The heritability of HbA1c and fasting blood glucose in different measurement settings

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Twin Research and Human Genetics, 2008, number 6: 597-602

Abstract

In an extended twin study we estimated the heritability of fasting HbA1c and blood glucose levels. Blood glucose was assessed in different settings (at home and in the clinic). We tested whether the genetic factors influencing fasting blood glucose levels overlapped with those influencing HbA1c and whether the same genetic factors were expressed across different settings. Fasting blood glucose was measured at home and during two visits to the clinic in 77 healthy families with same-sex twins and siblings, aged 20-45 years. HbA1c was measured during the first clinic visit. A 4-variate genetic structural equation model was used that estimated the heritability of each trait and the genetic correlations among traits.

Heritability explained 75% of the variance in HbA1c. The heritability of fasting blood glucose was estimated at 66% at home and lower in the clinic (57% and 38%). Fasting blood glucose levels were significantly correlated across settings ($0.34 < r < 0.54$), mostly due to a common set of genes that explained between 53% and 95% of these correlations. Correlations between HbA1c and fasting blood glucoses were low ($0.11 < r < 0.23$) and genetic factors influencing HbA1c and fasting glucose were uncorrelated. These results suggest that in healthy adults the genes influencing HbA1c and fasting blood glucose reflect different aspects of the glucose metabolism. As a consequence these two glycaemic parameters can not be used interchangeably in diagnostic procedures or in studies attempting to find genes for diabetes mellitus. Both contribute unique (genetic) information.

Introduction

World wide major efforts are ongoing to identify genetic variation underlying type 2 diabetes mellitus, one of the fastest growing threats to health (1;2). To establish the diagnosis of type 2 diabetes mellitus in the early stage, clinicians often rely on measurement of fasting blood glucose (FBG) (3) although in some countries HbA1c levels are used instead (4). During the course of the disease, type 2 diabetic patients may control their glucose homeostasis by measuring their fasting blood glucose, whereas health professionals mainly use HbA1c to monitor long-term glycaemia (5;6). Taken the heritability of type 2 diabetes mellitus (7) it is likely that both these indicators are themselves heritable.

Heritability of FBG has indeed been well-established, but the existing family and twin studies show large variation in the estimated contribution of genetic factors. A high heritability (77%) for FBG was reported in non diabetic first-degree relatives of type 2 diabetic patients (8). In contrast a mere heritability of 21% for the same variable was found in a large community based study of healthy families (9). The lowest genetic influence on the variability of FBG was found by Schousboe (10). Heritability in adult non-diabetic females was only 12%, although somewhat higher estimates were found for males (38%). Other twin studies in Western European populations showed heritabilities ranging from 38 to 67% (11-15).

A possible explanation for the discrepant heritability estimates for FBG is the potential influence of measurement setting on the relative contribution of genes and environment to FBG levels. Often the dietary state of the study participants is well-controlled, but blood glucose levels may be sensitive to many other behavioural factors like recent physical activity, psychological expectation, and degree of adaptation to blood letting procedures. These factors may be determined in part by the setting of blood letting. Collecting blood during a home visit, for instance, may lead to quite different behavioural antecedents than an active visit of the participant to a clinic. As part of an extended twin study addressing the genetic and environmental contribution to the variance of the beta-cell function in Dutch twin families, the first aim of the present study was to estimate the contribution of genes to the variance of FBG, obtained in different measurement settings, including a home visit and two visits to the clinic.

Despite its frequent use as an indicator of long-term glycaemic control and its established relation to diabetic complications (5;6) the genetics of HbA1c has been much less studied than that of FBG. Only two studies have reported heritability estimates. Snieder (16) found a heritability of 62% in healthy (only female) twins and an important contribution of age (14%). The heritability of HbA1c in non-diabetic first-degree relatives of type 2 diabetic patients was estimated at 55% (8). No heritability studies of HbA1c in male twins are known. A second aim of the present study was to estimate the heritability of HbA1c in both sexes.

Based on the idea that FBG and HbA1c are used interchangeably in the diagnosis and monitoring of diabetes mellitus it is expected that the genes influencing FBG and HbA1c should be largely overlapping. This has important consequences for large scale gene finding efforts, that could then pool samples using either one of these quantitative endophenotypes for diabetes mellitus risk. As a third aim, the present study provides a test of the expectation that the genes influencing FBG and HbA1c are largely overlapping.

Materials and Methods

Study population

Between September 2004 and December 2006 the Netherlands Twin Register (17) invited 154 twin families by mail to participate in a study addressing various aspects of beta-cell function, according to the following inclusion criteria: Caucasian origin, good general health, aged 20-45 years, and having a sibling in the family of the same-sex as the twin pair with a maximum age difference of 5 years. Exclusion criteria were diabetes mellitus, other relevant metabolic disorders, use of drugs that affect insulin secretion and/or insulin sensitivity, pregnancy and the first 6 months after childbirth. A minimum of two persons of one family (including one of the twins) was required. The twin-sibling design offers the opportunity to distinguish genetic and environmental sources of variation based on a comparison of the resemblance in family members of different genetic relatedness (18). Including an additional sibling to the classical twin design significantly increases the power to detect the sources of variation (19).

The study protocol consisted of one home screening visit to exclude diabetes mellitus by a 75-g oral glucose tolerance test (OGTT) and one visit to the clinical research

unit; a second visit to the research unit was optional. The procedures during the respective visits are outlined below. Fifty percent of the invited families agreed to participate. Reasons for non-participating included the lack of time (45%), only one member of the family wanted to participate (16%) and fear of needles (13%).

The 77 twin families included consisted of: 51 MZ pairs (22 male) and 21 DZ pairs (7 male). There were 2 MZ (1 male) and 3 DZ (2 male) incomplete twin pairs. Thirty one siblings took part in this study (15 male**).** FBG results of the optional second visit to the clinic were obtained for 123 subjects (57 male) of 54 families, comprising 34 MZ pairs (15 male), 13 DZ pairs (6 male) and 7 incomplete twin pairs (3 male) and 22 sibs (12 male). The two groups were comparable in zygosity, sex and BMI, but the group that also participated in the second visit was 1.5 years younger (P = 0.043). Twin zygosity was determined from DNA polymorphisms (20).

All subjects gave written informed consent. The study was approved by the local Ethics Committee and performed in accordance with the Declaration of Helsinki (21).

Measurements

During the screening visit at home an OGTT was performed. At all test occasions participants were instructed to fast overnight during 12- hours prior to the visit and to refrain from heavy physical exercise, alcohol use and smoking. The OGTT was started between 8.00 and 10.00 am. Fasting and 2-h post-load capillary blood was obtained and analyzed by a glucose dehydrogenase method (HemoCue 201+, Ängelstrom, Sweden) for glucose level (FGBG-O). All measurements were below diabetic levels (FBG-O < 6.1, 2-h blood glucose < 11.1 mmol/l)

After a median period of 33 days participants arrived at the clinical research unit at 8:00 a.m. to undergo a meal test. First, weight (balance scale Seca, The Netherlands), height (LOG Harpenden fixed Stadiometer, Great Britain) and waist- and hip circumference were measured. Second, subjects assumed a semi-recumbent position with their non-dominant hand resting in a heating box (50^0 Celsius) to obtain arterialized blood from a dorsal hand vein for measurement of among others fasting blood glucose and HbA1c. Whereas the 3cc test tube (containing Potassium EDTA (7.5%, 0.072 ml)) for HbA1c determination was immediately transported to the clinical chemistry department (see below), fasting blood glucose was assessed at bedside using a glucose oxidase method (YSI

2300 Stat plus, Yellow Springs, OH, USA). At an interval of 5 minutes, a second blood sample was taken for baseline fasting glucose (FBG-M) and hormonal levels. After the initial blood sampling the meal-test was started.

Sixty-nine percent of the participants returned to the clinic after a median period of 43 days at 8:00 a.m. for an optional (combined euglycaemic-hyperinsulinaemic and hyperglycaemic) clamp test. As before, two arterialized blood samples were drawn from the dorsal hand vein for measurement of fasting glucose (FBG-C) and hormonal levels at a 5 minute interval. After this, the clamp test was started.

Laboratory analysis

Analyses of HbA1c were performed at the VU University Medical Centre (department of Clinical Chemistry), Amsterdam, The Netherlands, using a DCCT standardized reversed-phase cation exchange chromatography (HA 8160 analyzer, Menarini, Florence, Italy). The HbA1c is detected by a dual-wavelength colorimetric (415-500). The intra-assay coefficient of variation (CV) is 0.6% at a mean of 4.9% and the inter-assay CV is 0.8 % at a mean of 5.5%. The HemoCue method has a CV of 1.5-2.5 % and correlates strongly with the YSI ($r = 0.978$) (22). The YSI has a within run CV of 2 % and a day-to-day CV of 6% (23). The two repeated measurements of FBG at each of the clinic visits showed strong test-retest correlations across the 5-minute intervals ($0.90 < r < 0.93$) and the mean value across the two measurements was used in all FBG analyses.

Data Analyses

Structural equation modelling was carried out in Mx (24). In a first step, a 4-variate unconstraint model was used to estimate means, variances and regression coefficients for covariates sex and age for each phenotype (HbA1c, FBG-O, FBG-M, FBG-C). Estimates of within trait and cross-traits correlations for MZ, DZ and twin-sib pairs were also obtained from this model. In the 4-variate analysis the following tests were carried out: 1) test of equality of means and variances for MZ and DZ twins and siblings 2) equality of covariances for DZ twins and siblings and 3) test of significance of age and sex regressions on the means. Likelihood-ratio tests were employed to identify the best model for the 4-variate data. MZ and DZ twin and twin-sib correlations, within person correlations between traits and cross-twin cross-trait correlations (e.g. between HbA1c level of the oldest twin

and FBG-O level of the youngest twin) were estimated in the most parsimonious model. Next, a genetic triangular decomposition was fitted to the data (Fig. 3.1). An ACE model consisting of Additive genetic, Common environmental and unique Environmental factors were used. The raw data option in Mx was used and the influence of covariates sex and age was incorporated as fixed effects on the mean.

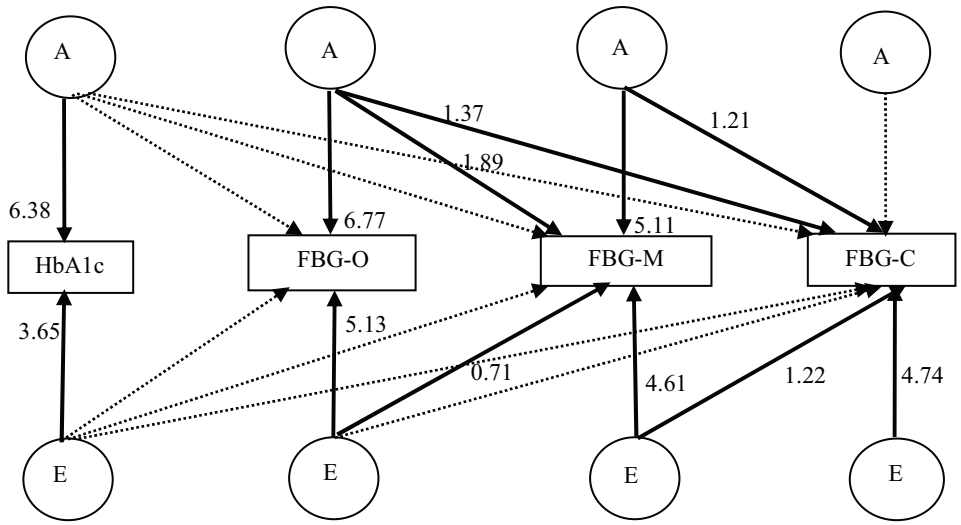

Figure 3.1.Genetic model for HbA1c and three FBG measures with factor loadings of observed variables on the latent Additive genetic and unique Environmental factors. FBG-O = fasting blood glucose at home; FBG-M = fasting blood glucose pre-meal; FBG-C = fasting blood glucose pre-clamp. Bold line = significant; dotted line = non-significant.

Results

FBG and HbA1c results on the OGTT and mixed meal test were obtained for 180 subjects (76 male) from 77 twin families, from which 51 MZ pairs and 60 DZ/sibling pairs (21 DZ) could be formed. FBG results on the optional clamp test were obtained for 123 subjects (57 male) from 54 twin families, from which 33 MZ pairs and 40 DZ/sibling pairs (14 DZ)

could be formed. Table 3.1 lists the mean values of the glycaemic parameters separately for men and women.

Table 3.1. Maximum likelihood estimates of means and standard deviations (SD)

variable	Mean male	Mean female	SD
Age (years)	30.32	30.84	4.63
HbA1c (%)	5.29	5.20	0.25
FBG-O (mmol/l)	4.71	4.56	0.45
FBG-M (mmol/l)	4.53	4.27	0.37
FBG-C (mmol/l)	4.61	4.29	0.31

FBG-O = fasting blood glucose at home; FBG-M = fasting blood glucose before meal; FBG-C = fasting blood glucose before clamp.

There was a significant sex effect on FBG in the hospital settings ($p<0.001$), with men having higher values than women. Age had a significant positive influence on HbA1c (β = 0.015 ; p 0.003) and FBG-C (β = 0.014 ; p 0.032).

The phenotypic correlations between the FBG levels in different settings were significant, albeit to a modest extent: r=0.49 (CI = 0.35 - 0.61) between FBG-O and FBG-M, r=0.34 (CI = 0.16 - 0.49) between FBG-O and FBG-C and r= 0.54 (CI = 0.39 - 0.67) between FBG levels in the two clinical settings. In contrast, the correlations for HbA1c with FBG-O (r=0.11, CI =-0.06 - 0.23), FBG-M (r=0.15, CI = -0.02 – 0.31) and FBG-C (r=0.23, CI = 0.05 - 0.41) were low and achieved significance only for FBG-C.

Table 3.2 shows the MZ and DZ/Sibling correlations with the 95% confidence intervals on the diagonal. MZ twin pairs resembled each other more strongly than the same-sex DZ twin and sibling pairs for all indicators of glycaemia, except for the FBG-C. The lower part of Table 2 gives the cross-trait cross-twin correlations. For the various FBG measurements these cross-trait correlations were generally higher in MZ pairs than in DZ pairs, suggesting that genetic factors contribute to the correlation between FBG in the three different settings. No shared genetic contribution is evident for HbA1c and FBG in any setting.

Table 3.2

MZ and DZ / sibling correlations and cross-trait correlations (95% confidence interval).

	HbA1c	FBG-O	FBG-M	FBG-C
MZ				
HbA1c	0.75 (0.61 to 0.84)			
FBG-O	0.14 (-0.05 to 0.32)	0.63 (0.42 to 0.76)		
FBG-M	0.12 (-0.06 to 0.30)	0.36 (0.19 to 0.51)	0.56 (0.37 to 0.70)	
FBG-C	0.16 (-0.04 to 0.35)	0.27 (0.06 to 0.46)	0.24 (0.02 to 0.44)	0.35 (0.05 to 0.58)
DZ /sibling				
HbA1c	0.47 (0.22 to 0.65)			
FBG-O	0.08 (-0.12 to 0.65)	0.53 (0.31 to 0.69)		
FBG-M	0.07 (-0.13 to 0.26)	0.31 (0.12 to 0.47)	0.37 (0.12 to0.56)	
FBG-C	0.18 (-0.03 to 0.37)	0.26 (0.05 to 0.45)	0.32 (0.10 to0.56)	0.39 (0.11 to 0.60)

FBG-O = fasting blood glucose at home; FBG-M = fasting blood glucose before meal; FBG-C = fasting blood glucose before clamp.
Twin correlations on the diagonal; cross-trait cross-twin correlations off-diagonal.

Table 3.3

Model fitting results for multivariate analyses of FBG in different settings and HbA1c

Test	model	-2LL	df	vs.	Δdf	Δ X^2	*P*
1	ACE	4.325.946	623				
2	CE	4.340.964	633	1	10	15.018	0.131
3	AE	4.333.074	633	1	10	7.128	0.713
4	E	4.455.775	643	1	20	129.829	<0.001
5	**AE, no non-significant parameters**	**4.340.422**	**640**	**3**	**7**	**7.348**	**0.394**

FBG= fasting blood glucose; -2LL = -2 log likelihood; df = degrees of freedom; vs .= compared to model; A = additive genetic influences; C = shared environmental; E = non shared environment. Δ = difference ; Significant age and sex covariates are included in all models. Preferred model in bold.

Table 3.3 shows the model fitting results, starting with the full ACE model and ending in the most parsimonious AE model. Figure 3.1 illustrates this final model and presents the factor loadings of the observed variables on the different latent factors. This model resulted in heritability estimates of 66% (CI = 50 – 77%) for FBG obtained during the test at home, 57% (CI = 40 – 71%) for FBG determined before the meal test and 38% (CI = 11-58%) for FBG measured during the pre-clamp baseline condition. Heritability of HbA1c was 75% (CI = 62 – 84%). The model showed that correlation of FBG across the three different settings was due to shared genetic as well as unique environmental influences (bold arrows). However, the contribution of the genetic factors was most striking, accounting for 78% of the covariance between FBG-O and FBG-M, 95% for FBG-O and FBG-C and 53% for FBG-M and FBG-C respectively. In keeping with the low phenotypic correlations, no significant genetic or environmental correlations were found between HbA1c and the FBG in any of the three settings.

Discussion

The present study shows substantial contribution of genetic influences to the variance in fasting blood glucose levels although heritability estimates varied across different measurement settings. The highest heritability estimate (66%) was found in the most natural environment, when FBG was assessed at home. Comparable heritability (57%) was found in the clinic during the pre-meal test. The lowest heritability (38%) was found on the final and most demanding test day, obtained pre-clamp when subjects knew they had to undergo invasive tests during the whole day. These different heritability estimates across settings may account for part of the discrepancy in heritability estimates found in the literature.

Inspection of the estimates of the variance components showed that the lower heritability of FBG on the pre-clamp assessment was mainly caused by differences in genetic variances in the three settings, whereas estimates of environmental variances were largely similar. We cannot rule out, however, that these 'setting' differences simply reflect the substantial day-to-day variation reported for FBG (25). Such day to day variation is also evident in the modest phenotypic correlations between the repeated measurements of FBG across the three different settings ($0.34 < r < 0.54$). Importantly, the stable part of the individual differences in FBG across settings could be largely attributed to common genetic factors that influence FBG irrespective of the setting of the blood collection. Large collaborative gene finding efforts that pool FBG samples across many different studies and countries have tacitly assumed that the exact setting in which FBG was obtained (clinic vs home; OGTT , meal or clamp studies) should not matter. The data from the present study confirm that such gene-finding efforts may safely pool samples from different settings.

Heritability of HbA1c was estimated at 75%, which is higher than reported by two previous studies (8;16). Because FBG and HbA1c are used interchangeably in the diagnosis and monitoring of diabetes mellitus it was expected that the genes influencing FBG and HbA1c should be largely overlapping. This expectation was not confirmed. Phenotypic correlations between HbA1c and FBG were either small or non-significant and no evidence was found for common genetic factors influencing FBG and HbA1c.

The small correlation among FBG and HbA1c and the lack of common genetic influences are in line with the study of Monnier (26) that showed only modest contribution

of fasting glucose levels to the variance of HbA1c. On the contrary, the correlation between mean blood glucose (measured by continuous glucose monitoring over the preceding 12 weeks) and HbA1c is much higher reaching up to a correlation of 0.9 (27). This suggests that non-fasting glucose levels are important determinants of HbA1c, allowing genetic factors influencing dietary habits, and behavioural and physical activity patterns to enter into the heritability of HbA1c In addition, non-glucose related factors may contribute to the heritability of HbA1c as there are substantial individual differences in glycation rate and intra-erythrocyte metabolism (28-30). Importantly, a recent Japanese study (31) suggests that both HbA1c and FBG contribute information on diabetes mellitus risk. HbA1c and fasting plasma glucose independently predicted the progression to diabetes mellitus in a healthy population, particularly when the FBG was ≥ 5.55 mmol/l.

In summary, the results of the present study suggest that in healthy adults the genes influencing FBG in different settings are largely overlapping. HbA1c and FBG, however, reflect different aspects of the genetics of glucose metabolism. As a consequence, these two glycaemic parameters can not be used interchangeably in diagnostic procedures or in studies attempting to find genes for diabetes mellitus. Both contribute unique (genetic) information.

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Chapter 4

The heritability of β-cell function parameters in a mixed meal test design

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Submitted

Abstract

Aims

Heritability of the individual differences in β-cell function has been estimated in different research settings, but never with the most physiological challenge: the mixed meal test. Here we assessed the heritability of classical and model-derived β-cell function parameters in a mixed meal test design.

Methods

A total of 183 healthy subjects (77 male), recruited from the Netherlands Twin Register, underwent a 4-h mixed meal test. Participants were same-sex twin pairs and their siblings, aged 20-50 years and of European origin. Insulin sensitivity, insulinogenic index, insulin response and post-prandial glycemia were assessed as well as model derived parameters of beta-cell function, in particular β-cell glucose sensitivity and insulin secretion rates. Univariate genetic analyses were used to estimate the heritability of each variable. Multivariate analyses were performed to test overlap in the genetic factors influencing β-cell function, waist circumference and insulin sensitivity.

Results

Significant heritabilities were found for insulinogenic index (63%), β-cell glucose sensitivity (50%), insulin secretion during the first 2 post-prandial hours (42-47%) and post-prandial glycemia (43-52%). Genetic factors influencing β-cell glucose sensitivity and insulin secretion during the first 30 post-prandial minutes showed only negligible overlap with those influencing waist circumference and insulin sensitivity.

Conclusions

The highest heritability for postprandial β-cell function was found for the classical insulinogenic index, but the most specific indices of the heritability of β-cell function appeared to be model-derived β-cell glucose sensitivity and the incremental ISR during the first 30 minutes after a mixed meal.

Introduction

The pathophysiology of Type 2 diabetes is characterized by insulin resistance, but the development of hyperglycaemia is mainly determined by deterioration of β-cell function (1). Twin and family studies have confirmed a clear cut genetic contribution to type 2 diabetes (2-4). Experimental probing of β-cell function by the intravenous glucose tolerance test (3) and the hyperglycaemic clamp test (5) revealed significant heritability of insulin response after different intravenous secretagogues. Recent genome wide association (GWA) studies have uncovered 26 confirmed gene variants that are associated with a higher risk for the development of type 2 diabetes (6;7) and at least fifteen of these genes affect β-cell function.

Previous findings regarding β-cell function have mostly been based on the calculation of surrogate indices of insulin secretion including fasting- and oral glucose tolerance test (OGTT)-derived plasma insulin and C-peptide levels. The validity of these measures can be expected to be different from the response to a real physiological assessment of the β-cell function as provided by the mixed meal test. This test combines the effect of different natural secretagogues (carbohydrates, proteins and fatty acids) with their contributing effects on gut function, including secretion of incretins and neural signals. To the best of our knowledge no previous study has specifically assessed the heritability of mixed meal test parameters.

The aim of this twin family study is to explore the heritability of classical and model derived β-cell function parameters and of postprandial glycemia indices obtained from a mixed meal test. We used a mathematical model (8;9) that clearly represents different aspects of β-cell function and is frequently applied in intervention studies. The main parameters are insulin secretion rate (ISR), calculated by means of deconvolution of C-peptide levels (10) and β-cell glucose sensitivity, representing the dose-response relation between insulin secretion and glucose concentration. As β-cell function is closely associated with (abdominal) overweight and insulin sensitivity, waist circumference (11) and Oral Glucose Insulin Sensitivity (OGIS)(12), were measured simultaneously. This allowed us to test to which extent genetic factors influencing the most important markers of postprandial insulin secretion (ISR, β-cell glucose sensitivity and insulinogenic index) overlap with those influencing waist circumference and insulin sensitivity.

Research Design and Methods

Participants

This study used a twin/same-sex sibling design to address genetic and environmental contribution to the variance of β-cell function in Dutch twin families recruited from the Netherlands Twin Register (13) as described previously (14). Mixed meal tests were performed in 183 (77 male) healthy participants of European origin, aged 20 to 49 years. In the weeks prior to these mixed meal tests, the presence of diabetes mellitus was excluded on the basis of a 75 g OGTT. There were 51 MZ twin pairs and 21 same-sex DZ twin pairs from 72 families. Fifteen MZ twin pairs and 8 DZ twin pairs had one additional same-sex sibling. Two MZ twin pairs and one DZ twin pair had 2 additional same-sex siblings. In five more families only one twin of the pair participated together with a same-sex sibling. In total 149 twins and 34 siblings participated. Twin zygosity was determined from DNA polymorphisms. In total 68 dizygotic/sibling pairs could be formed. The mean age difference between twins and their siblings was 3.2 years with a range of 1 to 9 years. Including additional siblings in the classical twin design significantly increases the power to detect the genetic and environmental sources of variation (15). All participants gave written informed consent. The study was approved by the local Ethics Committee and performed in accordance with the Declaration of Helsinki.

Meal test

The nutrient composition of the test meal was calculated from the Dutch Food Composition Table (16) and each portion of the ingredients was weighed before the meal was prepared. The meal for men consisted of 110 g brown bread, 20 g margarine, 25 g fat-rich cheese, 30 g jam, 19 g honey cake and 200cc semi-skimmed milk (721 kcal, 89 g carbohydrates [50% energy], 30 g fat [37% energy] and 24.4 g protein [13% energy]). Women consumed 79% of that meal with the same proportions of nutrients and energy (570 kcal, 71 g carbohydrates, 23 g fat and 19.4 g protein).

After a 12 h fast the mixed meal test procedure was started at the research unit at 08.00 hour. Anthropometric measurements were performed as described previously (5). A cannula was placed retrogradely in a heated dorsal hand vein to obtain arterialized blood. After baseline samples had been taken twice, the test meal was consumed between t=0 and t=10 min. Blood samples for glucose and hormonal levels were drawn at t=10, 20, 30, 40,

50, 60, 90, 120, 150, 180, 210, and 240 min. During the test the participants were confined to bed and were, besides the test meal, only allowed to consume water.

Laboratory analysis

Blood glucose was assessed at bedside using a glucose oxidase method (YSI 2300 Stat plus; Yellow Springs Instruments, Yellow Springs, OH, USA). This device has a within run CV of 2% and a day- to-day CV of 6%. Blood for hormonal levels was centrifuged (1,469 g) at 4^0 C and the serum stored at -80^0 C. All serum specimens were assessed for insulin and C-peptide levels at the VU University Medical Centre (Department of Clinical Chemistry, Amsterdam, The Netherlands) using an immunometric assay luminescence method (ACS: Centaur; Bayer Diagnostics, Mijdrecht, The Netherlands). There was no cross-reactivity with proinsulin or split products. The inter assay CV of insulin and C-peptide measurement was 6.5% and 6% respectively. The intra-assay CV was 4% for both.

Classical β-cell function parameters

Fasting and 2-h insulin levels and the insulin IAUC (incremental area under the curve: area under the curve by the trapezium rule minus the fasting level) during the entire 4-h test were measured. The glucose level at 30 min minus fasting level, the glucose level at 120 min and the glucose IAUC during the period from 0 to 120 min and. during the entire 4-h test were analyzed. As estimate of early insulin response the insulinogenic index (insulin level t30-t0/glucose level t30-t0) was calculated.

Model based β-cell function parameters

ISR and model based β-cell function parameters were calculated using a mathematical model developed by Mari et al.(8;9) with ISR calculated by means of deconvolution of C-peptide level (10). The fasting ISR, the ISR at 4.5 mmol glucose/l and the integral of incremental ISR during three periods: from 0 to 30 min, from 30 to 120 min and during the entire 4-h test were used.

In this model the ISR is composed by the sum of two components that is $P(t) f(G) + S_d(t)$, which account for different aspects of the β-cell function. $f(G)$ is the dose response that represents the static relationship between insulin and glucose concentration during the test. The average slope of this dose-response is denoted as β-cell glucose sensitivity. From

the dose response, insulin secretion at a glucose concentration of 4.5 mmol/l (approximately fasting glucose concentration in the whole group) was also calculated. P(t) is the potentiation factor which modulates this dose-response relation and has been constrained to have a time average of 1 during the experimental test. It represents a relative potentiation. The second component of insulin secretion ($S_d(t)$) represents the enhancement of insulin secretion proportional to the rate of rising of the plasma glucose concentration and is denoted as rate sensitivity, accounting for an initial fast rise in insulin secretion. Of this model we analyzed rate sensitivity, β-cell glucose sensitivity and the excursions of the potentiation factor using ratios between mean values at different time intervals (e.g. P (t100-t120)/P(t0-t20) and P(t220-t240)/P(t0-t20).

Insulin sensitivity

Insulin sensitivity (OGIS) was estimated using the meal carbohydrate dose and glucose and insulin levels during the first two hours of the meal test (12).

Statistical analysis

All genetic analyses were carried out in Mx (17), a structural equation modelling program specifically designed for the analysis of twin and family data. In the univariate analyses raw data were used while in the multivariate analyses all variables (waist circumference, OGIS, β-cell glucose sensitivity, insulinogenic index, ISR (0-30) and ISR (30-120) were Z transformed, prior to analysis (mean=0, SD=1) to reduce the large differences in variance across the variables. This transformation does not affect the estimates of familial correlations or heritabilities.

At first, we confirmed that the variances of the variables were comparable for twins and siblings, and that the covariances between DZ twins could be equated to those between a twin and a singleton sibling. The latter allowed us to treat all sibling pairs sharing 50% of their genetic material, whether DZ twin or twin-sibling pair, in the same way.

In the univariate analyses the within-variable cross-person correlations were assessed with age and sex as covariates. The MZ and DZ/sibling correlations describe the resemblance for a variable in MZ twins and in all other pairs of first-degree relatives (DZ twins, twin-sibling, sibling-sibling). These correlations form the basis to estimate the relative contribution of genetic and environmental factors to individual differences in each

variable. MZ twin pairs have all, or nearly all, genes in common, and DZ twin pairs, twin-sibling pairs and sibling-sibling pairs share on average half of their segregating genes. If MZ correlations are twice the DZ/sibling correlations or larger, genetic influences are suggested as the main source of familial resemblance. These genetic influences can be additive (A) or can act in a non-additive manner (D). If the MZ correlations are less than twice the DZ/sibling correlations, common environmental (C) influences shared by family members are suggested in addition to genetic influences. The remaining source of individual differences in the variable are unique environmental influences (E) including measurement error.

In a genetic univariate model (18) the total variance was decomposed into A, C or D, and E components for each trait. We tested if the contributions of these A, C and D factors were all significant using likelihood-ratio tests. The difference in minus two times the log-likelihood (-2LL) between two nested models has a χ^2 distribution. A corresponding p-value > 0.05 indicated that the more parsimonious model did not fit the data less well than the full model. This procedure was repeated for each variable to arrive at the most parsimonious model that fitted the data. Under this model we estimated the heritability of each variable individually.

Multivariate analyses were performed to asses the phenotypic correlations between selected variables and to reveal the overlapping and separate genetic influences on these variables. The three sets of variables selected were: 1) waist circumference, OGIS and β-cell glucose sensitivity, 2) waist circumference, OGIS, and the insulinogenic index, 3) waist circumference, OGIS, ISR (0-30) and ISR (30-120). All multivariate analyses were performed following the same procedure, based on the results of the univariate analyses. First we estimated the cross variable, within person correlations (e.g. the phenotypic correlations among waist circumference, OGIS and β-cell glucose sensitivity) and then the cross variable cross person correlations in MZ and DZ/sibling pairs for example between the waist circumference of a twin and the OGIS of her co-twin. When the cross-variable cross-person correlation is larger in MZ twin than in DZ/sibling pairs, this indicates that part of the association between the variables is explained by overlapping genetic factors.

Next, a multivariate genetic ACE model was fitted to the data. For instance the full 3 variate models included a set of 3 factors for A, C and E. The first factor influences all 3 variables; the next factor influences 2 variables and the last factor only one variable. We tested if the contributions of these A and C factors were all significant using likelihood-

ratio tests. Next the paths of each A and E factor to the 3 variables were tested for significance (figure 4.1 illustrates this for waist circumference, OGIS and β-cell glucose sensitivity). Under this model we estimated the heritability of each variable individually, and decomposed this heritability into components that are specific to each variable (e.g. waist circumference, OGIS and β-cell glucose sensitivity) and components that overlap (e.g. β-cell glucose sensitivity with waist circumference and OGIS).

Due to the statistical procedure, there may be slight differences in heritability estimates between the univariate and multivariate analyses.

Results

Sample characteristics are shown in Table 4.1. Waist circumference, weight, β-cell glucose sensitivity, ISR during the first 30 minutes and the insulinogenic index were significantly larger in men; OGIS, ISR at 4.5mmol/l glucose and the IAUC of glucose during the first 2 hours and during the total 4-h mixed meal test were significantly larger in women. The influence of age was significant for waist circumference (β = +0.51, p=0.003), ISR during the period 30 to 120 min (β = +0.31, p=0.035) and OGIS (β = -2.6, p=0.003).

MZ and DZ/sib correlations are shown in Table 4.2, as well as the univariate estimates of A, C and E under the most parsimonious genetic model. Significant heritability was found for 13 variables, and significant contribution of shared environmental factors for 4 variables. No significant family resemblance was found for the potentiation factor ratios and rate sensitivity.

There were significant phenotypic correlations between β-cell glucose sensitivity and waist circumference (r =0.21, p=0.01) and between β-cell glucose sensitivity and OGIS (r =-0.33, p=<0.01). Figure 4.1 illustrates the most parsimonious AE model resulting from this 3 variate analysis.

Table 4.1. Sample Characteristics

Variable	total	male	female
Number	183	77	106
Age (years)	31.0 ± 5.1	30.7 ± 4.8	31.3 ± 5.3
Waist circumference (cm)	84.7 ± 9.8	87.4 ± 8**	82.8 ± 10.3
Weight (kg)	72.7 ± 11.4	79.0 ± 9**	68.2 ± 10.6
Oral Glucose Insulin Sensitivity (ml min^{-1} m^{-2})	487 ± 51	471 ± 50**	498 ± 50
Model derived β-cell function parameters			
β-cell glucose sensitivity (pmol $min^{-1}m^{-2}$ mmol l^{-1})	131 ± 52	149 ± 57**	119 ± 44
Fasting Insulin Secretion Rate (pmol $min^{-1}m^{-2}$)	55.3 ± 16.2	54.6 ± 17.3	55.8 ± 15.4
ISR integral of increment (0-30) (nmol m^{-2})	4.83 ± 2.27	5.37 ± 2.31*	4.46 ± 2.18
ISR integral of increment (30-120) (nmol m^{-2})	20.0 ± 8.8	19.1 ± 9.1	20.6 ± 8.6
ISR integral of increment (0-240) (nmol m^{-2})	34.1 ± 12.9	32.2 ± 12.6	35.5 ± 13.0
ISR at 4.5mmol/l glucose (pmol $min^{-1}m^{-2}$)	81.8 ± 34.5	69.3 ± 29.1*	91.1 ± 35.4
Potentiation factor ratio (100-120) /(0-20)	1.26 ± 0.34	1.25 ± 0.35	1.27 ± 0.34
Potentiation factor ratio (220-240) / (0-20)	1.00 ± 0.27	1.02 ± 0.26	0.99 ± 0.27
Rate sensitivity (pmol $min^{-1}m^{-2}$ mmol l^{-1})	1135 ± 607	1137 ± 657	1135 ± 573
Classical β-cell function parameters			
Insulinogenic index (pmol l^{-1}/mmol l^{-1})	171 ± 88	194 ± 106*	156 ± 70
Fasting serum insulin (pmol/l)	34.8 ± 15.0	33.9 ± 16.7	35.4 ± 13.7
Serum insulin at t120 (pmol/l)	20.7 ± 12.3	19.9 ± 13.0	21.2 ± 11.8
Serum insulin IAUC (0-240) (pmol x hr/l)	613 ± 270	588 ± 269	630 ± 271
Glucose t30 minus t0 (mmol/l)	2.08 ± 0.67	2.11 ± 0.59	2.05 ± 0.72
Glucose at t120 (mmol/l)	5.39 ± 0.65	5.29 ± 0.55	5.46 ± 0.71
Glucose IAUC (0-120) (mmol x hr/l)	2.59 ± 1.05	2.31 ± 0.96*	2.80 ± 1.07
Glucose IAUC (0-240) (mmol x hr/l)	3.70 ± 1.66	2.98 ±1.3**	4.23 ± 1.71

Data are means ± SD. *p<0.05, **p<0.01 vs female; ISR= Insulin Secretion Rate;
IAUC= Area Under the Curve minus fasting level.

Table 4.2.Twin/sib correlations and heritability estimates with CI(95%) under the most parsimonious model

variable	Correlation		A: Heritability	C: Shared environment	E: Unique environment
	MZ	DZ/sib			
Waist circumference	0.60	0.30	60 (40, 74)		40 (26, 60)
Weight	0.59	0.25	57 (34, 73)		43 (27, 67)
Oral Glucose Insulin Sensitivity	0.47	0.30	49 (28, 65)		51 (35, 72)
Model derived β-cell function parameters					
β-cell glucose sensitivity	0.51	0.23	50 (26, 68)		50 (32, 74)
Fasting Insulin Secretion Rate	0.43	0.21	43 (21, 61)		57 (39, 79)
ISR integral of increment (0-30)	0.42	0.31	45 (24, 62)		55 (38, 76)
ISR integral of increment (30-120)	0.41	0.25	40 (16, 58)		60 (42, 84)
ISR integral of increment (0-240)	0.43	0.36		40 (21, 56)	60 (44, 79)
ISR at 4.5 mmol/l glucose	0.57	0.45		50 (34, 64)	50 (36, 66)

Potentiation factor ratio$_{(100-120)/(0-20)}$	0.04	0.09		7 (0, 52)	93 (75, 1)
Potentiation factor ratio$_{(220-240)/(0-20)}$	0.20	0.02	15 (0, 39)		85 (61, 1)
Rate sensitivity	0.18	0.16		17 (0, 37)	83 (63, 1)
Classical β–cell function parameters					
Insulinogenic index	0.63	0.31	63 (43, 77)		37 (23, 57)
Fasting serum insulin	0.37	0.20	38 (11, 59)		62 (41, 89)
Serum insulin at t120	0.26	0.21		24 (4, 43)	76 (57, 96)
Serum insulin IAUC (0-240)	0.45	0.41		44 (25, 59)	56 (41, 75)
Glucose (t30 minus t0)	0.54	0.17	52 (30, 68)		48 (32, 70)
Glucose at t120	0.51	0.13	50 (30, 66)		50 (34, 70)
Glucose IAUC (0-120)	0.58	-0.10	50 (31, 75)		50 (31, 75)
Glucose IAUC (0-240)	0.48	0.02	43 (19, 62)		57 (38, 81)

A = additive genetic influence ; C = shared environmental influence; E = Unique environmental influence; A,C and E values in %.

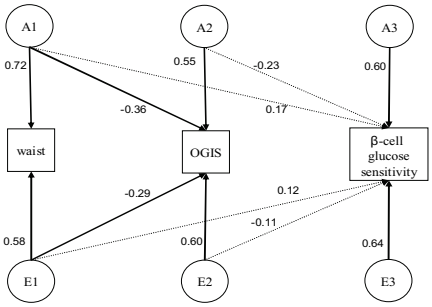

Figure .4.1.
Genetic model for waist, OGIS and β-cell glucose Sensitivity with path loadings of observed variables on the latent additive genetic(A) and unique environment (E) factors. Dotted line = non significant.

Table 4.3 shows the decomposition of heritabilities of these three parameters under this model. Out of the 50% of the variance in OGIS due to genetic factors, about one third (15%) is due to the same genetic factors that also influence waist circumference, whereas the remaining two thirds (35%) of the variance is due to genetic factors unique to OGIS. The overlap in the genetic factors influencing β-cell glucose sensitivity and those that influence waist circumference or OGIS, in contrast, is negligible.

Table 4.3 Heritability of waist, OGIS and β-cell glucose sensitivity .

Variable	Heritability			
	Total heritability	Part of the heritability deriving from the genetic factor for		
		waist	OGIS	β-cell glucose sensitivity
waist	60 (40, 74)	60		
OGIS	50 (30, 66)	15	35	
β-cell glucose sensitivity	50 (27, 68)	3	6	41

Values are per cent (95% CI); OGIS= oral glucose insulin sensitivity.

The phenotypic correlation of insulinogenic index with waist circumference and with OGIS was highly significant (r = 0.35 and -0.40, respectively, both p=<0.01). A second 3-variate analysis (waist circumference, OGIS and insulinogenic index) showed that this phenotypic correlation was mainly based on genetic factors, shared by waist circumference, OGIS and insulinogenic index. Table 4.4 shows the decomposition of heritabilities of these three parameters under the most parsimonious model which was again an AE model. Of the 63% of the variance in the insulinogenic index that was due to genetic factors, about one third

Table 4.4 Heritability of waist, OGIS and insulinogenic index

Variable	Heritability			
	Total heritability	Part of the heritability deriving from the genetic factor for		
		waist	OGIS	insulinogenic index
waist	60 (40, 74)	60		
OGIS	50 (30, 66)	15	35	
insulinogenic index	63 (43, 77)	10	10	43

Values are per cent (95% CI); OGIS= oral glucose insulin sensitivity.

is due to the same genetic factors that also influence waist circumference (10%) or OGIS (10%), whereas the remaining two thirds (43%) of the variance are due to genetic factors unique to the insulinogenic index.

Phenotypic correlations between waist circumference, OGIS, ISR (0-30) and ISR (30-120) are given in Table 4.5 and were all significant. The correlations between waist circumference and insulin secretion during the first two postprandial hours were less than those between OGIS and insulin secretion.

Table 4.5
Phenotypic correlations CI(95%) between waist, OGIS, ISR(0-30) and ISR(30-120)

	waist	OGIS	ISR (0-30)
OGIS	-0.50 (-0.61, -0.36)		
ISR (0-30)	0.13 (-0.03, 0.29)	-0.22 (-0.37, -0.06)	
ISR (30-120)	0.17 (0.01, 0.32)	-0.50 (-0.61, -0.37)	0.41 (0.27, 0.54)

OGIS = oral glucose insulin sensitivity; ISR (0-30) = integral of incremental insulin secretion from 0-30 min; ISR (30-120) = integral of incremental insulin secretion from 30 to 120 min.

According to the results of the 4-variate decomposition shown in Table 4.6 only a very small part of the total heritability of ISR (0-30) is derived from genetic factors shared with waist circumference and OGIS, while nearly one third of the total heritability of ISR (30-120) is derived from genetic factors influencing waist circumference and OGIS.

Table 4.6 Heritability of waist, OGIS, ISR(0-30) and ISR(30-120)

Variable	Heritability				
	Total	Part of the heritability deriving from the genetic factor for			
	Heritability	waist	OGIS	ISR(0-30)	ISR(30-120)
waist	62 (42, 75)	62			
OGIS	50 (30, 66)	15	35		
ISR(0-30)	47 (26, 63)	2	3	42	
ISR(30-120)	42 (19, 61)	6	7	8	21

Values are per cent (95% CI); OGIS= oral glucose insulin sensitivity; ISR (0-30)= integral of incremental insulin secretion from 0 to 30 min; ISR (30-120) = integral of incremental insulin secretion from 30 to 120 min.

Discussion

This same-sex twin family study is the first that estimated the heritability of β-cell function parameters derived from a mixed meal test, using classical as well as model derived parameters (8).

Our study shows that the model derived β-cell glucose sensitivity has a high heritability (50%) with a negligible overlap with genetic factors for waist circumference and insulin sensitivity. This replicates and extends findings in non genetically related samples (19) that also showed β-cell glucose sensitivity to be largely unrelated to waist circumference and insulin sensitivity. The β-cell glucose sensitivity is the most important parameter of the model because it quantifies the ability of the β-cell to respond to changes in glucose concentration and is a significant independent predictor of glucose intolerance. Recent findings (20;21) suggest a number of variants in several genetic loci (near genes *MTNR1B*, *CDKAL1*, *HHEX/IDE* and *TCF7L2*) that can account for the substantial heritability in β-cell glucose sensitivity.

The insulinogenic index is a classically calculated estimate of early insulin response in OGTT, already described in 1967 (22). It is strongly associated with the Acute Insulin Response after intravenous glucose administration (23) and is an independent predictor of worsening of glucose tolerance (24). In this study the insulinogenic index (insulin level t30-t0/glucose level t30-t0) was the postprandial parameter with the largest heritability (63%). This is substantially higher than the 36% heritability found when the insulinogenic index was estimated during an OGTT (25). In spite of this lower heritability, the insulinogenic index as derived from OGTT data has led to the identification of at least sixteen independent loci in genetic association studies (26-30).

The insulinogenic index and β-cell glucose sensitivity have a strong phenotypic correlation (0.68), of which 66% is explained by common genetic factors (data not shown). However, they appear to represent partly different aspects of the beta cell function. Murphy (31) showed that carriers of glucokinase gene mutations have a normal insulinogenic index but reduced β-cell glucose sensitivity with a large right shift. Tura (32) revealed that normal glucose tolerant women, who had previous gestational diabetes, had decreased β-cell glucose sensitivity but a normal insulinogenic index. Mari et al (33) demonstrated that the insulin secretagogue nateglinide improved β-cell glucose sensitivity in patients with type 2

diabetes, while the insulinogenic index did not change significantly. Despite its higher heritability, the insulinogenic index shared more genetic factors with waist circumference and insulin sensitivity than β-cell glucose sensitivity. This makes model derived β-cell glucose sensitivity a more specific genetic marker of the β-cell function.

Although the heritability of fasting insulin levels has been estimated in many studies (ranging from 8% (34) to 54% (35)), to our knowledge the heritability of fasting ISR, which takes insulin clearance into account, has never been assessed. The heritability of the fasting ISR and of the fasting insulin level show the same order of magnitude (43% and 38% respectively) and there is a high correlation between the two insulin measurements (0.80). However, fasting ISR is a better measure of the activity of the β-cell than insulin level, because insulin level is strongly co-determined by insulin clearance. The ISR (0-30) was less correlated with waist circumference and OGIS than the ISR (30-120) and the genetic variation influencing early insulin secretion also overlapped less with that for waist circumference and OGIS than later insulin secretion. This is again compatible with the relatively high number of genetic loci found in GWA studies, that are associated with early insulin secretion (28) while only two genetic loci are found to be significantly associated with reduced insulin secretion during the 2-h OGTT (29;36).

All postprandial glycemia parameters were significantly influenced by genetic factors, with heritability estimates ranging between 43% (4 hours) and 52% (first 30 min). Heritability of the other mixed meal test parameters ISR(0-240), ISR at 4.5 mmol glucose, potentiation factor ratios (100-120)/(0-20) and (220-240)/(0-20), rate sensitivity , serum insulin at t120 and serum insulin IAUC(0-240) was not significant although in many instances the MZ twin pair correlation was larger than the DZ/sib pair correlation. This may reflect the major limitation of the approach used in this study, namely the limited sample size of only 183 participants. Because meal size (37), meal composition (38) and rate of gastric emptying (39) have influence on postprandial insulin secretion and glycemia, a strict protocol was required with adequate trained assistance and researchers. Because our total study design consisted of several tests, it was time consuming and had relatively high costs thereby prohibiting a larger sample size. However, our results show a two-hour meal test to be sufficient for genetic testing, which makes this test really suitable for larger studies.

Up till now GWA studies have only used results of OGTT to test β-cell function and glucose tolerance after a glucose challenge. The advantage of an OGTT test over the mixed meal test is that it is cheaper, it has a simple protocol and it is feasible in the large samples

required for GWA. The disadvantage is that it gives only information of the effect of one secretagogue and is not a reflection of daily life. The mixed meal test is a real physiological challenge with different natural types of secretagogues with influence on incretin secretion and neural activation and in this way will give more relevant information. Moreover, with the same amount of CH intake (40), the β-cell glucose sensitivity and the insulinogenic index are larger in a 2hr mixed meal test than in an OGTT. This makes the mixed meal test a powerful method to study the effects of candidate genetic variants deriving from GWA studies in more detail.

In summary, we find that the highest heritability for postprandial β-cell function was found for the classical insulinogenic index, but the most specific β-cell function parameters appeared to be model derived β-cell glucose sensitivity and the integral of incremental ISR during the first 30 postprandial minutes. We conclude that the mixed meal test provides multiple heritable aspects of the β-cell function that can help us examine the biology underlying the wealth of genetic variants produced by GWA studies.

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Chapter 5

Genetic influences on the insulin response of the β-cell to different secretagogues

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Abstract

Aims

The aim of the present study was to estimate the heritability of the β-cell insulin response to glucose and to glucose combined with glucagon-like peptide-1 (GLP-1) or with GLP-1 plus Arginine.

Methods

This was a twin-family study that included 54 families from the Netherlands Twin Register. The participants were healthy twin pairs and their siblings of the same-sex, aged 20 to 50 years. Insulin response of the β-cell was assessed by a modified hyperglycaemic clamp with additional GLP-1 and arginine. Insulin sensitivity index (ISI) was assessed by the euglycaemic-hyperinsulinaemic clamp. Multivariate structural equation modelling was used to obtain heritabilities and the genetic factors underlying individual differences in BMI, ISI and secretory responses of the β-cell.

Results

The heritability of insulin levels in response to glucose was 52% and 77% for the first and second phase, respectively, 53% in response to glucose + GLP-1 and 80% in response to an additional arginine bolus. Insulin responses to the administration of glucose, glucose + GLP-1 and glucose + GLP-1 + arginine were highly correlated ($0.62 < r < 0.79$). Heritability of BMI and ISI was 74% and 60% respectively. The genetic factors that influenced BMI and ISI explained about half of the heritability of insulin levels in response to the three secretagogues. The other half was due to genetic factors specific to the β-cell.

Conclusions

In healthy adults, genetic factors explain most of the individual differences in the secretory capacity of the β-cell. These genetic influences are partly independent from the genes that influence BMI and ISI.

Introduction

Type 2 diabetes mellitus is a multifactorial disease, characterised by decreased insulin sensitivity and inadequate insulin secretion by the pancreatic β-cell (1). Twin (2-5) and family studies (6-10) support the notion that people who develop type 2 diabetes mellitus have a strong genetic predisposition, which may be partly conveyed through genetic effects on insulin resistance. Two twin studies, for instance, showed a significant genetic contribution to insulin sensitivity, assessed by euglycaemic-hyperinsulinaemic clamp with heritability estimates of 37% and 55% (11;12). Notwithstanding the importance of insulin sensitivity, genetic effects on β-cell function are likely to play a major role in the development of type 2 diabetes mellitus. Thus many of the genetic variants in and near genes recently found to be associated with risk of type 2 diabetes mellitus (13) influence β-cell function (14). In twin studies, the genetic contribution to β-cell function has been tested mostly by examining surrogate measurements of insulin secretion derived from fasting blood levels or in response to oral glucose. In five studies using IVGTT, heritability estimates of the acute insulin response to glucose ranged from 35% to 76% (6;11;15-17). The heritability of the second-phase insulin response to glucose in an IVGTT has been investigated in two of these studies only (11;17) and was estimated to be 28% and 58%.

Glucose is the key regulator of insulin secretion by the β-cell, which occurs through activation of the glycolytic flux, followed by mitochondrial activation, membrane depolarisation and finally release of insulin. However, under typical physiological conditions various non-glucose secretagogues also affect insulin secretion. These include incretins like glucagon-like peptide-1 (GLP-1), which is immediately secreted by the gut in reaction to a meal (18) and causes G-protein-coupled receptor activation of adenylate cyclase, and amino acids like arginine, which depolarises the β-cell membrane (19). Abnormal insulin responses after administration of these secretagogues may be a first sign of development of type 2 diabetes mellitus (20). To date, no studies have estimated the heritability of insulin response to glucose combined with GLP-1 and arginine. Furthermore, it is unclear whether the secretory responses to glucose + GLP-1 and glucose + GLP-1 + arginine are governed by the same genetic factors as the response to glucose alone. Although there is overlap in the mechanism by which they stimulate insulin secretion, each

of these secretagogues also has a specific intracellular pathway that may be influenced by different sets of genes (18, 19).

Increased understanding of the genetics of the different aspects of β-cell function and dysfunction may identify new targets for glucose-lowering drugs or preventive measures. The aim of this twin-family study was to explore the heritability of β-cell insulin response by using a modified hyperglycaemic clamp based on the procedure by Fritsche and colleagues (21). Apart from estimating the heritability of insulin response to glucose (first and second phase), GLP-1 and arginine, we also tested whether different genetic factors influence insulin response to each of these three secretagogues. Since the insulin response is strongly correlated to BMI and insulin sensitivity (22;23), measures of BMI and insulin sensitivity were assessed on the same day, the latter by the euglycaemic-hyperinsulinaemic clamp. This allowed us to test to which extent genetic factors influencing insulin response overlap with those influencing BMI and insulin sensitivity.

Methods

Design and participants

This study used a twin/same-sex sibling design to address the genetic and environmental contribution to the variance of β-cell function in Dutch twin families recruited from the Netherlands Twin Register (24) as described previously (25). The clamp tests were performed in 125 (58 men) healthy participants, aged 20 to 50 years and of European origin. In the weeks prior to these clamp tests, participants had been assessed with a 75 g screening OGTT to exclude the presence of diabetes mellitus. There were 34 monozygotic twin pairs and 13 same-sex dizygotic twin pairs from 47 families. Eight monozygotic twin pairs and five dizygotic twin pairs had one additional same-sex sibling. Two monozygotic twin pairs had two additional same-sex siblings. In six more families only one twin of the pair participated, together with a same-sex sibling. In one family no twin pair but two female siblings took part in the study. In total, 100 twins and 25 siblings participated. Twin zygosity was determined from DNA polymorphisms. In total 44 dizygotic/sibling pairs could be formed. The median age difference between twins and sibs was 3.5 years with a range of 1.8 to 9.1 years. Including an additional sibling in the classical twin design

significantly increases the power to detect the genetic and environmental sources of variation (26).

All participants gave written informed consent. The study was approved by the local Ethics Committee and performed in accordance with the Declaration of Helsinki.

Euglycaemic-hyperinsulinaemic clamp

After a 12 hours fast the clamp procedure (27) started in the clinic at 08.00 hours. Anthropometric measurements of weight (Seca balance scale; Schinkel, Nieuwegein, The Netherlands), height (Harpenden Stadiometer; Holtain, Crosswell, UK) and waist and hip circumference (28) were performed. One cannula was retrogradely placed in a heated dorsal hand vein to obtain arterialised blood. A second cannula was inserted into the antecubital vein of the arm for infusion of 0.9% saline, glucose 20% and insulin. After baseline samples had been taken twice, a primed, continuous (40 mU m^{-2} min^{-1}) insulin infusion (Velosuline/Actrapid; Novo Nordisk, Bagsvaer, Denmark) was given for 120 min. Glucose 20% was infused at a variable rate to maintain blood glucose at 0.3 mmol/l below the fasting level and within the range of 4.5 to 5.5 mmol/l. Blood glucose was monitored at 5 min intervals; blood samples for hormonal levels were obtained at 60, 90, 105 and 120 min.

Modified hyperglycaemic clamp

At 1 hour after completion of the euglycaemic-hyperinsulinaemic clamp, the hyperglycaemic clamp was performed at 10 mmol/l as described by Fritsche et al. (21). The blood glucose level was frequently monitored (at least every 5 min) and the infusion rate of glucose 20% accordingly adjusted. Blood samples for measurement of insulin and C-peptide were drawn at fixed time points, i.e. (1) at −5 and −2 min before the start of the hyperglycaemic clamp; (2) every min for 10 min after a bolus of each secretagogue; and (3) at 5 to 30 min intervals in the periods in-between. At t = 0 min the participant received an intravenous bolus of glucose for 1 min to acutely raise glucose level to 10 mmol/l (bolus calculated as follows: weight in kg × [10 − prehyperglycaemic glucose level in mmol/l] × 27/200 = ml glucose 20%). At 120 min (i.e. 2 hours later) GLP-1 (7-36 Amide Human; Polypeptide Laboratories, Wolfenbuettel, Germany) was given as a bolus injection (1.5 pmol/kg) for 1 min, followed by continuous infusion of 0.5 pmol kg^{-1} min^{-1}. At 180 min, a

bolus of 5 g arginine was injected over 50 seconds on top of the GLP-1 infusion. The GLP-1 infusion was terminated 20 min after the arginine bolus and the clamp finished.

Analytical procedures

Blood glucose was assessed at bedside using a glucose oxidase method (YSI 2300 Stat plus; Yellow Springs Instruments, Yellow Springs, OH, USA). This device has a within-run CV of 2% and a day-to-day CV of 6% (29). Blood for hormonal levels was centrifuged (1,469g) and the serum stored at −80°C. All serum specimens were assessed for insulin and C-peptide levels at the VU University Medical Centre (department of Clinical Chemistry, Amsterdam, the Netherlands) using an immunometric assay luminescence method (ACS: Centaur; Bayer Diagnostics, Mijdrecht, the Netherlands). There was no cross-reactivity with proinsulin or split products. The inter assay CV of insulin and C-peptide measurement was 6.5% and 6% respectively. The intra-assay CV was 4% for both.

Calculations

BMI was calculated as weight in kg/(height in metres)^2. The insulin sensitivity index (ISI) was defined as the glucose infusion rate per kg of body weight during the second h of the euglycaemic-hyperinsulinaemic clamp per unit of serum insulin concentration (μmol kg^{-1} min^{-1} (pmol/l)$^{-1}$). The incremental insulin response to the secretagogues was calculated in four periods named first-phase, second-phase, GLP-1-stimulated insulin response (GLP-1IR) and arginine-induced insulin response (ARG-IR), using the AUCs of the measured insulin levels as depicted in Figure 5.2. The first phase comprised the acute response to hyperglycaemia and was computed as the AUC of the insulin level during min 1 to 10, using the mean baseline level from −5 to −2 min. The second phase comprised the AUC from 80 to 120 min, again using the mean from −5 to −2 min as the baseline level. The GLP-1IR was calculated as the AUC from 160 to 180 min, using the last period of the second phase from 100 to 120 min as the baseline. The acute ARG-IR was calculated as the AUC from 182 to 185 min minus the pre bolus level (at 180 min). C-peptide responses to the three secretagogues were calculated in the same way.

Statistical analyses

All genetic analyses were carried out in Mx (30), a structural equation modelling program specifically designed for the analysis of twin and family data. All variables (BMI, ISI, first-phase, second-phase, GLP-1IR and ARG-IR) were *Z* transformed prior to analysis (mean=0, SD=1) to reduce the large differences in variances across the variables. This transformation does not affect the estimates of familial correlations or of heritabilities.

In a first step, we confirmed that the variances of the variables were comparable for twins and siblings and that the covariances between dizygotic twins could be equated to those between a twin and a singleton sibling. The latter allowed us to treat all sibling pairs sharing 50% of their genetic material, whether dizygotic twin or twin-sibling pair, in the same way. Next we estimated correlations among family members and among variables in a model that included the fixed effects of sex and age on each variable. Broadly, we can distinguish between three types of correlations: cross-variable within-person correlations (phenotypic correlations), within-variable cross-person correlations (familial correlations, i.e. monozygotic and dizygotic/sibling correlations) and cross-variable cross-person correlations. The phenotypic correlations describe the correlations among the four secretory responses (first-phase, second-phase, GLP-1IR and ARG-IR), BMI and insulin sensitivity. The monozygotic and dizygotic/sibling correlations describe the resemblance for these variables in monozygotic twins and in all other pairs of first-degree relatives (dizygotic twins, twin–sibling, sibling–sibling). These correlations form the basis for estimating the relative contributions of genetic and environmental factors to individual differences in each variable. Monozygotic twin pairs have all or nearly all genes in common; dizygotic twin pairs, twin–sibling pairs and sibling–sibling pairs share on average half of their segregating genes. If monozygotic correlations are larger than the dizygotic/sibling correlations, genetic influences are suggested.

Monozygotic and dizygotic/sibling correlations can also be computed across variables (cross-variable cross-person correlations), for example between the first-phase secretory response of a twin and the second- phase secretory response of his or her co-twin. When the cross-variable cross-person correlation is larger in monozygotic twin than in dizygotic/sibling pairs, this indicates that part of the association between variables is explained by overlapping genetic factors.

Next, a multivariate genetic model (31) was fitted to the data. For each of the six variables in the model, the total variance was broken down into additive genetic variance (A), common environmental variance shared by family members (C) and unique environmental variance (E). The full multivariate model included a set of six factors for A, C and E (Fig. 5.1). The first factor influences all six variables; the next factor influences five variables and the last factor only one variable. We tested whether the contributions of these A and C factors were all significant using likelihood-ratio tests. The difference in minus two times the log-likelihood (−2LL) between two nested models has a χ^2 distribution. A corresponding p value > 0.05 indicated that the more parsimonious model did not fit the data less well than the full model. Next the paths of each A and E factor to the six variables were tested for significance. This procedure was repeated to arrive at the most parsimonious model that fitted the data. Under this model, we estimated the heritability of each variable individually and broke this heritability down into: (1) components that overlap with BMI and ISI; (2) components that are specific to insulin response but shared among secretagogues; and (3) components that are specific to each secretagogue.

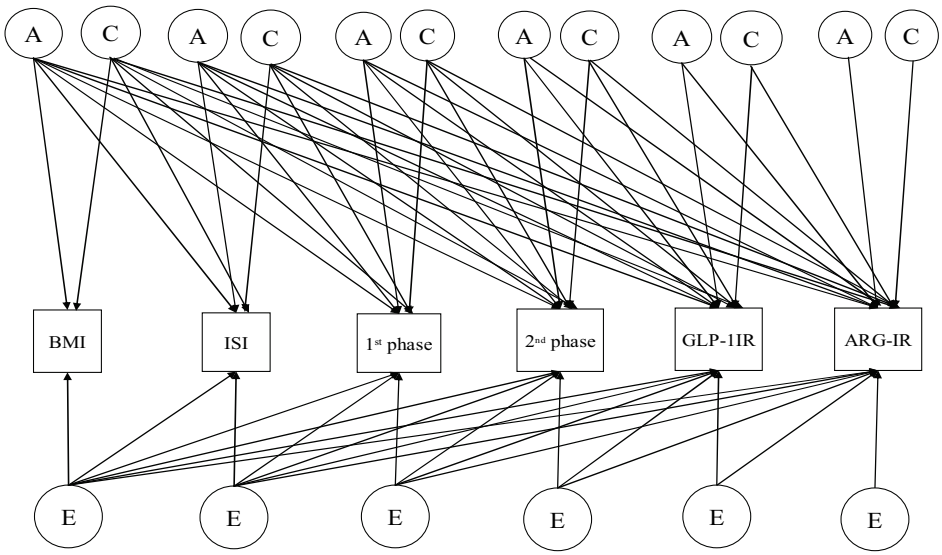

Figure 5.1 The full 6-variate ACE genetic model

Results

Sample characteristics are shown in Table 5.1 and the insulin levels attained during the hyperglycaemic clamp and GLP-1 and arginine additions in Fig. 5.2. The mean insulin secretion in response to the secretagogues was larger in men. This difference was highly significant after stimulation with GLP-1 (χ^2=18.44, Δdf=1, p<0.001) and arginine (χ^2=9.89, Δdf=1, p=0.002). The mean waist circumference was significantly larger in men (χ^2=7.05, Δdf=1, p=0.01). There was no significant influence of age on the means of BMI, ISI and the four secretory responses (χ^2=11.03, Δdf=6, p=0.09).

Table 5.1 Sample characteristics

Variable	Mean total	SD	Mean male	SD	Mean female	SD
Number	125		58		67	
Age (years)	30.8	5.6	30.4	5.2	31.2	5.9
Waist (cm)	85.5	10.2	88.2**	9.7	83.2	10.2
Weight (kg)	74.2	12.0	80.5**	10.4	68.8	10.5
BMI (kg/m$^{2)}$	24.0	3.4	24.1	3.0	23.96	3.8
ISI	.091	.039	0.098	0.047	0.084	0.029
Insulin response [a]						
First-phase	1,700	1,114	1,942*	1,247	1,484	938
Second-phase	9,337	7,471	10,830*	8,566	8,043	6,153
GLP-1	34,498	30,433	49,448*	35,333	21,361	16,829
Arginine	6,602	3,037	7,758**	3,147	5,554	2,533
C-peptide response						
First-phase	5.713	3.030	6.649**	3.383	4.878	2.409
Second-phase	66.68	26.65	74.74**	29.16	59.70	22.21
GLP-1	72.63	35.49	93.15**	36.05	54.60	23.20
Arginine	7.913	2.908	8.332	3.240	7.534	2.538

[a]Responses during the hyperglycaemic clamp are given as incremental responses, as defined in the Methods.
ISI in µmol min^{-1} kg^{-1}[pmol/l]$^{-1}$; Insulin response in pmol min^{-1} l^{-1}; C-peptide response in nmol min^{-1} l^{-1}; *p<0.05 vs female; **p<0.01 vs female.

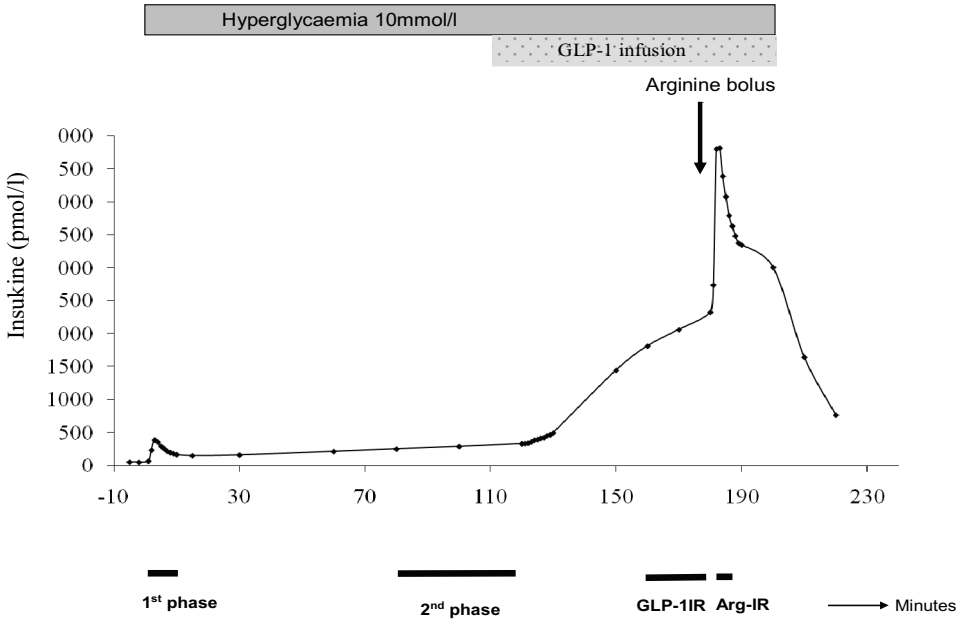

Figure 5.2 Insulin levels during the hyoperglycaemic clamp. The bars indicate the time periods of the calculated insuline responses

Phenotypic correlations are given in Table 5.2 and were all significantly larger than zero. The insulin secretion in response to glucose (first and second phase), glucose + GLP-1 and glucose + GLP-1 + arginine was highly correlated ($0.62 < r < 0.79$).

Table 5.2 Phenotypic correlations (95% CI) between BMI, insulin sensitivity, and the insulin responses of the β-cell.

Variable	BMI	ISI	first phase	second phase	GLP
ISI	-0.45 (-0.60;-0.24)				
1st phase	0.42 (0.23;0.58)	-0.56 (-0.69;-0.40)			
2nd phase	0.48 (0.30;0.63)	-0.57 (-0.69;-0.41)	0.79 (0.71;0.86)		
GLP-1IR	0.46 (0.27;0.61)	-0.56 (-0.69;-0.41)	0.67 (0.54;0.76)	0.79 (0.71;0.86)	
ARG-IR	0.44 (0.26;0.59)	-0.55 (-0.68;-0.39)	0.62 (0.48;0.73)	0.67 (0.54;0.77)	0.70 (0.61;0.80)

Within-variable cross-person correlations are presented in Table 5.3. Monozygotic twin correlations were all larger than dizygotic twin/sibling correlations. This indicates that genetic factors play a role in the variability of BMI, insulin sensitivity and insulin secretion after intravenous administration of glucose, glucose + GLP-1 and glucose + GLP-1 + arginine.

Table 5.3 Within-variable cross-person correlations (95% CI) for monozygotic twin and dizygotic twin/sibling pairs

Variable	Monozygotic twin pairs	Dizygotic twin /sibling pairs
BMI	0.75 (0.57, 0.83)	0.44 (0.17, 0.65)
ISI	0.76 (0.53, 0.87)	0.12 (−0.15, 0.38)
First-phase	0.63 (0.38, 0.78)	0.22 (−0.16, 0.51)
Second-phase	0.76 (0.61, 0.86)	0.31 (−0.12, 0.60)
GLP-1IR	0.57 (0.33, 0.74)	0.37 (−0.00, 0.67)
ARG-IR	0.82 (0.68, 0.89)	0.22 (−0.11, 0.50)

Cross-variable cross-person correlations are shown in Table 5.4. Monozygotic cross-twin cross-variable correlations were all larger than the dizygotic twin/sibling cross-variable correlations. These correlations indicate that overlapping genes contribute to the phenotypic correlations between BMI, insulin sensitivity and the insulin response to the different secretagogues.

In the full multivariate genetic ACE model, the contribution of A (combination of all additive genetic factors) proved to be significant (χ^2=48.77, Δdf=21, p=0.001), while C (combination of all common environmental factors) could be dropped from the model (χ^2=4.77, Δdf=21, p=1.00).

Table 5.4 Cross-variable cross-person correlations (95% CI), with monozygotic twin-pairs in lower diagonal and dizygotic twin/sibling pairs in upper diagonal

Variable	BMI	ISI	First-phase	Second-phase	GLP-1IR	ARG-IR
BMI		−0.10 (−0.32, 0.12)	0.10 (−0.16, 0.33)	0.17 (−0.11, 0.40)	0.17 (−0.10, 0.40)	0.19 (−0.06, 0.41)
ISI	−0.33 (−0.51,−0.18)		−0.25 (−0.46, −0.0)	−0.28 (−0.49, −0.02)	−0.20 (−0.42, 0.05)	−0.13 (−0.36, 0.11)
First-phase	0.31 (0.09, 0.50)	−0.43 (−0.60, −0.21)		0.27 (−0.12, 0.53)	0.24 (−0.11, 0.48)	0.14 (−0.17, 0.40)
Second-phase	0.33 (0.13, 0.51)	−0.45 (−0.61, −0.26)	0.60 (0.42, 0.72)		0.33 (−0.04, 0.59)	0.19 (−0.15, 0.45)
GLP-1IR	0.25 (0.03, 0.45)	−0.37 (−0.55, −0.14)	0.46 (0.25, 0.62)	0.56 (0.38, 0.70)		0.24 (−0.09, 0.49)
ARG-IR	0.34 (0.14, 0.51)	−0.46 (−0.61, −0.27)	0.46 (0.26, 0.61)	0.56 (0.40, 0.69)	0.52 (0.34, 0.66)	

Further testing showed that in the resulting AE model, four single A paths and four single E paths could be constrained to zero (χ^2=6.64, Δdf=8, p=0.58). The most parsimonious AE model with path loadings is presented in Fig. 5.3.

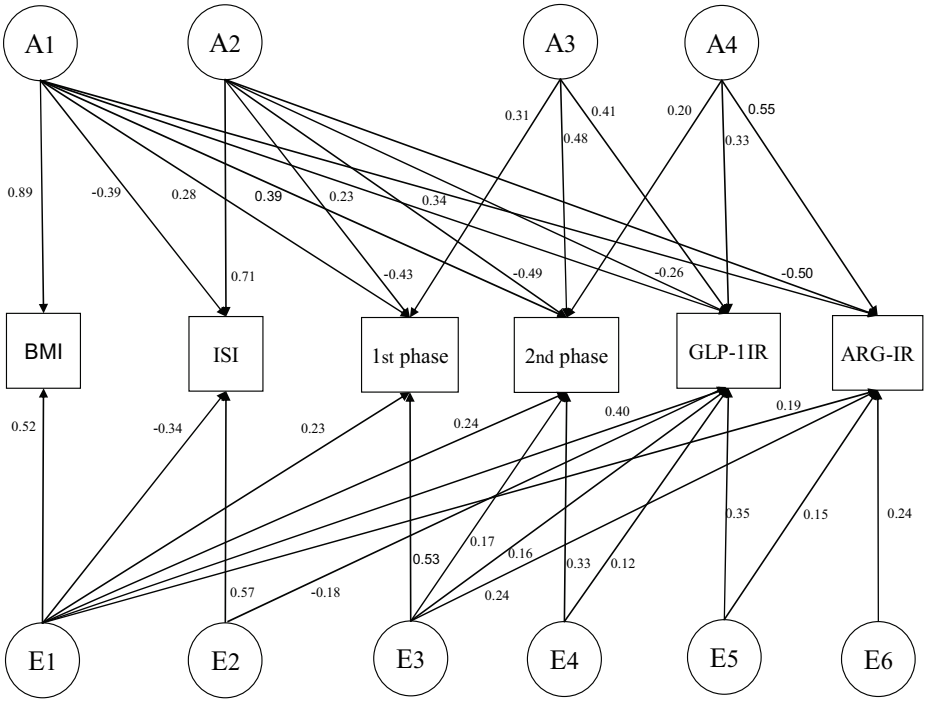

Figure 5.3 The most parsimonious model (AE) for genetic and environmental effects on BMI, ISI and the insulin responses of the beta cell. Factor loadings for observed variables on the latent additive genetic (A) and unique environmental factors (E) are shown.

In this best-fitting model there are two sets of genetic factors that influence the insulin response to the four stimulations independently of BMI and ISI. The genetic factors that influence BMI and ISI also influence the variability of the four secretory responses. The heritability estimates of the six variables are presented in Table 5.5. The heritability of BMI is estimated at 74%. The heritability of insulin sensitivity is 60%. Part of the genes influencing BMI also influences ISI. The first-phase response has a heritability of 52%, of which a small part (14%) is due to genetic factors specific for the insulin response and independent of the genetic factors for BMI and ISI heritability. The second-phase response has a heritability of 77%, of which nearly half can be attributed to the genes specific for the

insulin response. The heritability of insulin secretion in response to GLP-1 was 53%, mainly determined by genes specific for the insulin response. The secretory response to arginine had a heritability of 80%, of which 44% reflects genetic overlap with BMI and ISI, while 36% is specific for the insulin response. The maximum insulin level during the test was generated directly after the arginine bolus. The heritability of this total peak level was 77%.

Table 5.5 Heritability of BMI, ISI and the insulin responses of the β cell

Variable	Heritability			
	Total heritability	Overlap with BMI	Overlap with ISI	Overlap with insulin secretion
BMI	74 (56, 85)	74		
ISI	60 (32, 80)	14	46	
First-phase	52 (29, 70)	12	26	14
Second-phase	77 (60, 86)	18	29	30
GLP-1IR	53 (33, 70)	7	9	37
ARG-IR	80 (65, 88)	14	30	36

Values are per cent (95% CI)

Three alternative analyses were performed to bolster our confidence in these results (data not shown). First, correlations in Tables 5.2 to 5.5 were recomputed using C-peptide instead of insulin. This yielded virtually identical results. Second, we used the total AUC with zero as a baseline for the GLP-1 and arginine phases, rather than the incremental AUC with the previous levels as a baseline. As expected from the high correlation between absolute and incremental GLP-1 (r = 0.99) and arginine (r = 0.91) responses, highly similar patterns of twin sibling correlations were obtained when using the total AUCs. Finally, because waist circumference is seen by some researchers as more discriminating for the risk of type 2 diabetes mellitus (32), we repeated the analyses replacing BMI by waist. The heritability for waist circumference was 75%, its correlation to BMI r =0.84. Again highly comparable patterns of twin sibling correlations were obtained when using waist circumference instead of BMI.

Discussion

This study used a twin/sibling design to explore heritability of the insulin response of the β-cell in healthy individuals during a modified version of the hyperglycaemic clamp test used by Fritsche and colleagues (21). The heritability of first- and second-phase insulin secretion in response to glucose was 52% and 77% respectively. For the first phase our results fit neatly in the 35% to 76% range of heritability estimates previously reported using the IVGTT test (6;11;15-17). However, previous heritability estimates (28% and 58%) of second-phase insulin secretion in response to IVGTT were considerably lower (11;17) than those found in our hyperglycaemic clamp. This may reflect the greater precision inherent in the clamp method in comparison to the IVGTT, but may also be due to the different stimulation of the β-cell during the second phase of both tests (maintaining 10 mmol/l glucose vs decreasing glucose level).

Insulin secretion in response to administration of GLP-1 or GLP-1 + arginine in the presence of hyperglycaemia has been tentatively suggested as an endophenotype for type 2 diabetes mellitus risk (20). Here we show for the first time that these secretory responses do indeed show significant heritability (GLP-1 53%, arginine 80%). Moreover, the genetic information contained in the insulin response to these two secretagogues is only partially captured by the first-phase insulin response during the hyperglycaemic clamp. The multivariate analysis of the insulin responses to the three secretagogues and BMI and ISI showed that the genetic variance unique to β-cell function (factors A3+A4) contributed less strongly to individual differences in the first-phase response (14%) than in the second-phase response or in the responses to GLP-1 and GLP-1 + arginine (30–37%).

Overall, the findings clearly show that the genetic variation in β-cell function is only partly attributable to genes influencing BMI and ISI. These findings cannot simply be attributed to a restricted range of BMI or ISI values in this healthy sample, since our heritability estimates of BMI and insulin sensitivity are very comparable to previous estimates (11;12;33). Furthermore, the partial independence of genetic factors influencing β-cell function from those influencing BMI is congruent with the outcome of previous genome-wide association (GWA) studies addressing the genetic risk of diabetes mellitus. These show that correction for BMI can sometimes reduce the significance of SNP associations with type 2 diabetes mellitus, with the *FTO* gene being the most prominent

example (34). This suggests that obesity genes like *FTO* may belong to the first genetic factor (A1) in Figure 5.3. This raises the question of whether correction for BMI is appropriate if the goal is to identify genes 'that influence diabetes mellitus risk. Part of the genetic risk for insulin sensitivity and β-cell function truly overlaps with the risk for obesity.

The partial independence of the genetic factors influencing the β-cell response from those influencing insulin sensitivity is also congruent with the outcome of previous GWA studies and the follow-up of their major candidate genes in experimental studies. The majority of the ~20 loci shown in GWA studies to be associated with type 2 diabetes mellitus or fasting glucose are almost all implicated in defective β-cell function (14;35). Almost none of these genes were found to affect peripheral insulin sensitivity. Most notably, all previous studies that used a hyperglycaemic clamp procedure comparable to the one used here confirm the independence of genetic effects on insulin secretion and insulin sensitivity. For instance, the risk variants of *CDKAL1*, *IGF2BP2* (36), *TCF7L2* (37) or *WFS1* (38) clearly impaired glucose- or GLP-1-induced insulin secretion, but did not impact on insulin sensitivity.

It has often been suggested that β-cell dysfunction is uncovered only when insulin resistance creates a strongly increased insulin demand. This implies an interactive effect between insulin resistance and β-cell function. In support of such an effect it was recently shown that the summed effects of the risk alleles in *TCF7L2*, *CDKAL1*, *HHEX; SLC30A8* , *IGF2BP2, CDKN2A/B, JAZF1*and *WFS1* had a stronger effect on β-cell function in participants with low insulin sensitivity than in participants with high insulin sensitivity (39). Because we found that the genetic variation in β-cell function is partly independent of the genetic variation in ISI, the interactive effect between insulin resistance and β-cell function could be partly due to gene–gene interaction. Failing to account for such gene–gene interactions may explain some of the missing heritability plaguing GWA studies in diabetes mellitus (40).

In the near future, new candidate genes can be expected to derive from the ongoing collaborative GWA studies on diabetes mellitus or fasting glucose, which are still growing in scale. We assert that functional annotation of these genes should employ rigorous β-cell function tests including the hyperglycaemic clamp procedure used here. For instance, Schafer et al. (37), using a comparable hyperglycaemic clamp + GLP-1 + arginine as used in the current study, showed that carriers of the risk allele in two variants (rs7903146,

rs12255372) of the *TCF7L2* gene had unchanged GLP-1 secretion, but significantly reduced GLP-1-induced insulin secretion. This finding narrows the possible role for *TCF7L2* in type 2 diabetes mellitus, an often replicated association, to a functional defect in GLP-1 in the β-cells.

The major strength of our study, the clamp-based measurement of insulin sensitivity and insulin responses under a strict and uniform protocol, comes with a major limitation. Due to the expensive, time-consuming and demanding protocols, the sample was relatively small. This is reflected in the fairly broad confidence intervals around the estimates in Tables 5.2 to 5.5. As a consequence of the modest sample size, the a priori power to detect common environmental effects, for instance shared dietary practices in childhood, or genetic non-additivity (dominance, epistasis) was very poor (26). Also, the two-factor structure now found for β-cell responses might prove more complex, i.e. with GLP1-specific and arginine-specific genetic factors, when larger samples are assessed. Further caution is needed in generalising these data beyond the sample of relatively young, healthy Europeans used here.

In conclusion, genetic factors explain most of the individual differences in insulin response after administration of glucose and glucose combined with GLP-1 or GLP-1 + arginine in healthy adults. Our results show that the often used first-phase response may give an incomplete picture of the genes that are specific to β-cell function. They also show that the genetic factors influencing β-cell function are partly independent of the factors that influence BMI and ISI, and that in genetic designs ‘correction’ for BMI and ISI may not always be desirable. To chart the biological effects of (new) candidate genes from GWA studies on type 2 diabetes mellitus, the hyperglycaemic GLP-1/arginine challenge test may be a powerful tool.

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Chapter 6

A combined risk allele score of eight type 2 diabetes genes is associated with reduced first-phase glucose stimulated insulin secretion during hyperglycaemic clamps

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Abstract

Aims

At least twenty type 2 diabetes loci have now been identified and several of these are associated with altered β-cell function. In this study we have investigated the combined effects of eight known β-cell loci on insulin secretion stimulated by three different secretagogues during hyperglycaemic clamps.

Methods

447 subjects originating from four independent studies in the Netherlands and Germany (256 NGT/191 IGT) underwent a hyperglycaemic clamp. A subset had an extended clamp with additional GLP-1 and arginine (n=224). We next genotyped SNPs in *TCF7L2, KCNJ11, CDKAL1, IGF2BP2, HHEX/IDE, CDKN2A/B, SLC30A8* and *MTNR1B* and calculated a risk allele score by risk allele counting.

Results

The risk allele score was associated with lower first-phase glucose stimulated insulin secretion (GSIS) ($p=7.1*10^{-6}$). The effect size was equal in NGT and IGT subjects. We also noted an inverse correlation with the disposition index ($p=1.6*10^{-3}$). When we stratified the study population according to the number of risk alleles into three groups those with a medium or high risk allele score had 9% and 23% lower first-phase GSIS. Second-phase GSIS, ISI and GLP-1 or arginine stimulated insulin release were not significantly different.

Conclusions

A combined risk allele score for eight known β-cell genes is associated with the rapid first-phase GSIS and the disposition index. The slower second-phase GSIS, GLP-1 and arginine stimulated insulin secretion are not associated suggesting that especially processes involved in rapid granule recruitment and exocytosis are affected in the majority of risk loci.

Introduction

Type 2 diabetes mellitus is a polygenic disease in which the contribution of a number of detrimental gene variants in combination with environmental factors is thought to be necessary for the development of this disease. In the past two years results of several genome wide association studies (GWAS) have been published (1-5), leading to a rapidly increasing number of detrimental type 2 diabetes mellitus susceptibility loci. And more recently it has indeed been shown that combining information from these diabetes mellitus loci into a risk allele score for all loci enhances diabetes mellitus risk (6-9). However, the predictive power of this combined risk allele score is yet insufficient to substitute or largely improve predictive power of known clinical risk factors (7;8). At present little is known about how these gene variants in combination affect insulin secretion or insulin resistance. Based on recent data, mainly obtained from oral glucose tolerance tests (OGTT), it was shown that a combined risk allele score from gene variants associated with type 2 diabetes mellitus is associated with insulin secretion, and not with insulin sensitivity (10-13). However, the OGTT is unable to distinguish between first and second-phase insulin secretion. Furthermore other secretagogues like GLP-1 and arginine were not included in these studies.

It is thought that the rapid recruitment and release of insulin granules from the readily releasable pool (RRP) is responsible for the first-phase of insulin secretion whereas the slower prolonged second-phase involves recruitment to the membrane of more distant granules and *de novo* insulin synthesis. Although the exact pathways regulating both phases of glucose stimulated insulin secretion are not completely resolved it seems logical that they are at least in part different. This is further corroborated by our recent observation that the heritability for both phases of GSIS in twins is derived from partly non-overlapping sets of genes (14).

Also other, non-glucose, stimuli like incretins and amino acids can evoke an insulin response. Detailed phenotypic investigations of the response to these different stimuli may help to elucidate which processes are primarily affected by these loci. Previously we have already shown that type 2 diabetes mellitus genes/loci can have different effects on first and second-phase GSIS as measured using hyperglycaemic clamps. Also based on the method

of stimulation, i.e. oral versus intravenous the outcome may differ substantially (15-18), which provides further clues about the mechanism by which they affect insulin secretion.

In this study we genotyped gene variants in *TCF7L2*, *KCNJ11*, *HHEX/IDE*, *CDKAL1*, *IGF2BP2*, *SLC30A8, CDKN2A/CDKN2B* and *MTNR1B* in 447 hyperglycaemic clamped subjects (256 with normal glucose tolerance and 191 with impaired glucose tolerance) from four independent studies in the Netherlands and Germany. These eight loci were chosen based on the fact that they were reproducibly associated with β cell function in various studies (reviewed in (19;20)). A combined risk allele score of all eight gene variants was calculated for each individual and tested against the various detailed measurements of β-cell function using the hyperglycaemic clamp, generally considered to be the gold standard for quantification of first and second-phase glucose stimulated insulin secretion (GSIS) (21). Furthermore we also assessed the combined effect of these eight genes on two other stimuli; GLP-1 and arginine stimulated insulin secretion during hyperglycaemia, in a subset of the study sample (n=224). The latter test provides an estimation of the maximal insulin secretion capacity of a subject and may, according to animal studies, serve as a proxy for β-cell mass (22).

Research Design and Methods

Hyperglycaemic clamp cohorts

Four independent studies from the Netherlands (NL) and Germany (D) were used. The clinical characteristics of the study groups are given in Table 6.1. Details of three of the four samples have previously been described (Hoorn (NL, 137 IGT); Utrecht (NL, 60 NGT/12 IGT); Tübingen (D, 83 NGT/35 IGT)) (16). We have extended our study sample with a cohort selected from the Netherlands Twin Register (NTR, 113 NGT/7 IGT) (26). This cohort consists of a mixed sample of twins and non-twin sibs recruited from 50 families (family size 1-9). In total the NTR twin sample includes 66 monozygotic twins (31 pairs), 25 dizygotic twins (11 pairs) and 29 non-twin sibs.

Table 6.1 Clinical characteristics of the hyperglycaemic clamp cohorts

	The Netherlands			Germany
	Hoorn[a]	Utrecht[a]	NTR Twins[a]	Tübingen[a]
N (NGT/IGT)	137 (0/137)	72 (60/12)	120 (113/7)	118 (83/35)
Sex (M/F)	64/73	17/55	55/65	51/67
Age (y)	60.5 ± 8.7	46.6 ± 6.7	31.6 ± 6.4	39.2 ± 13.2
BMI (kg/m^2)	28.0 ± 4.0	25.9 ± 3.8	24.1 ± 3.5	25.5 ± 5.4
Fasting plasma glucose (mmol/l)	6.3 ± 0.7	4.7 ± 0.5	4.6 ± 0.4	5.1 ± 0.7
2-hr plasma glucose (mmol/l)	8.8 ± 1.7	5.7 ± 1.6	5.4 ± 1.2	6.5 ± 2.0
Fasting plasma insulin (pmol/l)	62 (46-91)	36 (24-54)	34 (26-47)	43 (30-66)

Data are means ± SD or median (interquartile range). [a] Original population from which the cohort originated (22-27)

Hyperglycaemic clamp procedure

All participants underwent a hyperglycaemic clamp at 10 mmol/l glucose for at least two hours (22-25;27). After a priming infusion of glucose to acutely raise blood glucose levels, blood glucose levels were measured with a glucose analyser and kept constant at 10 mmol/l during the whole clamp. Insulin levels were measured with immunoassays as previously described (22-25;27). In order to correct for this and possible other differences between centres we introduced a dummy variable (study centre) in our statistical analyses. First-phase insulin secretion was determined as the sum of the insulin levels during the first 10 minutes of the clamp. Second-phase insulin secretion was determined as the mean of the insulin levels during the last 40 minutes of the second hour of the clamp (80-120 min). The insulin sensitivity index (ISI) was calculated by relating the glucose infusion rate (M) to the plasma insulin concentration (I) during the last 40 min of the second hour of the clamp (M/I). Mitrakou et al (28) compared the insulin sensitivity index (ISI) determined with a hyperglycaemic clamp with insulin sensitivity as determined using the euglycaemic-hyperinsulinaemic clamp in the same subjects, and found a good agreement between the

two methods. The disposition index (DI) was calculated by multiplication of first-phase insulin secretion and ISI, in order to quantify insulin secretion in relation to the ambient insulin sensitivity (29;30).

Subjects from Tübingen and the NTR twin sample both underwent an extended clamp using additional GLP-1 and arginine stimulation as described previously (22). GLP-1 stimulated insulin release was measured as the mean incremental area under the curve (160 to 180 min) following GLP-1 stimulation (4.5 pmol kg^{-1} bolus for 1 min at t=120 followed by a continuous infusion of 1.5 pmol kg^{-1} min^{-1}). In the Dutch NTR twin cohort slightly lower GLP-1 concentrations were used (1.5 pmol kg^{-1} and 0.5 pmol kg^{-1} min^{-1} respectively). Arginine stimulated acute insulin release was measured by injecting a bolus of 5 grams arginine hydrochloride at t=180 as described previously (22). The acute insulin response to arginine was calculated as the mean incremental area under the curve from 182 to 185 min.

Genotyping

Based on the available literature regarding GSIS and the novel type 2 diabetes mellitus genes we selected gene variants in *TCF7L2* (rs7903146), *KCNJ11* (rs5219), *CDKAL1* (rs7754840), *IGF2BP2* (rs4402960), *HHEX/IDE* (rs1111875), *SLC30A8* (rs13266634), *CDKN2A/B* (rs10811661) and *MTNR1B* (rs10830963) for genotyping. Results of the association analysis of the effect of the individual genes on GSIS during hyperglycaemic clamps in the Dutch Hoorn and Utrecht study and the German Tübingen study have been published previously (15-18). β- and *P*-values for all four samples combined, including the Dutch NTR twins are given in Table 6.2. All SNPs were measured using either the Sequenom platform (Sequenom, San Diego, USA) or Taqman SNP genotyping assays (Applied Biosystems, Foster City, USA) in all subjects. The genotyping success rate was above 97% for all SNPs and samples measured in duplicate (~5%) revealed no errors.

Statistics

We combined the information of the SNPs using an allele count model (9). We summed the number of risk alleles carried by each individual assuming an equal and additive effect of each allele. The effect of the sum score of risk alleles on the responses was examined by calculating the beta's for the risk allele score with linear generalised estimating equations (GEE) which takes into account the family relatedness when computing the standard errors

(i.e. in the twin sample). For ease of interpretation the exponent beta's (10^{β}) are given throughout the manuscript. For analyses of first and second-phase GSIS, GLP-1 and Arginine stimulated insulin secretion adjustment for age, gender, BMI, study centre, glucose tolerance status and ISI was used. For the analysis of ISI and DI, ISI was removed from the model. All outcome variables were log-transformed prior to analysis. Logistic regression with adjustment for age, gender and BMI was used to test associations with dichotomous endpoints like the absence of a first-phase insulin peak and type 2 diabetes mellitus. A priori power calculations showed that the design used in this study would allow the detection of a difference in insulin secretion between 10 (glucose) to 25% (GLP-1, Arginine) with 80% power ($\alpha<0.05$) depending on the stimulus used and allele frequency. All data are given as estimated mean (95%-CI) unless otherwise stated. After correction for multiple hypothesis testing results were regarded significant at $p \leq 0.008$ (six tests). For all statistical analyses SPSS version 16.0 software (SPSS, Chicago, USA) was used.

Results

The risk allele counts for the eight β-cell genes were normally distributed in our participants (figure 6.1).

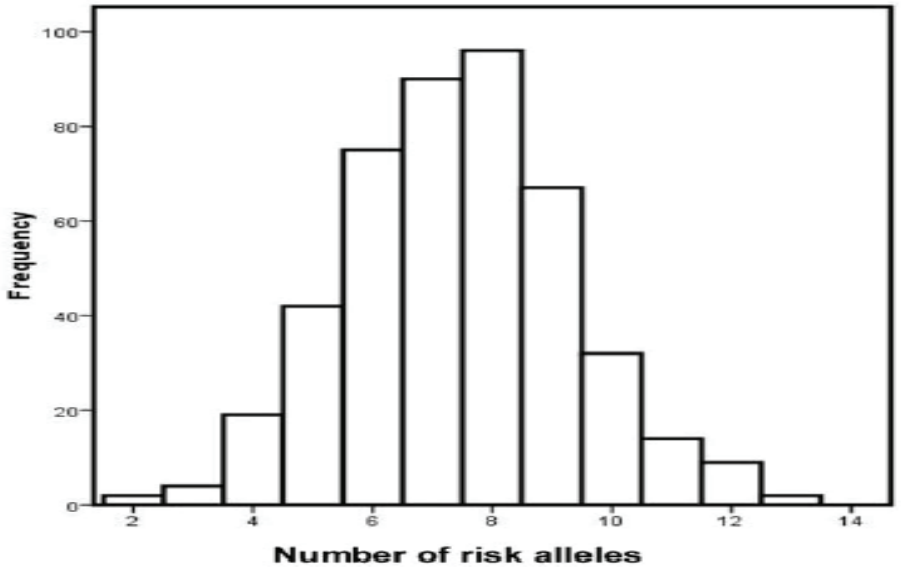

Fig 6.1 Distribution of risk alleles in the study sample

There was a significant inverse correlation between the number of risk alleles and first-phase GSIS (β=0.95 [95% CI 0.93 – 0.97], p=7.1 * 10^{-6}) (figure 6.2), indicating that first-phase GSIS decreases with a factor 0.95 with each additional risk allele.

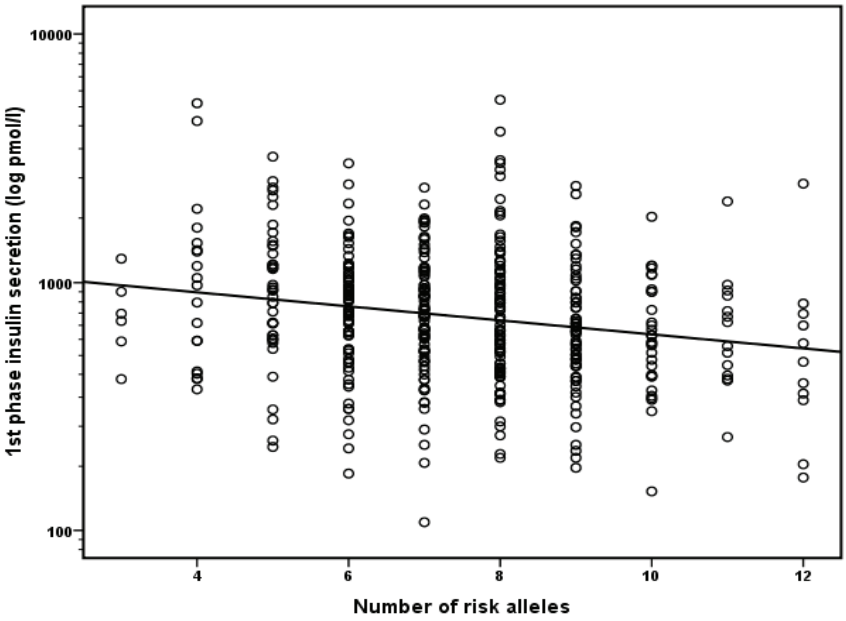

Figure 6.2 First-phase GSIS in relation to the risk alleles counts for the eight loci. Each circle represents an independent participant. The line represents the regression line after adjustment for age, sex, BMI, study centre, glucose tolerance status, and ISI.
β = 0.95 (95% CI 0.93-0.97; P = 7.1 x 10^{-6}.

The observed effect size on first-phase GSIS was equal in both normal and impaired glucose tolerant subjects (β_{NGT}=0.95, p=4.6 * 10^{-5} and β_{IGT}=0.95, p=0.015 respectively). Furthermore the effect was present in each of the separate study samples (β range 0.93 to 0.96, all p≤0.08). There was no significant effect of the number of risk alleles on second-phase GSIS or ISI (both p≥0.13). However there was also an inverse correlation with the DI as measured by the clamp (β=0.96 [95% CI 0.94 – 0.99], p=1.6 * 10^{-3}). The risk allele score explains 4% of the variance in first-phase GSIS and 5% of the variance in the disposition index.

To examine whether our results can be attributed to the effect of one or more single loci we also added the single loci to the model with the risk allele score, however none of the single loci remained significant in this analysis (all p>0.3). Previously we showed that three single loci are significantly associated with first-phase GSIS (*CDKAL1, IGF2BP2* and *MTNR1B*, (Table 6.2) (15-18;31). Therefore we also tested a model which includes the three significant single loci and a combined risk allele score for the remaining five loci (*TCF7L2, KCNJ11, HHEX, SLC30A8* and *CDKN2A/B*). In this analysis the five gene risk allele score still added significant information to the model (p<0.05).

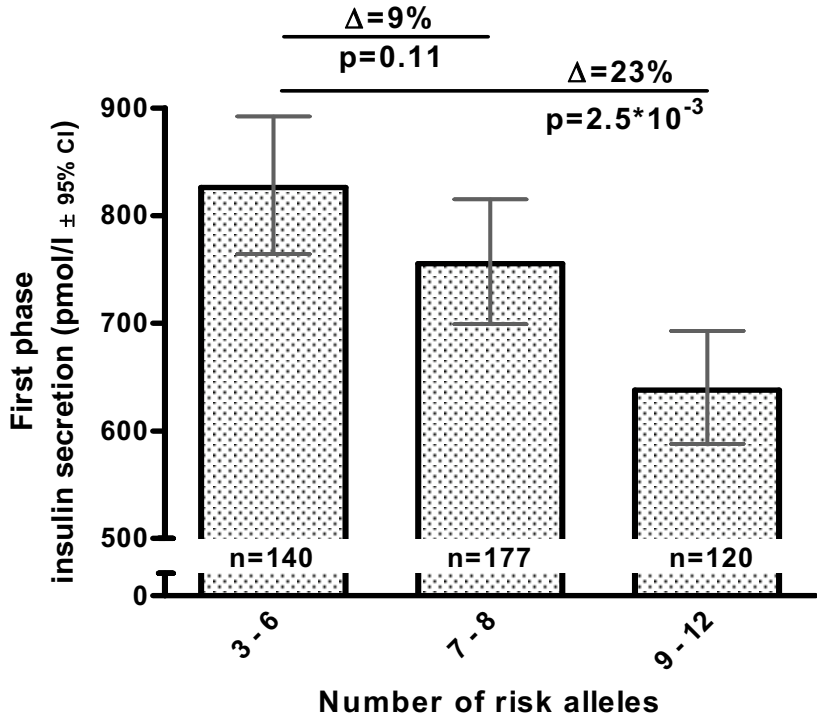

Figure 6.3 Mean estimated first-phase GSIS in three different risk allele strata. Those with three to six risk alleles were used as a reference group

For ease of interpretation we next stratified the participants into three approximately equally sized strata; carriers of a low (less than 7 risk alleles, n=140, 32%), medium (7-8 risk alleles, n=177, 40%) and high number of risk alleles (more than 8 risk alleles, n=120, 28%).

Table 6.2 Results in the individual cohorts.

	Hoorn study cohort				
	Low (≤6 risk alleles	Medium (7-8 risk alleles)	High (≥9 risk alleles)	P_{unadj}	P_{adj}
N (NGT/IGT)	43 (0/43)	63 (0/63)	36 (0/36)		
Age (years)	61 ± 1	61 ± 1	59 ± 1	0.26	
Sex (male/female)	19/24	34/29	13/23	0.22	
BMI (kg/m^2)	28.0 ± 0.6	28.0 ± 0.5	27.7 ± 0.6	0.94	
First-phase insulin secretion	691 (409-956)	587 (375-900)	488 (352-733)	0.23	0.14
Second phase insulin secretion	220 (186-352)	257 (169-342)	238 (141-389)	0.89	0.63
ISI	0.10 (0.07-0.16)	0.10 (0.07-0.17)	0.12 (0.08-0.18)	0.63	0.39
Disposition index (μmol min^{-1} kg^{-1})	69 (43-94)	66 (45-111)	59 (38-89)	0.54	0.46
	Utrecht Cohort				
N (NGT/IGT)	19 (14/5)	33 (29/4)	20 (17/3)		
Age (years)	47± 2	45± 1	49± 1	0.17	
Sex (male/female)	3/16	10/23	4/16	0.46	
BMI (kg/m^2)	26.7 ± 0.9	25.2 ± 0.7	26.2 ± 0.7	0.39	
First-phase insulin secretion	954 (738-1332)	780 (564-1023)	852 (372-1073)	0.09	$5.7*10^{-4}$
Second phase insulin secretion	278 (194-366)	246 (183-320)	281 (215-341)	0.96	0.84
ISI	0.17 (0.12-0.30)	0.18 (0.1-0.28)	0.19 (0.14-0.27)	0.95	0.90
Disposition index (μmol min^{-1} kg^{-1})	213 (93-242)	143 (91-206)	154 (79-192)	0.24	$7.2*10^{-3}$

Table 6.2 Continued. Results in the individual cohorts.

	Tübingen study cohort				
	Low (≤6 risk alleles	Medium (7-8 risk alleles)	High (≥9 risk alleles)	P_{unadj}	P_{adj}
N (NGT/IGT)	34 (28/6)	47 (36/11)	37 (19/18)		
Age (years)	40 ± 3	35 ± 2	44 ± 2	0.007	
Sex (male/female)	15/19	22/25	14/23	0.71	
BMI (kg/m^2)	25.9 ± 0.9	24.8 ± 0.8	26.2 ± 0.9	0.44	
First-phase insulin secretion	928 (510-1198)	658 (478-1119)	568 (421-892)	0.06	0.02
Second phase insulin secretion	226 (156-346)	238 (161-346)	197 (137-302)	0.43	0.38
ISI	0.13 (0.09-0.20)	0.12 (0.08-0.17)	0.13 (0.08-0.22)	0.82	0.49
Disposition index (μmol min^{-1} kg^{-1})	104 (68-176)	91 (57-155)	74 (53-113)	0.03	0.10
	NTR-VUmc Twin study Cohort				
N (NGT/IGT)	46 (45/1)	43 (39/4)	31 (29/2)		
Age (years)	32± 1	31± 1	31± 1	0.64	
Sex (male/female)	21/25	22/21	12/19	0.58	
BMI (kg/m^2)	24.0 ± 0.6	24.3 ± 0.5	24.0 ± 0.6	0.92	
First-phase insulin secretion	887 (634-1334)	844 (592-1233)	679 (520-839)	0.06	$9.4*10^{-4}$
Second phase insulin secretion	219 (157-363)	218 (177-380)	213 (162-305)	0.40	0.5
ISI	0.20 (0.15-0.31)	0.23 (0.12-0.32)	0.23 (0.13-0.34)	0.85	0.33
Disposition index (μmol min^{-1} kg^{-1})	182 (137-256)	178 (148-223)	167 (108-199)	0.10	0.039

Table 6.2 continued. Results in the individual cohorts

Other non-glucose secretagogues

	Low (≤6 risk alleles)	Medium (7-8 risk alleles)	High (≥9 risk alleles)	P_{unadj}	P_{adj}
Tübingen study Cohort					
GLP-1 stimulated insulin secr.	2941 (2065-5465)	2865 (1905-4110)	2021 (1093-2937)	0.01	$9.7*10^{-4}$
Arginine stimulated ins. secr.	2308 (1927-4242)	2405 (1217-3145)	1876 (1573-2752)	0.06	0.29
Peak level Arginine	6784 (5339-11911)	5957 (4218-8167)	4970 (3280-7167)	0.28	0.02
NTR-Vumc Twin study Cohort					
GLP-1 stimulated insulin secr.	1177 (617-2365)	1162 (794-2587)	1184 (726-2043)	0.75	0.40
Arginine stimulated ins. secr.	2245 (1368-2787)	2054 (1734-3032)	1888 (1175-3001)	0.43	0.84
Peak level Arginine	4529 (2670-7154)	4639 (3085-6939)	3868 (2627-5797)	0.59	0.89

insulin secretion in pmol/l; ISI = insulin sensitivity index (μmol min^{-1} kg^{-1}[pmol/l]$^{-1}$); Data are unadjusted mean ± SEM or median (interquartile range). Insulin secretion, insulin sensitivity index and disposition index were log-transformedbefore analysis. The table shows *P* values using an additive regression model, unadjusted (P_{unadj}) and adjusted (P_{adj}) for age, sex, BMI. ISI (where appropriate) and glucose tolerance status.

The characteristics of the three groups are given in Table 6.3 and the results per study sample in Table 6.4. Analysis of the difference in first-phase GSIS between these different strata showed a 9% and 23% lower first GSIS in the medium and high strata compared to the reference group (low) (P_{trend} $=5.9*10^{-6}$, Figure 6.3). Analysis of the differences in DI between these groups showed a 9% and 17% reduction in DI (P_{trend} $=2.9*10^{-3}$; Table 6.3). Again no significant difference between the strata was found for second-phase GSIS or ISI (both p>0.16). We did not observe an association of the number of risk alleles and GLP-1 stimulated insulin release during the clamp (Table 6.3). Furthermore the maximal insulin

Table 6.3 Clinical characteristics of three stratified groups for number of risk alleles.

Group	n	Sex (M/F)	Age (yrs)	BMI (kg/m^2)	First-phase insulin response (pmol/l)	Second-phase insulin response (pmol/l)	ISI	Disposition index (µmol/min/kg)	GLP-1 stimulated insulin release *	Arginine stimulated insulin release*
Low	141	58/83	45 ±15	26.0 ± 4.6	826 (764-892)	248 (232-265)	0.142 (0.130-0.156)	118 (108-129)	1792 (1541-2084)	2145 (1930-2385)
Medium	183	88/95	45 ±15	25.8 ± 4.5	755 (699-815)	252 (236-269)	0.140 (0.128-0.153)	108 (100-116)	1698 (1441-2002)	1982 (1747-2249)
High	123	42/81	45 ±13	26.1 ± 4.4	638 (588-693)	239 (221-258)	0.158 (0.142-0.174)	98 (90-107)	1614 (1354-1923)	2080 (1855-2332)
β_1					0.88 (0.83;0.93)	0.98 (0.93; 1.03)	ND	ND	0.95 (0.84; 1.07)	0.98 (0.91; 1.06)
P-					$5.9*10^{-6}$	0.50			0.38	0.65
β_2					0.87 (0.81;0.93)	0.96 (0.90; 1.03)	1.05 (0.98; 1.13)	0.91 (0.86;0.97)	0.92 (0.83;1.06)	0.97 (0.89; 1.06)
P-					$1.8*10^{-5}$	0.27	0.16	$2.9*10^{-3}$	0.28	0.48

Data are means ± SD or estimated means using model 1 (95% CI). Low = carriers of less than 7 risk alleles, medium = carriers of 7 or 8 risk alleles, high = carriers of more than 8 risk alleles. ISI = Insulin sensitivity index ((µmol/min/kg/pmol/l). Insulin release in pmol/l. All variables were log-transformed before analysis. *P*-values were computed for different additive models using linear generalised estimating equations (GEE) which take into account the family relatedness when computing the standard errors. Model 1; adjusted for study centre, glucose tolerance status, age, gender, BMI and ISI . Model 2; adjusted for study centre, glucose tolerance status, age, gender, BMI *available for 224 subjects from the Tübingen and NTR sample. .ND, not determined

secretion capacity as measured by arginine stimulation was not affected by the number of risk alleles present (p=0.65, Table 6.3).

Recently we have shown that a four gene risk allele score alters the age related decline in β-cell function in obese subjects as measured by OGTT (11). Although we have a limited number of obese subjects in the present study (BMI ≥30 kg/m^2, n=66) we noted a similar increased decline in β-cell function in obese subjects with a higher number of risk alleles (first-phase GSIS: β_{low}=1.01 [0.99 – 1.03], *P*=0.46); β_{medium}=0.98 [0.96 – 0.99], *P*=1.1*10^{-3}); β_{high}=0.97 [0.96 – 0.99], *P*=5.5*10^{-3}).

Previously we have shown that the absence of a first-phase insulin peak is a strong predictor of future development of type 2 diabetes mellitus in subjects with impaired glucose tolerance (24). In the present study subjects with IGT without a first-phase peak had on average 1.28 (95% CI 0.71-1.85) more risk alleles than those with a peak (*P*=1.0*10^{-5}). In the three strata the frequency of an absent first-phase peak increased from 12% in the low group to 40% in the high stratum (P_{trend}=6.9*10^{-4}, adjusted for age, gender and BMI, Table 6.5). Those with a medium or high number of risk alleles also had an increased risk of conversion to type 2 diabetes mellitus during follow-up, however due to the small numbers this was not significant.(Table 6.5).

Legend for Table 6.4 Association results for the single genes
Data are represented as β's (95% CI), RAF, risk allele frequency. All variables were log-transformed before analysis.β and *P* values were computed for additive models using linear generalised equations(GEE) which takes into account the family relatedness when computing the standard errors. First and second phase glucose stimulated insulin secretion, GLP-1 and arginine stimulated secretion were adjusted for study centre, family relatedness, glucose tolerance status, age, gender, BMI and ISI. ISI and Disposition index were adjusted for study centre, family relatedness, glucose tolerance status, age, gender and BMI. * available for 224 subjects from the Tübingen and NTR-VUmc twin sample. [1]Data for the Hoorn, Utrecht and Tübingen samples originated from 't Hart et al (15), [‡]Tschritter et al and 't Hart et al (18, 15) and [§]Groenewoud et al (16). Data for the Dutch sample from Simonis-Bik et al(14), Data from the Tübingen study are from Staiger et al (31).

Table 6.4 Association results for the single genes

Gene	RAF	First-phase insulin response (pmol/l)	Second-phase insulin response (pmol/l)	Insulin sensitivity index (µmol/min/kg/ pmol/l)	Disposition index (µmol/min/kg)	GLP-1 stimulated insulin release* (pmol/l)	Arginine stimulated insulin release* (pmol/l)
TCF7L2, rs7903146 †							
β	0.32	0.95 (0.89;1.02)	1.02 (0.96;1.09)	1.09 (0.99;1.19)	1.00 (0.93;1.07)	1.02 (0.89;1.16)	0.97 (0.87;1.09)
P		0.18	0.48	0.07	0.95	0.81	0.67
KCNJ11, rs5219 ‡							
β	0.37	0.96 (0.89;1.03)	0.98 (0.92;1.04)	1.03 (0.95;1.11)	0.96 (0.91;1.05)	0.94 (0.83;1.06)	0.97 (0.89;1.05)
P		0.22	0.49	0.49	0.56	0.32	0.44
CDKAL1, rs7754840 §							
β	0.33	0.89 (0.83;0.95)	0.98 (0.92;1.04)	0.96 (0.89;1.04)	0.86 (0.80;-0.93)	0.94 (0.83;1.06)	0.96 (0.86;1.06)
P		$6.3*10^{-4}$	0.45	0.33	$1.5*10^{-4}$	0.31	0.42
IGF2BP2, rs4402960 §							
β	0.31	0.94 (0.87;1.00)	0.96 (0.91;1.02)	1.05 (0.98;1.12)	0.97 (0.91;1.05)	0.86 (0.75;0.99)	0.95 (0.86;1.04)
P		0.05	0.16	0.16	0.49	0.04	0.24
HHEX/IDE, rs1111875 §							
β	0.60	0.94 (0.88;1.00)	0.97 (0.92;1.03)	1.06 (0.98;1.15)	0.96 (0.89;1.03)	1.00 (0.89;1.14)	1.02 (0.93;1.11)
P		0.07	0.40	0.17	0.26	0.94	0.74
SLC30A8, rs13266634 §							
β	0.72	0.97 (0.90;1.04)	0.99 (0.93;1.05)	1.07 (0.98;1.16)	1.02 (0.94;0.91)	0.97 (0.84;1.11)	0.96 (0.87;1.06)
P		0.38	0.69	0.13	0.64	0.63	0.43
CDKN2A/B, rs10811661 §							
β	0.81	1.01 (0.93;1.10)	1.03 (0.97;1.11)	0.94 (0.85;1.03)	0.98 (0.89;1.07)	1.08 (0.94;1.24)	1.06 (0.95;1.19)
P		0.83	0.37	0.18	0.63	0.29	0.31
MTNR1B, rs10830963 ‖							
β	0.26	0.92 (0.86;1.02	1.03 (0.97;1.09)	0.96 (0.88;1.05)	0.90 (0.84;0.97)	1.12 (0.99;1.27)	1.07 (0.99;1.17)
P		0.01	0.40	0.37	$4.9*10^{-3}$	0.07	0.10

Table 6.5 Impaired glucose tolerant group details and follow-up.

Group (number of risk alleles)	n	1st phase peak absent/present	Type 2 diabetes during follow-up (n=93) yes/no
Low (≤6)	51	6/45 (0.12)	9/20 (0.31)
Medium (7-8)	75	21/54 (0.28)	14/24 (0.37)
High (≥9)	47	20/30 (0.40)	13/13 (0.50)
P		$4.7*10^{-3}$	0.16
$P_{\text{model 1}}$		$6.9*10^{-4}$	0.19

Stratification according to the number of risk alleles in subjects with IGT only. Absence of the first-phase peak was defined according to the method of Nijpels et al (24). Numbers in parenthesis are percentages of total. *P* = unadjusted; $P_{\text{model 1}}$ is *P* value after logistic regression analysis adjusted for age, sex and BMI.

Discussion

In this study we have shown that a risk allele score for eight β-cell loci is associated with lower glucose-stimulated first-phase insulin secretion but not with other measures of β-cell function. Previously three other groups investigated the relationship between a risk allele score of β-cell loci and glucose stimulated insulin secretion. Pascoe et al. (12) used a risk allele score of 7 loci (*TCF7L2*, *KCNJ11*, *HHEX/IDE*, *CDKAL1*, *IGF2BP2*, *SLC30A8, CDKN2A/B*) whereas Haupt et al. used four loci for his main analyses (*TCF7L2, CDKAL1, HHEX/IDE, SLC30A8*) (10;11). Finally Stančáková et al. (13) recently reported the results of a risk allele score identical to the one used in this study All three groups mainly used data from OGTTs in non-diabetic volunteers and were able to show that their risk allele scores are inversely correlated with β-cell function. The novelty of our study is the fact that we used hyperglycaemic clamps with three different stimuli and the extended risk allele score including eight proven β-cell loci (*TCF7L2*, *KCNJ11*, *HHEX/IDE*, *CDKAL1*, *IGF2BP2*, *SLC30A8, CDKN2A/B* and *MTNR1B*, a gene for which it has recently been shown that it is associated with type 2 diabetes mellitus and reduced GSIS) (31-34). We were able to show that only the first-phase GSIS is associated with our combined risk allele score. In contrast, the other measures of β-cell function and insulin sensitivity were not associated. Furthermore we noted a significant association with a lower DI (which is the product of first-phase GSIS x ISI) suggesting that the investigated subjects are unable to compensate adequately for a diminished insulin sensitivity (30) . Previously it has been shown that a low DI is associated with glucose intolerance, and highly predictive for future diabetes mellitus (35). Remarkably the alterations in first-phase GSIS and DI are already present in normal glucose tolerant subjects suggesting that these defects are either present from birth on or develop well before the onset of hyperglycaemia. Interestingly it appears from our previous (11) and current data that environmental and or genetic factors acting on obesity interact with the genetic effects on β-cell function by altering the rate of the age related decline in β-cell function.

Our data highlight the importance of using different methods to investigate various aspects of insulin secretion. Whereas previous studies have shown that these genes together can affect overall insulin secretion during OGTTs, this report refines this important observation by showing that mainly the first-phase of glucose stimulated insulin secretion is

affected. This suggests that their combined effect primarily involves processes regulating the rapid recruitment and exocytosis of insulin granules following glucose stimulation. *SLC30A8* encodes a β-cell specific Zn transporter important for insulin storage, stability and granule exocytosis which may fit well with the observed defect (36). For the other genes it is less clear how they may affect the first-phase of GSIS. However, for one of the genes present in our risk allele score, *TCF7L2*, its role in insulin granule recruitment and exocytosis was recently supported by cell based studies using overexpression or knock-down of the gene (37).

As we and others have shown previously, the genetic variation in *TCF7L2,* mainly affects GLP-1 induced insulin secretion (17;38). In our current analysis, no resistance to GLP-1 induced insulin secretion with increasing number of risk alleles could be detected. This may have several reasons. First, this incretin resistance mediated by variation in *TCF7L2* is likely to be masked in the present analysis by the other 7 risk loci which have no known effect on incretin induced insulin secretion. This also suggests that the association of the risk allele score with first-phase GSIS is not dominated by the effects of a single locus, but rather reflects the addition of independent risks mechanisms from all loci together. This is further corroborated by the fact that when we tested for dominance of single genes, by adding them to the model, there were no associations with the single loci. Second, the power of the present analysis may be too low considering the relatively small subgroup in which we assessed the GLP-1 induced insulin secretion (n=224).

Several of the loci present in our risk allele score are putatively involved in transcriptional and/or cell cycle control, and it has been suggested that they may cause a reduced β-cell mass leading to the observed β-cell defects (20;39;40). However, our data show that our risk allele score of eight proven β-cell genes is not associated with arginine induced insulin secretion during hyperglycaemia, a marker of (near) maximal insulin secretion capacity which has been proposed as a proxy for β-cell mass (22).

The finding that a higher risk allele score has no effect on second-phase GSIS, incretin induced insulin secretion or maximal insulin secretion capacity in normal and impaired glucose tolerant subjects, does not exclude a relevant role of these mechanisms in the β-cell defects leading to type 2 diabetes mellitus. However, we may conclude that the reduced first-phase GSIS is the first and prominent β-cell defect leading to type 2 diabetes mellitus. This is in accordance with our recent finding that the absence of a first-phase insulin peak during hyperglycaemic clamps was the best predictor of future development of

type 2 diabetes mellitus in subjects with impaired glucose tolerance (HR 5.74 [95% CI 2.60-12.67]).(24). The strong correlation we observe between our risk allele score and the absence of a first-phase peak in our IGT subjects suggests that the eight genes we tested might be a better predictor of future type 2 diabetes mellitus compared to the generally used risk allele score of all known type 2 diabetes mellitus genes. However, due to the very small number of converters in our study this hypothesis should be tested in larger, more suitable, prospective study samples.

One of the strong aspects of our studies is the fact that we use four independent study samples from the Netherlands and Germany which largely reduced the chance of false positive findings. However, although this is the largest study sample available using hyperglycaemic clamps to test associations between diabetes mellitus loci and β-cell function we can not exclude that we have missed some of the more subtle alterations. Larger samples including type 2 diabetic subjects and perhaps other sophisticated tests of β-cell function would be needed to fully explore all aspects of β-cell function regarding these diabetes mellitus loci.

In conclusion we show that a combined score of risk alleles for eight β-cell loci is associated with reduced first but not second-phase GSIS or maximal insulin secretion capacity. Furthermore in IGT subjects there was a strong correlation with the absence of a first-phase insulin peak which is a strong predictor of future development of type 2 diabetes mellitus. Our data provide evidence that the β-cell loci identified thus far act mainly via detrimental effects on processes involved in the early, rapid recruitment and exocytosis of insulin granules after glucose stimulation rather than altering maximal insulin secretion capacity.

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Chapter 7

Gene variants in the novel type 2 diabetes loci *CDC123/CAMK1D, THADA, ADAMTS9, BCL11A* and *MTNR1B* affect different aspects of pancreatic β-cell function

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Abstract

Aims

Recently results from a meta-analysis of genome wide association studies have yielded a number of novel type 2 diabetes mellitus loci. However, conflicting results have been published regarding their effects on insulin secretion and insulin sensitivity. In this study we used hyperglycaemic clamps with three different stimuli to test associations between these novel loci and various measures of β cell function.

Methods

For this study, 336 participants, 180 normal glucose tolerant and 156 impaired glucose tolerant, underwent a two hour hyperglycaemic clamp. In a subset we also assessed the response to GLP-1 and arginine during an extended clamp (n=123). All subjects were genotyped for gene variants in *JAZF1, CDC123/CAMK1D, TSPAN8/LGR5, THADA, ADAMTS9, NOTCH2/ADAMS30, DCD, VEGFA, BCL11A, HNF1B, WFS1* and *MTNR1B.*

Results

Gene variants in *CDC123/CAMK1D, ADAMTS9, BCL11A* and *MTNR1B* affected various aspects of the insulin response to glucose (all $p<6.9 \times 10^{-3}$). The *THADA* gene variant was associated with lower β-cell response to GLP-1 and arginine (both $p<1.6 \times 10^{-3}$) suggesting lower β-cell mass as a possible pathogenic mechanism. Remarkably, we also noted a trend towards an increased insulin response to GLP-1 in carriers of *MTNR1B* (P = 0.03) which may offer new therapeutic possibilities. The other seven loci were not detectably associated with β- cell function.

Conclusions

Diabetes mellitus risk alleles in *CDC123/CAMK1D, THADA, ADAMTS9, BCL11A* and *MTNR1B* are associated with various specific aspects of β-cell function. These findings point to a clear diversity in the impact that these different gene variants may have on (dys)-function of pancreatic β-cells.

Introduction

Genome wide association studies (GWAS) have revealed a large number of novel type 2 diabetes mellitus susceptibility loci (1-4). Most of the genes identified during the first wave of GWAS results are shown to affect β-cell function as indicated by lower insulin responses to oral (OGTT) or intravenous (IVGTT) glucose tolerance tests (5). By applying the hyperglycaemic clamp methodology, considered the gold standard for measurements of β-cell function, we further refined the observed β-cell defects to defects in first but not second phase glucose stimulated insulin secretion (GSIS) (6) or incretin stimulated secretion (7). This differentiation is of importance to help resolve the pathogenic mechanism of the diabetes mellitus loci identified by GWA studies.

More recently the DIAGRAM consortium published at least six additional susceptibility loci, *JAZF1, CDC123/CAMK1D, TSPAN8/LGR5, THADA, ADAMTS9* and *NOTCH2/ADAM30* (8) and three putative susceptibility loci *DCD, VEGFA* and *BCL11A*. Studies using OGTTs have yielded conflicting results on the effects of these new loci on β-cell function and insulin sensitivity. Grarup et al. (9) reported β-cell dysfunction associated with gene variants in *JAZF1, TSPAN8/LGR5* and *CDC123/CAMK1D*. The results for *CDC123/CAMK1D* have only been replicated by Sanghera et al in Asian Indians (10) but not by three other studies in Caucasians. All of the other three studies also failed to replicate the results for *JAZF1* and *TSPAN8/LGR5* (11-13). Furthermore gene variants in three other loci have been established as true type 2 diabetes mellitus susceptibility loci, *HNF1B, WFS1* and *MTNR1B* (14-19). Although mutations in *HNF1B* are associated with β-cell defects in MODY it is unknown whether the type 2 diabetes mellitus associated common SNP is also associated with reduced β-cell function (14;15). It has been shown that *WFS1* associates with reduced oral (11;13;20-22) but not intravenous glucose stimulated insulin secretion (22). Schäfer et al. (22) further demonstrated that the *WFS1* gene affects GLP-1 stimulated insulin secretion during clamps. For the *MTNR1B* locus several studies have shown reduced insulin secretion in response to glucose (17-19;23;24).

In this study 180 normal and 156 impaired glucose tolerant (IGT) subjects originating from three independent studies in the Netherlands were genotyped for variants in *JAZF1, CDC123/CAMK1D, TSPAN8/LGR5, THADA, ADAMTS9, NOTCH2/ADAMS30, DCD, VEGFA, BCL11A, HNF1B, WFS1* and *MTNR1B*. We tested whether these loci are

associated with alterations in β-cell function as assessed by hyperglycaemic clamp methodology with, in a subset, two additional secretagogues, namely GLP-1 and arginine. Arginine stimulation during hyperglycaemia is a test of (near) maximal insulin secretion and has been proposed as a proxy for β-cell mass (25).

Research Design and Methods

Hyperglycaemic clamp cohorts

Participants originated from three independent studies in the Netherlands (26-30). The clinical characteristics of the study sample are given in table 7.1.

Table 7.1 Clinical characteristics of the individual study samples

	Hoorn*		Utrecht*		NTR Twins*
	IGT	NGT	IGT	NGT	IGT
N	137	64	12	116	7
Gender (M/F)	64/73	15/49	4/8	58/58	0/7
Age (y)	60.5 ± 8.6	45.9 ± 6.4	49.5 ± 7.7	31.5 ± 6.5	31.2 ± 3.2
BMI (kg/m^2)	28.1 ± 4.0	25.8 ± 3.8	26.7 ± 4.1	24.2 ± 3.5	24.5 ± 3.3
Fasting plasma glucose (mmol/l)	6.3 ± 0.7	4.6 ± 0.4	5.1 ± 0.4	4.6 ± 0.4	4.6 ± 0.6
2-hr plasma glucose (mmol/l)	8.8 ± 1.7	5.1 ± 1.0	8.5 ± 1.2	5.2 ± 1.1	8.1 ± 0.3
Fasting plasma insulin (pmol/l)	62 (46-91)	30 (24-42)	66 (42-78)	34 (27-51)	39 (29-60)
First-phase insulin response	587 (378-895)	885 (644-1217)	678 (461-909)	814 (589-1162)	795 (693-1210)
Second-phase insulin response	255 (176-354)	260 (191-365)	251 (186-307)	218 (162-358)	217 (210-434)
Insulin sensitivity index	0.108 (0.07-0.16)	0.190 (0.13-0.28)	0.111 (0.08-0.26)	0.227 (0.15-0.32)	0.123 (0.11-0.18)
Disposition index (μmol/min/kg)	65 (42-92)	172 (103-238)	72 (55-128)	180 (140-234)	138 (82-151)
GLP-1 stimulated insulin release	NA	NA.	NA	1225 (734-2587)	848 (577-1239)
Arginine stimulated insulin release	NA.	NA.	NA.	2188 (1526-2973)	1673 (1438-1908)

Data are means ± SD, median (interquartile range) or *n*.
* Original population from which the cohort originated (26;28-30). NA, not available. Insulin response in pmol/l. Insulin sensitivity index in μmol min $^{-1}$ kg $^{-1}$[pmol/l]$^{-1}$.

In short we recruited for this study 137 IGT subjects from the Hoorn study, 76 subjects (64 NGT/12 IGT) from Utrecht(27;28) and 123 twins and sibs (116 NGT/7 IGT) from the Netherlands Twin Register (NTR) (30). The NTR twin sample includes 66 monozygotic, 28 dizygotic twins and 29 of their non-twin sibs recruited from 50 families. Details of the three individual samples have previously been described (6;26-30).

Hyperglycaemic clamp procedure

All participants underwent a hyperglycaemic clamp at 10 mmol/l glucose for at least two hours (26;28-30). First-phase insulin secretion was determined as the sum of the insulin levels during the first 10 minutes of the clamp. Second phase insulin secretion was determined as the mean of the insulin levels during the last 40 minutes of the second hour of the clamp (80-120 min). The insulin sensitivity index (ISI) was defined as the glucose infusion rate (*M*, μmol · min^{-1} · kg^{-1}) necessary to maintain the hyperglycaemic clamp divided by the plasma insulin concentration (*I*, pmol/l) during the last 40 min of the second hour of the clamp (*M/I*). Mitrakou et al (31)compared the insulin sensitivity index (ISI) determined with a hyperglycaemic clamp with insulin sensitivity as determined using the euglycaemic- hyperinsulinaemic clamp in the same subjects, and found a good agreement between the two methods. The disposition index (DI) was calculated by multiplication of first-phase insulin secretion and ISI, in order to quantify insulin secretion in relation to the ambient insulin sensitivity (32;33).

Subjects from the NTR twin sample underwent a modification of the extended clamp using additional GLP-1 and arginine stimulation as described previously by Fritsche et al. (25). GLP-1 stimulated insulin release was measured as the mean incremental area under the curve (160 to 180 min) following GLP-1 stimulation (1.5 pmol kg^{-1} bolus for 1 min at t=120 followed by a continuous infusion of 0.5 pmol kg^{-1} min^{-1}). Arginine stimulated acute insulin release was measured by injecting a bolus of 5 g arginine hydrochloride at t=180 as described previously (25). The acute insulin response to arginine was calculated as the mean incremental area under the curve from 182 to 185 min.

Genotyping

Based on the available literature regarding the novel type 2 diabetes mellitus genes we selected gene variants in *JAZF1* (rs864745), *CDC123/CAMK1D* (rs12779790), *TSPAN8/LGR5* (rs7961581), *THADA* (rs7578597), *ADAMTS9* (rs4607103),

NOTCH2/ADAM30 (rs2641348) (8), the putative type 2 diabetes mellitus genes *DCD* (rs1153188), *VEGFA* (rs9472138) and *BCL11A* (rs10490072) (8), *HNF1B* (rs757210) (14;15), *WFS1* (rs10010131) (16) and *MTNR1B* (rs10830963) (17-19). All SNPs were measured using either the Sequenom platform (Sequenom, San Diego, USA) or Taqman SNP genotyping assays (Applied Biosystems, Foster City, USA) in all individual subjects. The genotyping success rate was above 96% for all SNPs and samples measured in duplicate (~5%) were in complete concordance. All genotype distributions obeyed Hardy Weinberg equilibrium ($p \geq 0.05$) except for *MTNR1B* ($p=0.01$). SNP genotypes were recoded as 0, 1 or 2 with the 2 genotype as the at risk genotype reported in the original publications.

Statistics

The effect of the gene variants on the β-cell responses was examined with linear regression assuming an additive model unless otherwise stated. To take into account the family relatedness (i.e. in the twin sample) empirical standard errors were used (using the generalised estimating equations (GEE)). The analyses of first and second-phase GSIS, GLP-1 and arginine stimulated insulin secretion were adjusted for age, gender, BMI, study centre, glucose tolerance status (NGT/IGT) and ISI. For the analysis of ISI and DI, ISI was removed from the covariates. All outcome variables were log-transformed prior to analysis. In addition to the analysis of the pooled data we also performed a random effects meta-analysis of the results obtained in the three separate cohorts using Comprehensive Meta-Analysis version 2 software (www. Meta-analysis.com). A priori power calculations showed that the design used in this study would allow the detection of a difference in insulin secretion of approximately 15% (glucose) to 30% (GLP-1, arginine) with 80% power ($\alpha<0.05$) depending on the stimulus used and allele frequency of the SNPs. All data are given as estimated mean (95% CI) unless otherwise stated. After correction for multiple hypothesis testing results were regarded significant at $P \leq 0.008$ (six tests). Apart from the meta-analysis SPSS version 16.0 software (SPSS, Chicago, Il, USA) was used for all statistical analyses.

Results

As previously shown second-phase insulin secretion as measured with the hyperglycaemic clamp was only slightly reduced in the subjects with IGT ($P > 0.1$) whereas all other measures of glucose stimulated insulin release and ISI were significantly lower (all $P < 0.0001$, table 7.1) (28). Genotype distributions for each of the tested gene variants are given in table 7.2. Genotype distributions were comparable to other Caucasian populations. First, no associations were found with insulin sensitivity with the sole exception of *THADA*, where we noted a significantly lower insulin sensitivity index ($P = 6.9 \times 10^{-3}$) in carriers of the T risk allele. Five loci, however, significantly affected β-cell function. These associations are shown in table 7.2 and will be briefly summarized below. Throughout, reported *P* values represent the values obtained for the full model which includes the genotype of interest and age, gender, BMI, glucose tolerance status, family relatedness and insulin sensitivity (where appropriate) as covariates. A model without BMI yielded essentially the same results (data not shown). A meta-analysis of the results in the three separate study samples instead of the analysis of the pooled data yielded virtually identical results (data not shown).

CDC123/CAMK1D. The rs12779790 variant in the *CDC123/CAMK1D* locus was not significantly associated with first-phase GSIS, however, we do note a significantly decreased second-phase GSIS in carriers of the at risk genotype (table 7.2, $P = 4.9 \times 10^{-3}$). The response to GLP-1, arginine stimulation and insulin sensitivity were not significantly different although we do note a trend towards a reduced response to arginine (-32%, P = 0.015).

THADA. Because the protective C/C genotype of the rs7578597 SNP is only present in three subjects we pooled the CC and CT genotype groups. The TT risk genotype was not significantly associated with first-phase GSIS (P=0.77) but all other measures of β-cell function were reduced (11 to 37%), although not always statistically significant: second-phase insulin response ($P = 0.019$), disposition index ($P = 0.039$), GLP-1 (P=1.6x10^{-3}) and arginine stimulated insulin response (2.3×10^{-4}; table 7.2). As stated above we also noted a significantly lower insulin sensitivity index (ISI, $P = 6.9 \times 10^{-3}$) in carriers of the at risk genotype.

Table 7.2 Insulin response according to genotype.

Gene	n	First-phase insulin response (pmol/l)	Second-phase insulin response (pmol/l)	Insulin sensitivity index (μmol/min/kg/pmol/l)	Disposition index (μmol/min/kg)	*n* (GLP-1 and Arg)	GLP-1 stimulated insulin release* pmol/l)	Arginine stimulated insulin release* (pmol/l)
JAZF1, rs864745								
C/C	73	727 (652-812)	262 (236-292)	0.141 (0.122-0.162)	107 (95-121)	26	1034 (799-1337)	1728 (1495-1998)
C/**T**	161	723 (672-778)	239 (223-255)	0.155 (0.142-0.170)	111 (103-120)	48	1374 (1122-1683)	1992 (1727-2297)
T/T	100	759 (686-841)	263 (243-286)	0.160 (0.145-0.177)	124 (111-139)	49	1200 (951-1514)	2233 (1969-2532)
P		0.54	0.80	0.15	0.07		0.63	0.018
CDC123/ CAMK1D, rs12779790								
A/A	212	755 (704-810)	260 (245-275)	0.155 (0.143-0.168)	117 (109-127)	74	1318 (1094-1588)	2181 (1979-2403)
A/**G**	110	713 (656-774)	238 (220-258)	0.153 (0.138-0.169)	112 (101-123)	48	1106 (881-1389)	1817 (1588-2078)
G/G	12	617 (478-797)	200 (176-228)	0.146 (0.108-0.198)	94 (71-125)	1	1142 (913-1428)	1486 (1322-1671)
P		0.10	0.0049	0.68	0.16		0.24	0.015
TSPAN8/LGR5, rs7961581								
T/T	159	738 (687-793)	253 (237-270)	0.149 (0.135-0.164)	113 (103-123)	47	1253 (1028-1529)	2094 (1860-2357)
T/**C**	141	724 (668-784)	247 (229-265)	0.158 (0.142-0.175)	113 (105-123)	65	1222 (994-1503)	2024 (1797-2280)
C/C	34	738 (613-889)	254 (219-295)	0.160 (0.135-0.190)	118 (97-142)	11	1148 (796-1657)	1710 (1362-2146)
P		0.88	0.84	0.34	0.72		0.73	0..24
THADA, rs7578597								
C/C	3	905 (484-1694)	365 (317-421)	0.125 (0.067-0.230)	121 (80-182)	0	n.a.	n.a.
C/**T**	72	739 (662-825)	271 (247-296)	0.180 (0.160-0.204)	127 (113-142)	25	1783 (1352-2352)	2605 (2236-3035)
T/T	261	732 (689-778)	244 (232-257)	0.147 (0.137-0.158)	110 (103-118)	98	1120 (970-1292)	1897 (1744-2064)
P		0.77†	0.019†	0.0069†	0.039†		0.0016†	0.00023†

Table 7.2 Continued. Insulin response according to genotype

Gene	n	First-phase insulin response (pmol/l)	Second-phase insulin response (pmol/l)	Insulin sensitivity index (µmol/min/kg/pmol/l)	Disposition index (µmol/min/kg)	*n* (GLP-1 and Arg)	GLP-1 stimulated insulin release* pmol/l)	Arginine stimulated insulin release* (pmol/l)
ADAMTS9, rs4607103								
T/T	20	549 (467-646)	206 (172-246)	0.136 (0.106-0.175)	83 (69-99)	7	777 (597-1011)	1632 (1335-1994)
T/**C**	119	725 (668-787)	256 (238-274)	0.152 (0.137-0.169)	111 (101-123)	47	1291 (1028-1621)	1990 (1753-2260)
C/C	187	767 (714-824)	252 (237-268)	0.157 (0.145-0.171)	121 (112-130)	69	1244 (1032-1498)	2094 (1866-2350)
P		0.0059	0.26	0.32	0.0026		0.38 (D 0.002)	0.18 (D 0.046)
NOTCH2/ADAM30, rs2641348								
A/A	253	736 (692-782)	248 (234-262)	0.152 (0.141-0.163)	114 (107-121)	94	1226 (1045-1438)	2035 (1858-2228)
A/**G**	73	746 (661-841)	256 (230-285)	0.154 (0.133-0.179)	113 (97-131)	27	1228 (896-1683)	2036 (1671-2482)
G/G	10	654 (502-852)	278 (242-319)	0.189 (0.156-0.229)	121 (96-152)	2	1323 (1100-1593)	1398 (1251-1563)
P		0.76	0.33	0.37	0.89		0.93	0.59
DCD, rs1153188								
T/T	24	811 (670-982)	279 (243-321)	0.169 (0.136-0.210)	128 (103-160)	5	1448 (1143-1834)	2068 (1467-2915)
T/**A**	120	726 (675-781)	248 (231-267)	0.154 (0.138-0.171)	113 (103-124)	40	1018 (757-1368)	1976 (1723-2268)
A/A	192	732 (678-790)	247 (232-263)	0.152 (0.140-0.165)	113 (104-123)	78	1336 (1151-1551)	2043 (1845-2262)
P		0.55	0.29	0.49	0.48		0.27	0.83
VEGFA, rs9472138								
C/C	176	722 (674-774)	245 (231-260)	0.156 (0.145-0.169)	114 (106-123)	68	1207 (1014-1436)	1908 (1715-2121)
C/**T**	131	765 (704-832)	263 (243-284)	0.153 (0.136-0.172)	117 (106-130)	48	1278 (989-1652)	2203 (1942-2498)
T/T	28	695 (578-835)	229 (197-268)	0.141 (0.115-0.174)	101 (83-123)	7	1096 (556-2161)	1922 (1267-2917)
P		0.77	0.80	0.44	0.55		0.97	0.35

Table 7.2 continued :Insulin response according to genotype

Gene	n	First-phase insulin response (pmol/l)	Second-phase insulin response (pmol/l)	Insulin sensitivity index (μmol/min/kg/pmol/l)	Disposition index (μmol/min/kg)	*n* (GLP-1 and Arg)	GLP-1 stimulated insulin release* pmol/l)	Arginine stimulated insulin release* (pmol/l)
BCL11A, rs10490072								
C/C	32	810 (703-934)	226 (199-256)	0.169 (0.141-0.201)	132 (111-158)	13	812 (595-1108)	1774 (1553-2028)
C/**T**	126	799 (738-866)	255 (237-274)	0.145 (0.131-0.161)	120 (110-132)	49	1266 (978-1639)	2073 (1814-2369)
T/T	178	685 (637-737)	251 (236-268)	0.157 (0.144-0.171)	107 (99-116)	61	1311 (1139-1508)	2040 (1810-2300)
P		0.0031	0.39	0.92	0.010		0.060 (D0.008)	0.41 (D 0.074)
HNF1B, rs757210								
C/C	118	746 (696-799)	255 (237-274)	0.149 (0.134-0.166)	112 (103-122)	51	1218 (966-1535)	2049 (1792-2342)
C/**T**	145	737 (672-809)	251 (233-270)	0.154 (0.139-0.170)	116 (105-128)	49	1265 (1034-1546)	2034 (1828-2263)
T/T	71	704 (634-782)	240 (218-263)	0.161 (0.144-0.179)	111 (99-125)	23	1174 (874-1577)	1946 (1586-2387)
P		0.38	0.33	0.35	0.99		0.93	0.70
WFS1, rs10010131								
A/A	39	623 (527-737)	258 (217-306)	0.160 (0.128-0.200)	99 (84-117)	11	1564 (1155-2120)	2311 (1773-3011)
A/**G**	176	751 (701-804)	257 (243-272)	0.149 (0.138-0.162)	114 (106-123)	66	1298 (1086-1551)	2066 (1854-2303)
G/G	119	749 (686-818)	238 (221-257)	0.158 (0.143-0.175)	119 (108-131)	46	1072 (848-1356)	1900 (1663-2171)
P		0.14	0.21	0.81	0.09		0.058	0.18
MTNR1B, rs10830963								
C/C	187	757 (706-813)	239 (226-253)	0.163 (0.150-0.177)	122 (112-131)	57	1044 (865-1259)	1869 (1675-2085)
C/**G**	113	758 (700-821)	270 (248-294)	0.139 (0.123-0.157)	110 (101-120)	49	1440 (1142-1814)	2157 (1868-2490)
G/G	35	561 (487-647)	239 (207-276)	0.158 (0.132-0.190)	90 (77-106)	17	1360 (1084-1705)	2231 (1973-2523)
P		0.010	0.27	0.22	0.0015		0.026	0.037

Data are estimated means (95% CI) unless otherwise indicated. Alleles identified as risk alleles for type 2 diabetes are indicated in bold. All variables were log-transformed before analysis. *P*-values were computed for additive models using linear generalised estimating equations, which takes into account the family relatedness when computing the standard errors. First and second phase GSIS, GLP-1 and arginine stimulated insulin secretion were adjusted for study centre, family relatedness, glucose tolerance status, age, sex, BMI and ISI. ISI and DI were adjusted for study centre, family relatedness, glucose tolerance status, age, sex and BMI.

*available for 123 subjects from the NTR twin sample.

† *P* values are for the recessive model.

Table 7.3 Insulin response according to genotype in NGT subjects (genes with significant effects only).

Gene	NGT				
	n	First-phase insulin response (pmol/l)	Second-phase insulin response (pmol/l)	Insulin sensitivity index (μmol/min/kg/pmol/l)	Disposition index (μmol/min/kg)
CDC123/ CAMK1D, rs12779790					
C/C	109	888 (812-971)	257 (239-276)	0.202 (0.182-0.225)	178 (160-199)
C/**T**	66	792 (714-877)	247 (226-269)	0.202 (0.177-0.230)	162 (146-181)
T/T	5	720 (540-962)	197 (164-237)	0.221 (0.166-0.295)	154 (122-193)
P		0.034	0.14	0.89	0.13
THADA, rs7578597					
C/C	1	1109(1005-1224)	388 (355-424)	0.280 (0.247-0.318)	264 (240-290)
C/**T**	43	843 (728-976)	272 (244-303)	0.249 (0.215-0.288)	192 (164-224)
T/T	136	840 (776-910)	243 (228-259)	0.190 (0.173-0.208)	164 (150-180)
P		0.91*	0.057*	0.0017*	0.073*
ADAMTS9, rs4607103					
T/T	12	694 (581-830)	207 (171-251)	0.182 (0.122-0.271)	137 (110-170)
T/**C**	71	832 (759-912)	245 (224-267)	0.204 (0.180-0.231)	169 (150-191)
C/C	97	867 (788-955)	259 (241-279)	0.204 (0.182-0.228)	176 (160-194)
P		0.11	0.054	0.71	0.12
BCL11A, rs10490072					
C/C	18	976 (828-1151)	230 (197-269)	0.213 (0.172-0.263)	210 (170-258)
C/**T**	71	885 (799-979)	261 (241-283)	0.191 (0.168-0.217)	175 (155-197)
T/T	91	785 (719-858)	246 (227-267)	0.211 (0.188-0.236)	161 (146-177)
P		0.0066	0.96	0.59	0.019
MTNR1B, rs10830963					
C/C	91	853 (778-936)	243 (226-260)	0.212 (0.189-0.237)	177 (159-196)
C/**G**	65	882 (802-970)	267 (241-295)	0.188 (0.160-0.221)	172 (154-193)
G/G	21	696 (593-818)	241 (210-276)	0.228 (0.191-0.272)	157 (135-183)
P		0.23	0.49	0.83	0.31

Table 7.3 continued Insulin response according to genotype in IGT subjects) (genes with significant effects only

		IGT			
Gene	n	First-phase insulin response (pmol/l)	Second-phase insulin response (pmol/l)	Insulin sensitivity index (μmol/min/kg/pmol/l)	Disposition index (μmol/min/kg)
CDC123/ CAMK1D, rs12779790					
C/C	103	717 (610-843)	249 (216-286)	0.109 (0.090-0.132)	79 (67-93)
C/**T**	44	715 (584-875)	208 (174-249)	0.102 (0.081-0.128)	77 (61-97)
T/T	7	564 (363-877)	186 (144-239)	0.096 (0.059-0.157)	60 (36-99)
P		0.42	0.0028	0.44	0.37
THADA, rs7578597					
C/C	2	920 (359-2358)	360 (270-478)	0.070 (0.035-0.138)	76 (46-124)
C/**T**	29	730 (570-936)	257 (209-316)	0.122 (0.092-0.162)	87 (68-112)
T/T	125	710 (606-833)	232 (202-266)	0.104 (0.087-0.125)	76 (64-90)
P		0.67*	0.14*	0.32*	0.21*
ADAMTS9, rs4607103					
T/T	8	487 (340-699)	193 (137-272)	0.093 (0.068-0.127)	52 (37-73)
T/**C**	54	699 (582-840)	252 (215-296)	0.102 (0.082-0.128)	74 (62-89)
C/C	94	747 (625-892)	227 (196-263)	0.111 (0.092-0.135)	84 (69-102)
P		0.051	0.75	0.22	0.012
BCL11A, rs10490072					
C/C	14	740 (553-990)	207 (159-268)	0.127 (0.091-0.177)	84 (60-119)
C/**T**	55	815 (663-1000)	231 (196-272)	0.101 (0.082-0.126)	85 (70-104)
T/T	87	670 (570-787)	238 (205-276)	0.108 (0.088-0.132)	74 (62-88)
P		0.10	0.33	0.73	0.19
MTNR1B, rs10830963					
C/C	96	762 (645-900)	226 (195-263)	0.115 (0.094-0.140)	88 (74-104)
C/**G**	48	732 (609-879)	260 (218-309)	0.093 (0.075-0.114)	73 (61-87)
G/G	14	509 (410-633)	223 (175-284)	0.096 (0.066-0.141)	53 (40-70)
P		0.0067	0.38	0.078	0.00036

Data are estimated means (95% CI) unless otherwise indicated. Alleles identified as risk alleles for type 2 diabetes are indicated in bold. All variables were log-transformed before analysis. *P*-values were computed for additive models using linear generalised estimating equations, which takes into account the family relatedness when computing the standard errors. First and second phase GSIS were adjusted for study centre, family relatedness, age, sex, BMI and ISI. ISI and DI were adjusted for study centre, family relatedness, glucose tolerance status, age, sex and BMI.

**P* values are for the recessive model

ADAMTS9. Analysis of rs4607103 in *ADAMTS9* provided evidence for an effect on first-phase GSIS. Carriers of the type 2 diabetes mellitus risk genotype 'CC' showed paradoxically a 40% increased first-phase GSIS compared to the non-risk 'TT' reference genotype (P = 5.9 x 10^{-3}). This effect was similar in direction in both NGT and IGT subjects (Table 7.3). Furthermore, the risk allele carriers also showed a higher disposition index (p=2.6x10^{-3}). Second-phase GSIS, the response to GLP-1 or arginine and ISI were not significantly affected by the *ADAMTS9* genotype.

BCL11A. Carriers of the rs10490072 'TT' risk genotype of the *BCL11A* locus had on average a 16% lower first-phase GSIS (P = 3.1 x 10^{-3}). The disposition index was also lower though not statistically significant (P = 0.010). Other measures of β-cell function and ISI were not significantly different (table 7.2).

MTNR1B. The risk allele for *MTNR1B* was significantly associated with a decreased disposition index (P = 1.5 x 10^{-3}) but not other measures of glucose stimulated insulin secretion. Although not statistically significant there were increased responses to GLP-1 (+30%, P = 0.026) and arginine stimulation (+19%, P = 0.037) in carriers of the risk allele for rs10830963.

Other novel type 2 diabetes mellitus loci. Gene variants in the *JAZF1, TSPAN8/LGR5, DCD, NOTCH2/ADAM30, VEGFA,* loci were not significantly associated with any of the β-cell measures or insulin sensitivity (Table 7.2).

Discussion

The DIAGRAM consortium and others recently showed that *JAZF1, CDC123/CAMK1D, TSPAN8/LGR5, THADA, ADAMTS9, NOTCH2/ADAMS30, HNF1B, WFS1, MTNR1B* and possibly also *DCD, VEGFA, BCL11A* should be added to the list of confirmed type 2 diabetes mellitus loci (8;14-19). In this study we have shown that gene variants in five of these loci are associated with measures of beta cell function obtained during hyperglycaemic clamps, either in response to glucose alone and/or in combination with other beta cell secretagogues during hyperglycaemia. In contrast to our previous work, which showed that most other known loci primarily affect first-phase GSIS (6;7); (34), the current set of loci also affected various other aspects of β-cell function.

***CDC123/CAMK1D,* rs12779790**. Previously Grarup et al (9) reported that the G risk allele of rs12779790 *CDC123/CAMK1D* was associated with a lower insulinogenic index, corrected insulin response (CIR) and area under the insulin/glucose curve during OGTTs . They also noted a lower disposition index in carriers of the G allele. The β-cell defect was confirmed in a study of subjects from Asian Indian descent (10). Three other studies in Caucasians failed to replicate the observation made by Grarup et al. However, in all three studies a similar, though not significant trend towards lower β-cell function could be observed (11-13). These results are in line with our observation of a lower insulin response to glucose stimulation. We also noted a trend towards a reduced insulin response after arginine stimulation (-32%, $P = 0.015$). Arginine stimulation during hyperglycaemia is a measure of (near) maximal insulin secretion and has been suggested as a proxy for β-cell mass. Given the putative role of *CAMK1D* in granulocyte function it seems plausible that this gene variant affects β-cell function by causing reduced β-cell mass due to enhanced apoptosis (35). Further research is, however, needed to verify this hypothesis.

***THADA,* rs7578597**. We have shown that homozygous carriers of the risk allele have lower levels of various measures of β-cell function. This was not previously reported in any of the OGTT based studies although Stancakova et al (13) showed some evidence for a reduced early phase insulin response ($P = 0.045$) . *THADA*, encoding Thyroid Adenoma Associated protein, has been suggested to be involved in the death receptor pathway and apoptosis (36). Given the fact that the gene variant is associated with reduced response to arginine stimulation during the clamp this could imply that those subjects with the

rs7578597 (T1187A) gene variant in *THADA* have a reduced β-cell mass due to increased apoptosis. Again further studies are needed to confirm our hypothesis of increased apoptosis and lower β-cell mass as the underlying disease mechanism. The *THADA* variant was the only variant associated with insulin sensitivity; this was however not corroborated by any of the other studies and may thus be a false positive association.

***ADAMTS9,* rs4607103**. Remarkably we noted a significantly increased first-phase GSIS and disposition index in carriers of the risk allele. The observed increased β-cell function was present in all separate samples and in NGT and IGT subjects when analysed separately, arguing against a chance finding. Also Lyssenko et al. (11) reported an increased DI during follow-up in carriers of the risk genotype. The other studies, however, did not report any changes in β-cell function or insulin sensitivity (9;10;12;13). Given these counterintuitive results and the unknown function of *ADAMTS9* in type 2 diabetes mellitus susceptibility and / or β-cell function our data warrant further replication and studies into the disease mechanism.

***BCL11A,* rs10490072**. For carriers of the risk allele in *BCL11A* we noted a significant reduction in first-phase GSIS. Only Staiger et al. (12) included *BCL11A* in their analyses and they did not corroborate our results. *BCL11A*, encoding B-cell CLL/lymphoma 11A, has been implicated in several blood related phenotypes and acts as a DNA-sequence specific transcriptional repressor, acting on genes like *BCL6, COUP-TF* and *SIRT1* (37). Sirtuins, like *SIRT1* have been implicated in several processes directly linked to type 2 diabetes mellitus (38) and one may speculate that *BCL11A* gene variants exert their effect via the regulation of *SIRT1* expression.

***MTNR1B,* rs10830963**. Recently the Melatonin receptor 1B gene has been identified as a novel type 2 diabetes mellitus and fasting plasma glucose gene (17-19). Also in this study the risk allele was associated with increased fasting plasma glucose levels (P = 0.004). Several studies have shown that gene variants in this locus are associated with lower oral and intravenous glucose stimulated insulin secretion (39). Our results regarding the lower disposition index seem to corroborate these previous findings. Though not formally statistically significant due to the smaller sample size we, surprisingly, also noted increased insulin responses towards GLP-1 (+30%) and arginine stimulation (+19%). This seems to contradict the observed decreased insulin response to oral glucose during OGTT in *MTNR1B* carriers since it is known that the insulin response to oral glucose is in part mediated via the positive effects of incretins, like GLP-1 (40). In vitro short term exposure

of β-cells and islets to melatonin results in a decreased insulin response to glucose and GLP-1 (39) but studies using INS-1E cells have also suggested that prolonged exposure to melatonin, in contrast to short term exposure, results in a potentiation of the response to GLP-1 (41). If replicated our results indicate that carriers of this gene variant may well benefit from treatment with GLP-1 agonists or DPP-IV inhibitors.

WFS1 Previously it has been reported that *WFS1* gene variants are associated with reduced insulin response to oral but not intravenous glucose (11;13;20-22). In line with those previous reports we also could not detect an effect of intravenous glucose. Furthermore, Schäfer et al (22) demonstrated a reduced response to GLP-1 stimulation during hyperglycaemic clamps. In this study with similar size and power we were unable to confirm this observation. Our data do not confirm previously reported β-cell defects in *JAZF1* and *TSPAN8* (9) which is in line with the other reports based on OGTTs (10-13).

One of the main limitations of the current study is the relatively small number of participants. Although this is the largest study applying the gold standard method for assessing β-cell function, the hyperglycaemic clamp, we cannot exclude that we have missed subtle defects associated with the different gene variants especially given the fact that their effects on type 2 diabetes mellitus risk are also small. Furthermore we have applied a rather lenient correction for multiple hypotheses testing which means that some of the current findings may be spurious. Our results should therefore be regarded exploratory and we fully subscribe the need for replication but such replication is non-trivial because the hyperglycaemic clamp methodology is demanding for both researchers and participants. However, our current results clearly justify these investments.

A further limitation is the inclusion of a mix of normal and impaired glucose tolerant subjects. It is well known that subjects with IGT often have insulin resistance and / or insufficient β-cell function to maintain normal glucose homeostasis and are thus at high risk to develop type 2 diabetes mellitus. One may argue that the observed associations with decreased β-cell function are thus due to the known association with type 2 diabetes mellitus and the risk implied by the IGT state. However our data analysing separately NGT and IGT subjects showed that the direction of the effects for the gene variants we found associated was in general similar in both groups and not mainly driven by the IGT subjects arguing against this potential bias. Furthermore we used a random effects meta-analysis approach to test whether the relationship between the genes and the outcome variables is homogeneous over the three cohorts. Also this analysis yielded virtually identical results

providing further evidence that our data are not influenced by the inclusion of the IGT subjects. However, although the associations we found are resistant to the above described analyses and present in both NGT and IGT subjects we cannot exclude that for other genes/loci this would not be the case.

In conclusion we found novel associations between gene variants in *THADA, ADAMTS9* and *BCL11A* loci and various aspects of β-cell function. In carriers of the *THADA* variant we observed decreases in both GLP-1 and arginine induced insulin release hinting at lower β-cell function and/or mass. Carriers of gene variants in *ADAMTS9* and *BCL11A* show alterations in first-phase GSIS suggesting they may primarily affect processes involved in the rapid recruitment and release of insulin from insulin granules.

In addition to the above mentioned associations we have confirmed that a gene variant in *CDC123/CAMK1D* is associated with reduced β-cell function and our data suggest it may do so via a reduced β-cell mass. Furthermore, our data suggest that carriers of the *MTNR1B* risk allele may be more sensitive towards the stimulatory effects of GLP-1 which may offer therapeutic possibilities if confirmed. These findings point to a clear diversity in the impact that these different gene variants may have on (dys)function of pancreatic beta cells and justify the use of the hyperglycaemic clamp methodology, especially with additional secretagogues, to resolve the pathogenic mechanisms of these loci.

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Chapter 8

Summary and conclusions

Summary

This thesis describes an experimental study in healthy MZ and same-sex DZ twins and siblings registered at the Netherlands Twin Register. The main aim was to estimate the heritability of different aspects of the β-cell function and to identify part of the genes causing this heritability. A total of 77 twin families were successfully included in the study. In well over 3 years (2004-2007), 190 screening OGTTs were performed at home and 189 mixed meal tests at the clinical research unit. Moreover, euglycaemic-hyperinsulinaemic clamps and modified extended hyperglycaemic clamps with three different stimuli (glucose, GLP-1 and arginine) were performed in 130 twins and siblings that were willing to participate in all three investigations. The first part of the thesis describes the procedures and analyses that were performed to obtain a heritability estimation of glycaemia and β-cell function parameters derived from above mentioned tests. By using the multivariate extension of the twin design it could be tested to what extent different glycaemia and β-cell function parameters are influenced by the same genetic factors, and to what extent β-cell genetic factors are independent of the genetic factors influencing body composition and insulin sensitivity. The second part describes two studies of the association between genetic variants that increase the risk for type 2 diabetes mellitus and some of the β-cell function parameters. This chapter provides a summary of the results, the main conclusions and suggestions for further research.

HbA1c and fasting blood glucose

HbA1c and fasting blood glucose are both used as diagnostic parameters for type 2 diabetes mellitus. Chapter 3 estimated the heritability of these traits and examined their phenotypic and genetic correlation structure. Heritability of HbA1c was estimated at 75%. Fasting blood glucose was measured in three different settings (pre-OGTT at home, pre-meal test and pre-clamp test in the clinic). The heritability of fasting blood glucose was different across these settings (range 38% to 66%). However, the genetic correlation between them was high ($0.53 < r < 0.95$) and I concluded that gene finding efforts may safely pool FBG samples from different settings. The most remarkable finding was the small and non-significant correlation between fasting blood glucose (FBG) and HbA1c, and that FBG assessed in the three different settings appeared to have no overlapping genetic influences

with HbA1c. I concluded that these two glycaemic parameters cannot be used interchangeably in diagnostic procedures or in studies attempting to find genes for diabetes. Both contribute unique (genetic) information.

The insulin response of the β-cell to a mixed meal

Chapter 4 used the mixed meal test to assess the heritability of classical and mathematical model derived β-cell function parameters during a real physiologic challenge. The heritabilities of waist circumference and insulin sensitivity, with the formula of Oral Glucose Insulin Sensitivity (OGIS), were also estimated because these variables are known to be associated with β-cell function. The results showed significant heritabilities of most of the classical but only some of the model derived β-cell function parameters. The insulinogenic index, an important parameter of early insulin response and an independent predictor of worsening glucose tolerance, had the highest heritability (63%) of the post prandial parameters, but one third of this heritability was shared with the genetic influences on waist and OGIS. The model derived β-cell glucose sensitivity, which quantifies the ability of the β-cell to respond to changes in glucose concentration and is a significant independent predictor of glucose intolerance, had a high heritability (50%) with a negligible overlap with waist and OGIS. Fasting insulin level and fasting insulin secretion rate (ISR) had comparable heritability estimates (38% and 43% respectively) but the fasting insulin secretion rate may be a better measure of the activity of the β-cell than fasting insulin level, because insulin level is strongly co-determined by insulin clearance. The incremental ISR during the first two of the four postprandial hours showed a significant heritability in the first 30 minutes (47%) as well the next one and a half hour (42%). However, the genetic influences on ISR in the first 30 minutes had only a negligible overlap with waist and OGIS, while one third of the heritability of the ISR during the next one and a half hour was shared with waist circumference and OGIS. My conclusion was that the mixed meal test provides multiple heritable aspects of the β-cell function that can help us examine the biology underlying the wealth of genetic variants produced by genome wide association studies. Most promising parameters are the model derived β-cell glucose sensitivity and the insulin secretion rate in the first 30 minutes, because they are relatively independent of body composition and insulin sensitivity.

The insulin response of the β-cell to different secretagogues

Chapter 5 explored the heritability of the insulin response of the beta cell during a modified version of the hyperglycaemic clamp test used by Fritsche and colleagues. The heritability of the first phase (52%) and second phase (77%) glucose stimulated insulin response were estimated, as well as the heritability of the insulin response to additional GLP-1 (53%) and GLP-1 + arginine (80%). From this, I concluded that genetic factors explain most of the individual differences in insulin response after administration of glucose and glucose combined with GLP-1 or GLP-1 + arginine in healthy adults.

The heritabilities of BMI and insulin sensitivity (ISI) were assessed on the same day (74% and 60% respectively), the latter by the euglycaemic-hyperinsulinaemic clamp. We found that the genetic variance unique to β-cell function, i.e. independent of the genetic factors influencing BMI and ISI, contributed less strongly to individual differences in the first-phase response (only 14%) than in the second-phase response (30%) or in the responses to additional GLP-1 (36%) and GLP-1 + arginine (37%). Hence, I concluded that the often used first-phase response may give an incomplete picture of the genes that are specific to beta cell function. I further concluded that the genetic factors influencing the β-cell function are partly the same as the factors that influence BMI and ISI, and that in genetic designs 'correction' for BMI and ISI may not always be desirable.

Association between type 2 diabetes mellitus related gene variants and β-cell function parameters

Chapter 6 and 7 describe two studies on the association between established (chapter 6) and new (chapter 7) type 2 diabetes mellitus related gene variants and β-cell function. Up till now mainly OGTT data were used in genetic association studies on β-cell function. The novelty of our studies was the use of hyperglycaemic clamps, including the clamp that combined three different stimuli (glucose, GLP-1 and arginine). Because of multiple hypothesis testing results were regarded significant at $P \leq 0.008$ (six tests).

In the first study the combined risk allele score based on eight proven beta cell loci (*TCF7L2*, *KCNJ11*, *HHEX/IDE*, *CDKAL1*, *IGF2BP2*, *SLC30A8, CDKN2A/B* and *MTNR1B*) was used. Data came from three independent studies in the Netherlands (Hoorn, Utrecht and NTR/VUmc Amsterdam) and one study from Tübingen, Germany. Only the rapid first phase glucose stimulated insulin secretion (GSIS) and the disposition index (DI =

first phase GSIS x ISI) were significantly inverse associated with this combined risk allele score. In contrast, the slower second phase GSIS, GLP-1 and arginine stimulated insulin secretion and insulin sensitivity were not associated. Furthermore we observed a strong correlation between our combined risk allele score and the absence of a first phase insulin peak in our subjects with IGT which is a strong predictor of future development of type 2 diabetes mellitus. We concluded that these eight β-cell loci seem to act mainly via detrimental effects on processes involved in the early, rapid recruitment and exocytosis of insulin granules after glucose stimulation.

The aim of the second association study, described in chapter 7, was to assess separately the association of each of the 12 genetic risk alleles in recently detected type 2 diabetes mellitus loci with β-cell function parameters. Data came from the above mentioned three independent Dutch clamp studies. The only association with insulin sensitivity was found in carriers of the T risk allele in *THADA,* who showed a significant lower ISI. An increased first phase GSIS was associated with the C risk allele of the *ADAMTS9* gene, while carriers of the TT risk genotype of the *BCL11A* locus had a lower first phase GSIS. Risk variants in the *CDC123/CAMK1D* gene and the T risk allele in *THADA* were associated with a significantly decreased second phase GSIS. GLP-1 and arginine induced insulin secretion were reduced in the homozygous *THADA* TT risk genotype, although not always statistically significant, suggesting lower beta cell mass as a possible pathogenic mechanism.

Remarkably, carriers of the risk allele of the *MTNR1B* gene had, although not statistically significant, increased responses to GLP-1 (+30%, p=0.03) and arginine stimulation (+19%, p=0.037). If replicated, these results indicate that carriers of the G risk allele may well benefit from treatment with GLP-1 agonists or dipeptidyl-IV inhibitors, which may offer new therapeutic possibilities. The *ADAMTS9* risk allele was associated with a higher disposition index in contrast to the risk alleles for *BCL11A* and *MTNR1B* that were significantly associated with a decreased disposition index. Seven of the 12 risk alleles in the recently discovered type 2 diabetes mellitus loci showed no association with any β-cell function parameter. We concluded that type 2 diabetes mellitus risk alleles in *CDC123/CAMK1D, THADA, ADAMTS9, BCL11A* and *MTNR1B* are associated with specific aspects of β-cell function. These findings point to a clear diversity in the impact that these different gene variants may have on (dys)function of pancreatic β-cells.

Main conclusions

- FBG and HbA1c are heritable traits but cannot be used interchangeably because both contribute unique (genetic) information.
- FBG samples from different settings may safely be pooled in gene finding efforts.
- Classical and model derived β-cell function parameters, used in a mixed meal test, show a significant heritability and represent different aspects of β-cell function.
- The β-cell glucose sensitivity and the insulin secretion rate during the first 30 post prandial minutes provide the most specific genetic information of the β-cell function after an oral challenge.
- In the hyperglycaemic GLP-1/arginine challenge test genetic factors explain most of the individual differences in insulin response after intra venous administration of the three different secretagogues.
- The responses to glucose combined with GLP-1 and GLP-1 + arginine are the best indicators of β-cell function, while the often used first phase GSIS may give an incomplete picture of the genes that are specific to β-cell function.
- The combined score of type 2 diabetes mellitus risk alleles in *TCF7L2*, *KCNJ11*, *CDKAL1*, *IGF2BP2*, *HHEX/IDE*, *CDKN2A/B, SLC30A8* and *MTNR1B* is mainly associated with a decreased first phase glucose induced insulin secretion and a lower disposition index.
- Type 2 diabetes mellitus risk alleles in *CDC123/CAMK1D, THADA, ADAMTS9, BCL11A* and *MTNR1B* are each associated with specific aspects of β cell function.
- There is not one single test that can give comprehensive genetic information about the function of the β-cell. Instead, the mixed meal test and the hyperglycaemic GLP-1/arginine challenge test each contribute unique genetic information about the function of the β-cell.

Suggestions for further research

In this twin-family study a tremendous amount of data was obtained, of which only a part could be analysed and presented in this thesis. It is important that these valuable data (including serum specimens in the freezer, autonomic nervous system and blood pressure measurements, food consumption questionnaires) are used in further research. Such future studies may reveal the genetic and environmental contribution to individual variation in hormone secretion during a meal (e.g. proinsulin, incretins and glucagon). In addition it could be investigated whether there is a relation between the autonomic nervous system responses (e.g. measured by blood pressure, heart rate variability or pre-ejection period responses) and β-cell function during meal and clamp tests, and whether genetic factors are involved in this relation. The questionnaires about food consumption give the possibility to investigate the relation between the consumption of different nutrients and β-cell function. The additional use of the research materials gathered during my studies will hopefully contribute to a better understanding of the development of type 2 diabetes mellitus. In view of the growing impact of this disease on daily life of millions of people, there is much to be gained if we can improve primary prevention and further optimize our treatment strategies.

Nederlandse samenvatting

Genetische invloeden op de β-cel functie

een Nederlandse tweeling-familie studie

Samenvatting

In dit proefschrift wordt een experimentele studie beschreven bij gezonde monozygote (eeneiige) en dizygote (twee-eiige) tweelingen van gelijk geslacht en hun broers of zusters, allen geregistreerd bij het Nederlands Tweelingen Register van de Vrije Universiteit te Amsterdam. Het voornaamste doel was om de erfelijkheid te schatten van verschillende aspecten van de β-cel functie (β-cellen liggen in de alvleesklier en produceren insuline). Tevens wilden we een deel van de genen identificeren, die deze erfelijkheid veroorzaken. In het totaal werden 77 tweeling families in deze studie geïncludeerd. In een tijdsperiode van ruim 3 jaar (2004-2007) werd bij 150 tweelingen en 40 van hun broers /zusters in de leeftijd van 20-50 jaar thuis een OGTT verricht om de aanwezigheid van latente diabetes mellitus uit te sluiten en kwamen 190 van hen naar de research afdeling van het VU medisch centrum te Amsterdam voor een maaltijd test. Bovendien hebben 130 van hen op een derde testdag deelgenomen aan een euglycemische hyperinsulinemische clamp en aan een gemodificeerde hyperglycemische clamp met drie verschillende stimuli (glucose, GLP-1 en arginine).

In het eerste deel van dit proefschrift worden de uitgevoerde procedures en analyses beschreven. Eerst werden bloedglucose en β-cel functie parameters berekend uit de testen (OGTT, maaltijdtest en clamptesten) om vervolgens te komen tot een schatting van de erfelijkheid van deze parameters. Naast univariate analyses werden in Mx, een computerprogramma speciaal ontworpen voor analyse van tweeling en familie data, ook multivariate analyses gedaan. Hierdoor konden we nagaan in hoeverre verschillende glycemische en β-cel functie parameters beïnvloed worden door dezelfde genetische factoren. Tegelijkertijd onderzochten we in hoeverre genetische factoren die de β-cel functie beïnvloeden onafhankelijk zijn van de genetische factoren die lichaamssamenstelling en insuline gevoeligheid beïnvloeden.

In het tweede deel worden twee studies beschreven naar de associatie tussen genetische varianten, die het risico op diabetes mellitus type 2 verhogen, en enkele β-cel functie parameters.

Deze samenvatting wordt afgesloten met suggesties voor verder onderzoek.

HbA1c en nuchtere bloedglucose

HbA1c en nuchtere bloedglucose worden beiden gebruikt als diagnosticum voor diabetes mellitus type 2. In hoofdstuk 3 is de erfelijkheidsschatting van deze parameters beschreven en is de structuur van hun fenotypische en genetische correlatie geanalyseerd. De erfelijkheid van HbA1c werd geschat op 75%. De nuchtere bloedglucose was gemeten in drie verschillende testsituaties (bij de OGTT, de maaltijd test en de clamp test). De erfelijkheid van de nuchtere bloedglucose was verschillend in deze drie situaties (range 38% tot 66 %). De genetische correlatie tussen hen was echter hoog ($0.53 < r < 0.95$) en ik kwam tot de conclusie dat nuchtere bloedglucose waarden gemeten in verschillende situaties gerust gecombineerd mogen worden in studies naar de betreffende genen. De meest opmerkelijke bevindingen waren de geringe en niet significante correlatie tussen nuchtere bloedglucose en HbA1c en het feit dat er vrijwel geen gemeenschappelijke genetische invloeden werden gevonden voor de nuchtere bloed glucose en de HbA1c. Ik concludeerde dat deze twee glycemische parameters niet uitwisselbaar zijn, noch als criterium voor de diagnose diabetes mellitus type 2 noch in studies die de genen proberen te vinden voor deze ziekte. Beide bevatten unieke (genetische) informatie.

Insuline secretie door de β-cel bij een maaltijd met gestandaardiseerde samenstelling

Hoofdstuk 4 laat zien hoe een echte fysiologische prikkel, namelijk een maaltijd met gestandaardiseerde samenstelling, gebruikt kan worden om de erfelijkheid te onderzoeken van zowel klassieke als van via een wiskundig model berekende β-cel functie parameters. De erfelijkheid van de middelomtrek en de insuline gevoeligheid (OGIS) werden ook geschat omdat bekend is dat deze eigenschappen gecorreleerd zijn met β-cel functie. De resultaten toonden een significante erfelijkheid van de meeste klassieke β-cel functie parameters maar slechts van enkele uit het model berekende waarden. De insulinogene index, een belangrijke parameter van vroege insuline productie en een onafhankelijke voorspeller van verslechtering van de glucose tolerantie, had met 63% de hoogste erfelijkheid. Echter, één derde van deze erfelijkheid was gemeenschappelijk met de genetische invloeden op middelomtrek en insuline gevoeligheid. De via het model berekende β-cel glucose gevoeligheid, welke het vermogen van de β-cel kwantificeert om te reageren op de veranderingen in glucose concentratie en een significante onafhankelijke

voorspeller is van glucose intolerantie, had een hoge erfelijkheid (50%). Deze erfelijkheid van β-cel glucose gevoeligheid was slechts voor een verwaarloosbaar deel gemeenschappelijk met de genetische invloeden op middelomtrek en OGIS. Nuchtere insuline concentratie en nuchtere insuline secretie snelheid (ISR) hadden vergelijkbare erfelijkheidsschattingen (respectievelijk 38% en 43%). Echter, de nuchtere ISR is waarschijnlijk een betere maat voor de activiteit van de β-cel dan de nuchtere insuline concentratie omdat de insuline concentratie in sterke mate mede bepaald wordt door de insuline klaring. De ISR gedurende de eerste twee uur na de maaltijd toonde zowel in de eerste 30 minuten als in de daaropvolgende anderhalf uur een significante erfelijkheid (resp. 47% en 42 %). De genetische invloeden op de ISR gedurende de eerste 30 minuten vielen echter nauwelijks samen met die voor middelomtrek en OGIS, terwijl één derde van de erfelijkheid van de ISR gedurende de daaropvolgende volgende anderhalf uur samenviel met die voor middelomtrek en OGIS. Mijn conclusie was dat de gestandaardiseerde maaltijd test meerdere erfelijke aspecten van de β-cel oplevert. Deze bevindingen kunnen ons helpen om de werking van genetische varianten, die gevonden worden bij genoom wijde associatie studies, te bestuderen. De meest belovende parameters van β-cel functie zijn de via een model berekende β-cel glucose gevoeligheid en de insuline secretie snelheid gedurende de eerste 30 minuten na de maaltijd, omdat deze maten relatief onafhankelijk zijn van lichaamssamenstelling en insuline gevoeligheid.

De insuline respons van de β-cel op verschillende intraveneuze stimuli

In hoofdstuk 5 wordt het onderzoek beschreven naar de erfelijkheid van de insuline respons van de β-cel gedurende een gemodificeerde versie van de hyperglycemische clamptest, gebruikt door Fritsche et al. De schatting voor de erfelijkheid van de door glucose geïnduceerde insuline respons (GSIS) gedurende de eerste fase was 52% en van de GSIS gedurende de tweede fase 77%. De erfelijkheid van de insuline respons bij een bloedglucose van 10 mmol/l was na intraveneuze toediening van GLP-1 53% en na GLP-1 + arginine 80%. Hieruit concludeerde ik dat bij gezonde personen genetische factoren het grootste deel verklaren van de individuele verschillen in insuline respons na toediening van intraveneuze glucose en glucose gecombineerd met GLP-1 of GLP-1 + arginine.

De erfelijkheid van BMI en insuline gevoeligheid (ISI) werden op dezelfde dag onderzocht (resp. 74% en 60%), de laatste d.m.v. de euglycemische hyperinsulinemische

clamp. Wij vonden dat de genetische variantie die uniek was voor de β-cel functie, d.w.z. onafhankelijk van de genetische factoren die BMI en insuline gevoeligheid beïnvloeden, in wisselende mate bijdroeg aan de individuele verschillen in de insuline respons. Gedurende de eerste fase was deze unieke genetische variantie voor de β-cel functie slechts 14%, terwijl die gedurende de tweede fase 30% was en in de insuline respons als reactie op additionele GLP-1 en GLP-1 + arginine zelfs 37%. Hieruit concludeerde ik dat de vaak gebruikte eerste fase insuline respons waarschijnlijk een onvolledig beeld geeft van de genen die specifiek zijn voor de functie van de β-cel. Ik concludeerde tevens dat genetische factoren die de functie van de β-cel beïnvloeden deels dezelfde zijn als de factoren die BMI en insuline gevoeligheid beïnvloeden. Hieruit volgt dat in genetische studies correctie voor BMI en insuline gevoeligheid niet altijd wenselijk is.

Associatie tussen gen varianten, gerelateerd aan diabetes mellitus type 2 en β-cel functie parameters

In hoofdstuk 6 en 7 worden twee studies beschreven over de associatie tussen bekende en nieuwe gen varianten die gerelateerd zijn aan diabetes mellitus type 2 en de functie van de β-cel. Tot op heden werden voornamelijk OGTT uitkomsten gebruikt in genetische associatie studies met β-cel functie. Het nieuwe van deze studies was het gebruik van de hyperglycemische clamp, met tevens de clamp die drie verschillende stimuli combineerde (glucose, GLP-1 en arginine).

In de eerste studie werd een gecombineerde risico allel score gebruikt, welke gebaseerd was op acht bewezen β-cel gerelateerde gen varianten (*TCF7L2*, *KCNJ11*, *HHEX/IDE*, *CDKAL1*, *IGF2BP2*, *SLC30A8, CDKN2A/B* and *MTNR1B*). De data kwamen van drie onafhankelijke studies in Nederland (Hoorn, Utrecht en NTR/VUmc Amsterdam) en een studie uit Tübingen, Duitsland. Alleen de snelle eerste fase van de glucose gestimuleerde insuline secretie (GSIS) en de dispositie index (= eerste fase insuline productie x insuline gevoeligheid) waren significant omgekeerd gecorreleerd met deze gecombineerde risico allel score. Daarentegen was er geen associatie met de insuline productie gedurende de langzamere tweede fase, de GLP-1 en arginine geïnduceerde insuline productie of met de insuline gevoeligheid. Verder zagen we dat er een sterke correlatie was tussen deze gecombineerde risico allel score en de afwezigheid van een eerste fase insuline piek in de proefpersonen met verminderde glucose tolerantie. Dit

fenomeen is een belangrijke voorspeller van toekomstige ontwikkeling van diabetes mellitus type 2. Wij concludeerden dat deze acht β-cel gerelateerde gen varianten vooral een schadelijke invloed hebben op de processen die betrokken zijn bij de vroege snelle werving en uitstoting van insuline na glucose stimulatie.

Het doel van de tweede associatiestudie, die beschreven wordt in hoofdstuk 7, was om van ieder van de 12 onlangs ontdekte genetische varianten gerelateerd aan diabetes melllitus type 2 de associatie met de β-cel functie parameters te onderzoeken. De data kwamen van de eerdere vermelde drie onafhankelijke Nederlandse clamp studies. De enige associatie met insuline gevoeligheid werd gevonden in dragers van het T risk allel in *THADA,* die een significant lagere insuline gevoeligheid toonden. Een verhoogde, eerste fase GSIS was geassocieerd met het C risk allel van het *ADAMTS9* gen, terwijl dragers van het TT risk genotype van de *BCL11A* locus een lagere eerste fase GSIS hadden. Risico varianten in het *CDC123/CAMK1D* gen en het T risk allel in *THADA* waren geassocieerd met een significant verminderde tweede fase GSIS. GLP-1 en arginine geïnduceerde insuline secretie waren, hoewel niet altijd statistisch significant, verminderd in het homozygote THADA TT risico genotype, wat suggereert dat een kleinere β-cel massa mogelijk het pathogene mechanisme is.

Het was opmerkelijk dat dragers van het risico allel van het *MTNR1B* gen een verhoogde insuline respons hadden na GLP-1 (+30%, p=0.03) en arginine stimulatie (+19%, p=0.037). Als dit gerepliceerd wordt, duiden deze resultaten er op dat dragers van het G risico allel goed zouden kunnen profiteren van behandeling met een GLP-1 agonist of dipeptidyl-IV remmers, hetgeen nieuwe mogelijkheden biedt voor therapie. Het *ADAMTS9* risico allel was geassocieerd met een hogere dispositie index in tegenstelling tot de risico allelen voor *BCL11A* en *MTNR1B* die significant geassocieerd waren met een verminderde dispositie index. Zeven van de 12 risico allelen in de onlangs ontdekte diabetes mellitus type 2 loci toonden geen associatie met enig β-cel functie parameter. Wij concludeerden dat diabetes mellitus type 2 risico allelen in *CDC123/CAMK1D, THADA, ADAMTS9, BCL11A* en *MTNR1B* zijn geassocieerd met specifieke aspecten van de β-cel functie. Deze bevindingen duiden op een duidelijke diversiteit van de impact die deze verschillende gen varianten kunnen hebben op de (dys)functie van de β-cellen van de pancreas.

Suggesties voor verder onderzoek

Tijdens deze tweeling-familie studie is een omvangrijke hoeveelheid data verkregen, waarvan slechts een deel kon worden geanalyseerd en in dit proefschrift is beschreven. Het is belangrijk dat deze waardevolle data (waaronder serum monsters in de diepvries, metingen van hartritme, ademhaling en bloeddruk, voedsel consumptie vragenlijsten) gebruikt worden voor verder onderzoek. Deze toekomstige analyses kunnen mogelijk onthullen in welke mate genetische en omgevingsfactoren bijdragen aan de individuele variatie in hormoon secretie gedurende een maaltijd (m.n. proinsuline, incretines en glucagon). Tevens kan zo onderzocht worden of er een relatie bestaat tussen reacties van het autonome zenuwstelsel en de β-cel functie gedurende een maaltijd en clamp test en of genetische factoren daarbij een rol spelen. De voedingsvragenlijsten geven de mogelijkheid om de relatie te onderzoeken tussen de consumptie van verschillende nutriënten en de β-cel functie. Het gebruik van zoveel mogelijk onderzoeksgegevens, verzameld tijdens mijn research in de afgelopen jaren, zal hopelijk bijdragen aan een verdieping van de kennis over de ontwikkeling van diabetes mellitus type 2. Met het oog op de enorme impact van deze ziekte op het dagelijks leven van een steeds groter wordende groep mensen, kan veel vooruitgang geboekt worden als we de primaire preventie kunnen verbeteren en onze behandel strategieën optimaliseren.