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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.

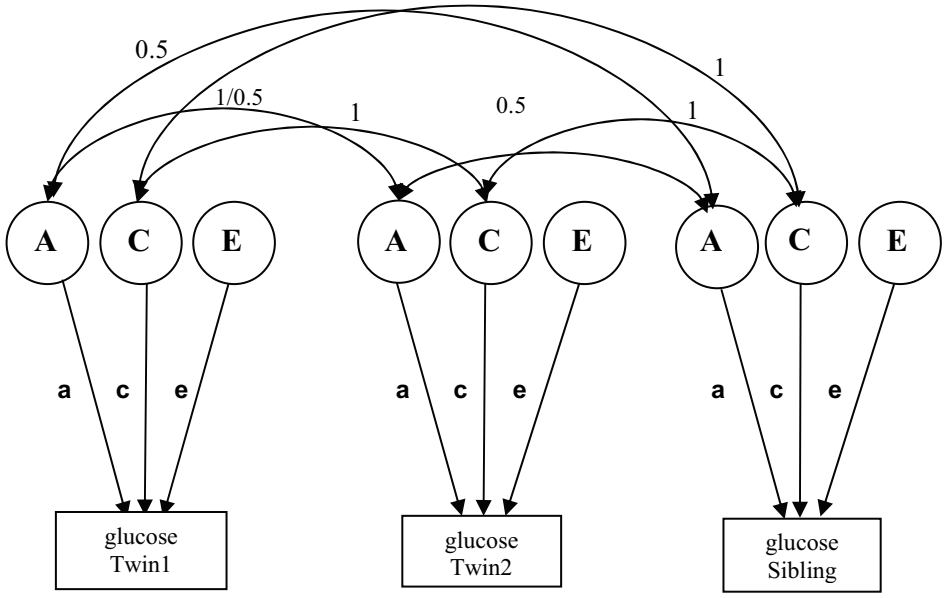

Figure 2.1: Univariate path diagram for a twin design with one additional sib
A = additive genetic, C = shared environmental E = non shared environmental influences to the trait. The factor loadings of these influences are a, c and e. In MZ twins the correlation of the additive genetic factors is 1.0, 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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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.

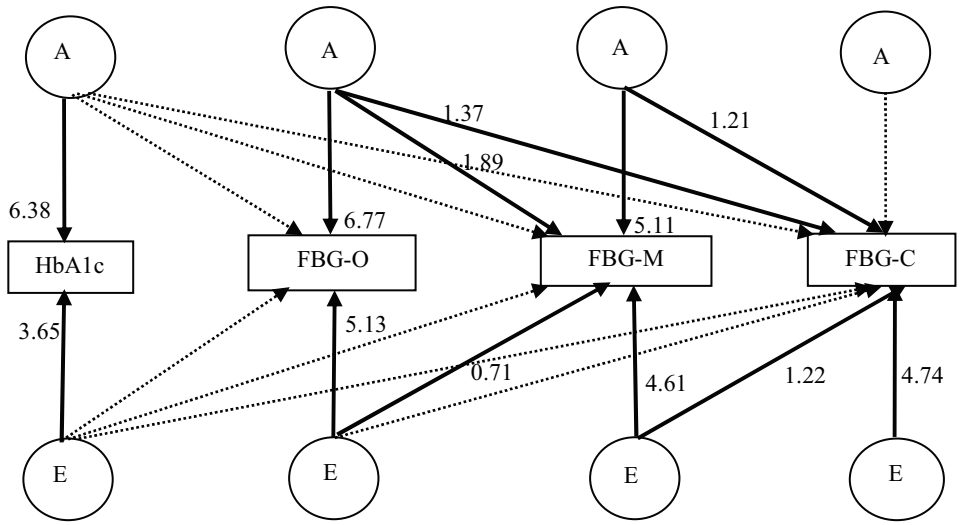

Figure 3.1.Genetic model for HbA1c and three FBG measures with factor loadings of observed variables on the latent Additive genetic and unique Environmental factors. FBG-O = fasting blood glucose at home; FBG-M = fasting blood glucose pre-meal; FBG-C = fasting blood glucose pre-clamp. Bold line = significant;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