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.

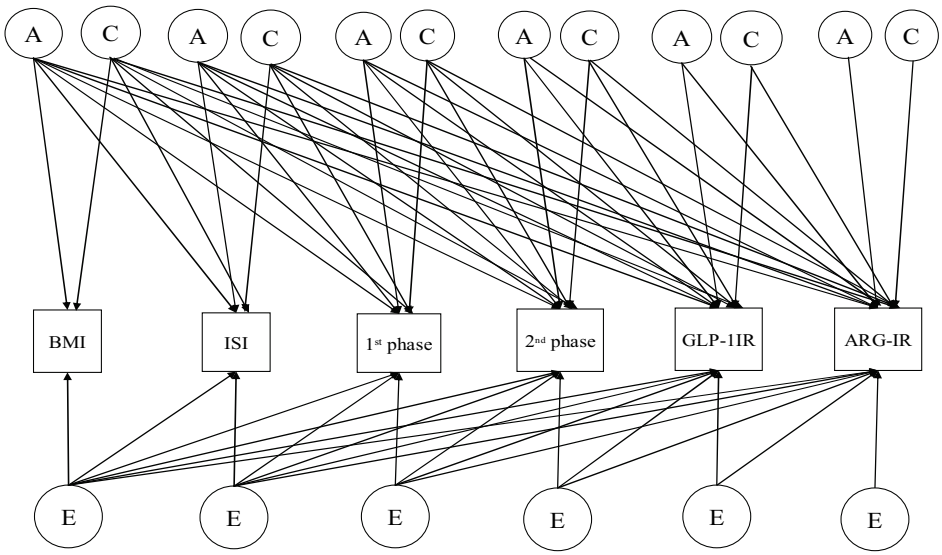

Figure 5.1 The full 6-variate ACE genetic model

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.

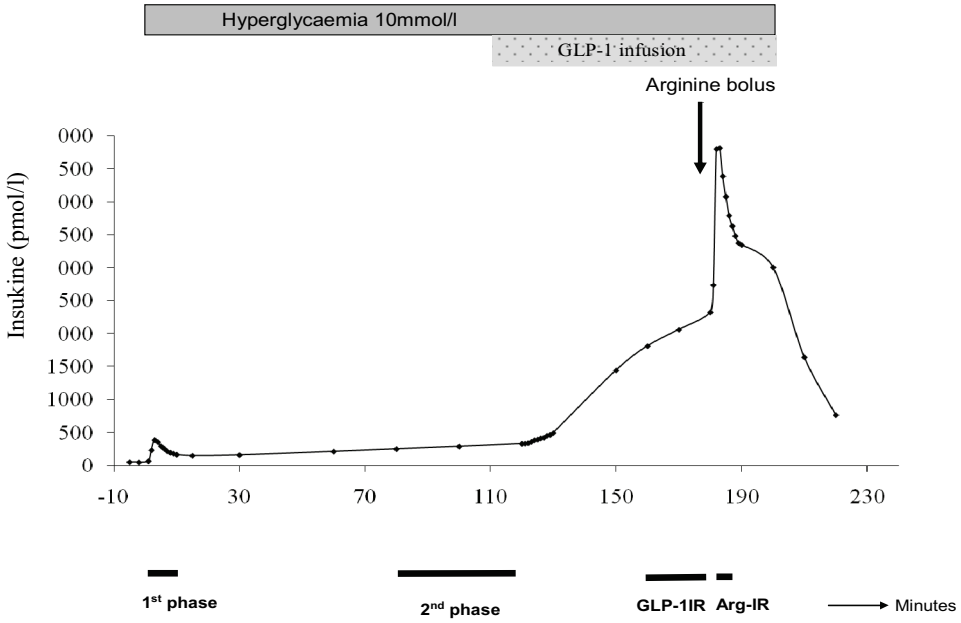

Figure 5.2 Insulin levels during the hyoperglycaemic clamp. The bars indicate the time periods of the calculated insuline responses

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.

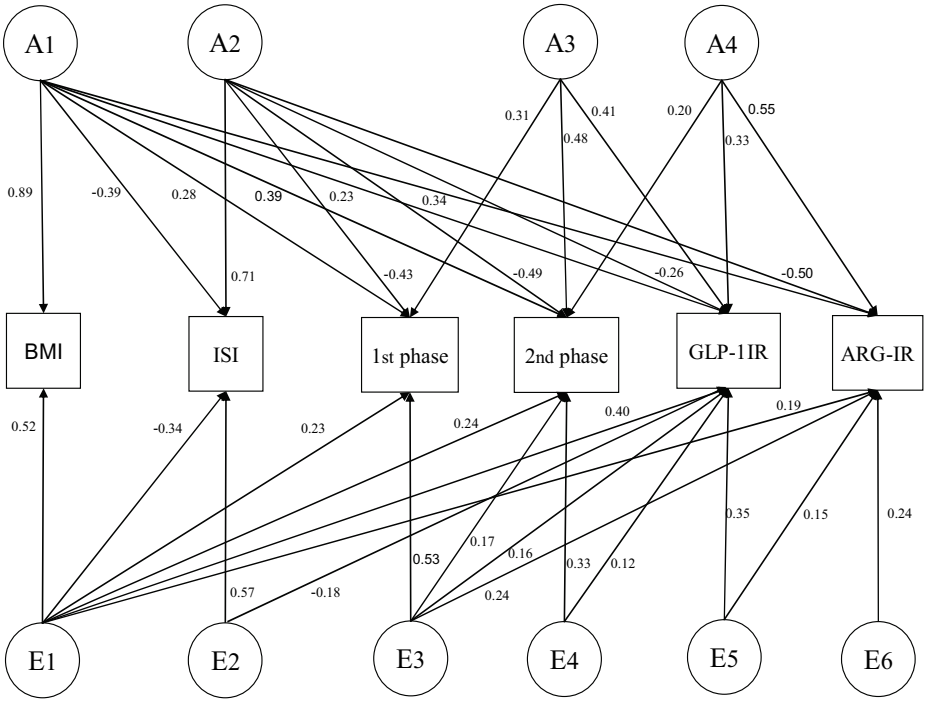

Figure 5.3 The most parsimonious model (AE) for genetic and environmental effects on BMI, ISI and the insulin responses of the beta cell. Factor loadings for observed variables on the latent additive genetic (A) and unique environmental factors (E) are shown.

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).

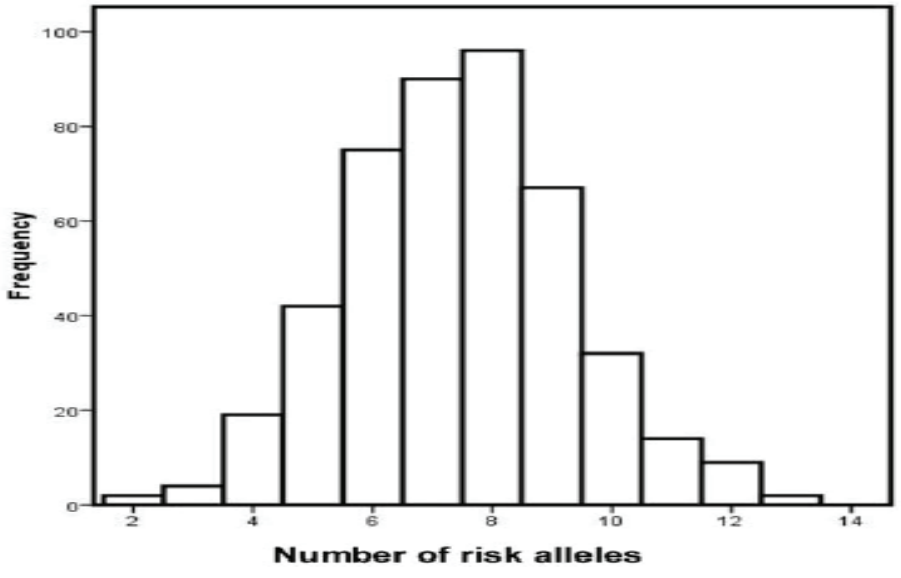

Fig 6.1 Distribution of risk alleles in the study sample

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.

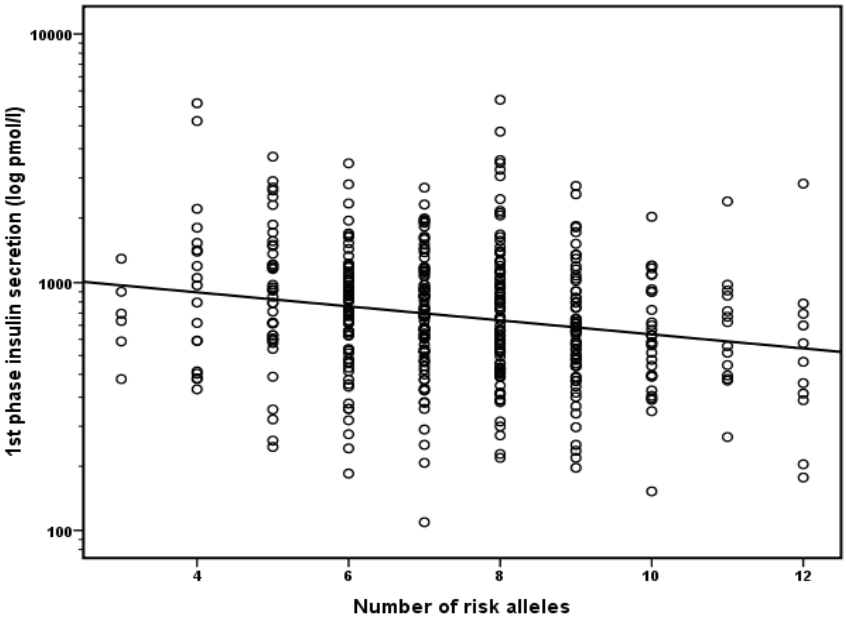

Figure 6.2 First-phase GSIS in relation to the risk alleles counts for the eight loci. Each circle represents an independent participant. The line represents the regression line after adjustment for age, sex, BMI, study centre, glucose tolerance status, and ISI.
β = 0.95 (95% CI 0.93-0.97; P = 7.1 x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

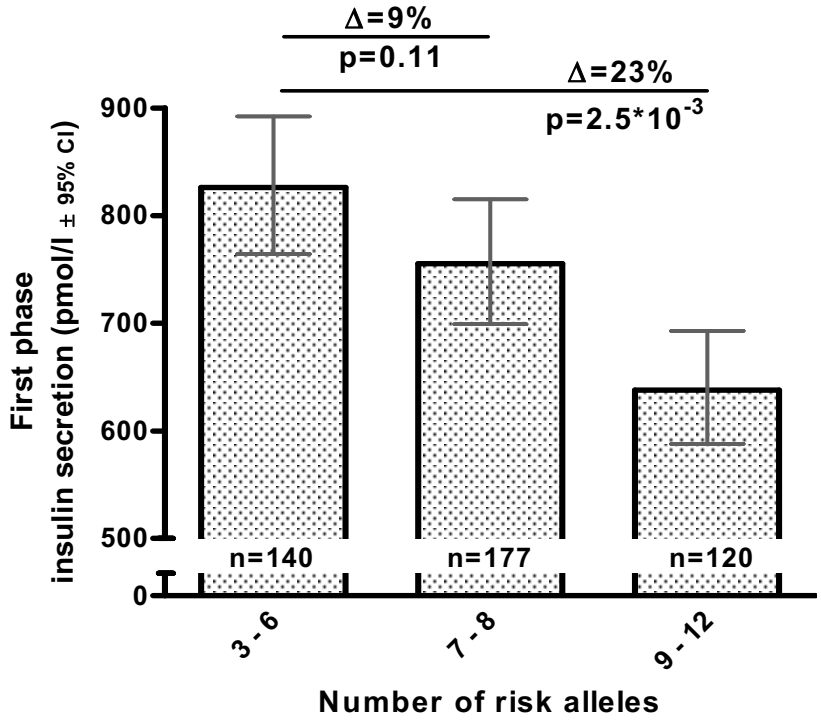

Figure 6.3 Mean estimated first-phase GSIS in three different risk allele strata. Those with three to six risk alleles were used as a reference group

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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