The genetics of type 2 diabetes

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Chapter 5

Combined effects of single-nucleotide polymorphisms in *GCK*, *GCKR*, *G6PC2* and *MTNR1B* on fasting plasma glucose and type 2 diabetes risk

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Abstract

Aims/hypothesis

Variation in fasting plasma glucose (FPG) within the normal range is a known risk factor for the development of type 2 diabetes. Several reports have shown that genetic variation in the genes for glucokinase (*GCK*), glucokinase regulatory protein (*GCKR*), islet-specific glucose 6 phosphatase catalytic subunit-related protein (*G6PC2*) and melatonin receptor type 1B (*MTNR1B*) is associated with FPG. In this study we examined whether these loci also contribute to type 2 diabetes susceptibility.

Methods

A random selection from the Dutch New Hoorn Study was used for replication of the association with FGP (2361 non-diabetic participants). For the genetic association study we extended the study sample with 2628 participants with type 2 diabetes. Risk allele counting was used to calculate a four-gene risk allele score for each individual.

Results

Variants of the *GCK*, *G6PC2* and *MTNR1B* genes but not *GCKR* were associated with FPG (all, $p \leq 0.001$; *GCKR*, $p = 0.23$). Combining these four genes in a risk allele score resulted in an increase of 0.05 mmol/l (0.04–0.07) per additional risk allele ($p = 2 \times 10^{-13}$). Furthermore, participants with less than three or more than five risk alleles showed significantly different type 2 diabetes susceptibility compared with the most common group with four risk alleles (OR 0.77 [0.65-0.93], $p = 0.005$ and OR 2.05 [1.50-2.80], $p = 4 \times 10^{-6}$ respectively). The age at diagnosis was also significantly associated with the number of risk alleles ($p = 0.009$).

Conclusion

A combined risk allele score for single-nucleotide polymorphisms in four known FPG loci is significantly associated with FPG and HbA_{1c} in a Dutch population-based sample of non-diabetic participants. Carriers of low or high numbers of risk alleles show significantly different risks for type 2 diabetes compared with the reference group.

Introduction

Variation in fasting plasma glucose (FPG) levels within the normal range are associated with an increased risk of developing type 2 diabetes and coronary heart disease (1;2). Furthermore, it is known that FPG is partially genetically determined (3). Several loci influencing FPG levels have been identified. These loci encode glucokinase (*GCK*), glucokinase regulatory protein (*GCKR*) and islet-specific glucose 6 phosphatase catalytic subunit-related protein (*G6PC2*) (4-10). Recently, the gene encoding melatonin receptor type 1B (*MTNR1B*) was identified as a fourth locus influencing FPG (11-13). In this study we investigated the combined effect of these loci on FPG levels in the Netherlands and analysed their single and combined effects on the risk of type 2 diabetes.

Methods

Study samples

Study sample for continuous trait analysis

For this part of the study we used participants from the ongoing New Hoorn Study, a population-based cohort study in the Netherlands, which examines potential determinants of glucose intolerance and related disorders (14;15). From this study, 2361 non-diabetic white participants (46% male, aged 53±7 years) were selected from the original random sample of the population register of the town of Hoorn, the Netherlands. Glucose tolerance status was assessed with OGTT using the 1999 WHO criteria (16).

Case–control sample for genetic association with type 2 diabetes

As a control sample we used all participants with normal glucose tolerance from the above-mentioned sample (n=2041). Subjects with impaired glucose tolerance (IGT) and/or impaired fasting glucose (IFG) (n=320) were excluded from the control group because they have an increased risk of type 2 diabetes.

For the case sample we used all known (n=90) and newly identified (n=90) cases from the New Hoorn Study. To improve power we added cases from Diabetes Care System West Friesland (DCS, n=1906) (17). The DCS aims to improve diabetes

care by coordinating diabetes care, involving all caregivers and providing education for patients in order to improve patient empowerment. Patients are referred to the DCS by their physicians and are from the same geographical region as those taking part in the New Hoorn Study. We also included 542 type 2 diabetes patients from the diabetes clinics at Leiden University Medical Centre (Leiden, the Netherlands) and VU University Medical Centre (Amsterdam, the Netherlands), who were referred to the clinic by their physicians. In total we selected 2628 participants with type 2 diabetes (55% males, aged 64±11 years) for the case–control study. All participants in our study were of white ethnicity. The study was approved by the appropriate medical ethics committees and was in accordance with the principles of the Declaration of Helsinki.

Genotyping and quality control

Based on previous publications, we selected the single-nucleotide polymorphisms (SNPs) rs1799884 in *GCK* (4), rs1260326 (P446L) in *GCKR* (7), rs560887 in *G6PC2* (9) and rs10830963 in *MTNR1B* (11-13) for genotyping with Taqman SNP genotyping assay (Applied Biosystems, Foster City, CA, USA). All genotype frequencies were similar between the case subgroups. For quality control the allelic discrimination plots were visually observed for good clustering. Plates with bad clustering or a success rate below 95% were repeated. Next, we assessed Hardy–Weinberg equilibrium ($p>0.05$) and genotyped approximately 5% duplicate samples, which all showed identical genotypes.

Statistical analysis

Differences in FPG and other clinical variables (HbA_{1c}, 2 h glucose, triacylglycerol, LDL, HDL, total cholesterol, BMI and waist–hip ratio) were analysed in non-diabetic participants using linear regression, adjusted for BMI, age and sex as possible confounders. All analyses were performed using an additive model, because previous studies had shown that this model was the best fit. In order to combine the effects of all SNPs, risk alleles were counted and used as a sum score (18). A risk allele was defined as an allele that results in an increased FPG as described in the literature. Differences in genotype distribution, allele frequency and risk allele

scores between participants with normal glucose tolerance and those with type 2 diabetes were compared using standard contingency tables with Fisher's exact test, and allelic ORs were calculated with logistic regression adjusted for age, sex and BMI. Subjects with either IGT or IFG were excluded from this analysis. Using Bonferroni correction for multiple hypothesis testing, $p<0.001$ was considered statistically significant for association of FPG loci with clinical variables (36 tests). For the case–control study, $p<0.01$ was considered significant (four tests). All statistics were calculated using SPSS 16.0 (SPSS, Chicago, IL, USA).

Power calculations

Power calculations were performed using Quanto (19). We had an estimated power of 80% to detect a minimal per allele effect in clinical variables between 0.056 and 0.069 mmol/l, depending on allele frequency (α=0.001). For the association study with type 2 diabetes we had an estimated power of 80% to detect a minimal OR between 1.13 and 1.16 for single gene effects (α=0.01). For all power calculations we assumed an additive model.

Table 1 Association of SNPs with FPG (n=2361) and type 2 diabetes (n=4669)

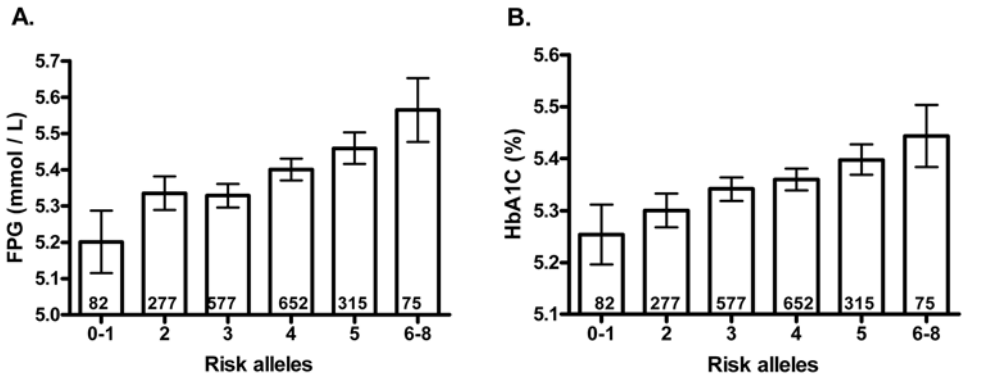
Locus	FPG, mmol/l (genotype count)			Effect/allele mmol/l	P_{add}	T2D OR 95% CI	P_{Add}
	AA	AB	BB				
GCK	5.39±0.01 (1523)	5.45±0.02 (620)	5.47±0.05 (65)	0.06 0.03 to 0.09	0.001	1.12 1.00–1.25	0.06
GCKR	5.35±0.03 (267)	5.39±0.01 (956)	5.38±0.01 (924)	0.01 −0.02 to 0.03	0.23	0.94 0.86–1.02	0.13
G6PC2	5.32±0.03 (218)	5.36±0.01 (930)	5.43±0.01 (1077)	0.06 0.04 to 0.09	5×10^{-6}	0.96 0.87–1.05	0.32
MTNR1B	5.37±0.01 (1269)	5.44±0.01 (891)	5.52±0.04 (135)	0.08 0.05 to 0.11	7×10^{-8}	1.12 1.02–1.23	0.02

Estimated FPG levels (mean±SD) per genotype are adjusted for age, sex and BMI
Effect per allele on FPG levels, 95% CI and *p* values, adjusted for age, sex and BMI, were generated by linear regression
The B genotype carries the risk allele
Odds ratios are for associations of independent SNPs with type 2 diabetes and were calculated based on allele frequency in 2041 controls and 2628 type 2 diabetes participants
T2D, type 2 diabetes

Results

All SNPs passed quality control guidelines. Associations between SNPs and clinical variables were analysed in the non-diabetic participants only. Results of association with FPG levels were comparable to those reported in the literature (all $p \leq 0.001$), except for *GCKR*, for which we could not detect a significant effect on FPG levels ($p=0.23$; results shown in Table 1). However, *GCKR* showed nominal evidence for decreased 2 h glucose, but did not reach a formally significant *p* value ($p=0.008$; see Electronic supplementary material [ESM] Table 1). Furthermore, *GCK* and *G6PC2* showed increased HbA_{1c} levels ($p=5\times10^{-8}$ and 3×10^{-5}; ESM Table 1). In line with our FPG results, *GCKR* was not associated with HbA_{1c} levels ($p=0.50$). However, we did confirm the previously reported association of the T allele of rs1260326 (*GCKR*) with increased triacylglycerol levels ($p=9\times10^{-7}$; ESM Table 1) (5). Other clinical variables were not associated with any of the analysed variants (ESM Table 1). We analysed the combined effect of all SNPs by calculating the risk allele score for each individual. We observed a combined effect of the risk alleles on FPG levels. The increase in FPG level per additional risk allele was 0.05 mmol/l (0.04–0.07 mmol/l), $p=2\times10^{-13}$ (Fig. 1a). A similar result was

Fig. 1 Combined effect of GCK, GCKR, G6PC2 and MTNR1B on FPG and HbA_{1c} in non-diabetic participants from the New Hoorn Study.



A Fasting plasma glucose. Numbers within the bars are numbers of participants per allele group. The per allele effect was 0.05 (0.04–0.07) mmol/l ($p=2\times10^{-13}$).
Error bars represent 95% CI.
B HbA_{1c}. Numbers within the bars represent the number of participants per allele group. The per allele effect was 0.03% (0.02–0.04) ($p=5\times10^{-10}$).
Error bars represent 95% CI

observed for HbA_{1c}: 0.03% (0.02–0.04) increase per additional risk allele, $p=5\times10^{-10}$ (Fig. 1b). We also analysed whether the rate of the age-related increase in FPG was affected by the number of risk alleles. However, we did not observe any differences in these rates between the risk allele scores in our cross-sectional data set (ESM Fig. 1). Separate analysis of only the participants with normal glucose tolerance (n=2041) did not alter any of the results (data not shown)

Next, we analysed the association of these single variants and the risk allele score with type 2 diabetes susceptibility. Only rs10830963 (*MTNR1B*) and rs1799884 (*GCK*) showed weak evidence for association with type 2 diabetes (p=0.02 and p=0.06 respectively; Table 1 and ESM Table 2). Risk allele scores were calculated for the participants with normal glucose tolerance and those with type 2 diabetes and all risk allele groups were compared with the reference group having four risk alleles, since this was the most common group (31%). The lower risk allele groups showed a protective effect on type 2 diabetes, while the risk allele groups with more than four risk alleles showed an increased risk of type 2 diabetes (Table 2). Those with fewer than three risk alleles had a significantly reduced risk of type 2 diabetes (OR 0.77 [0.65-0.93], p=0.005) whereas those with more than five had a significantly increased risk of type 2 diabetes compared with the reference group (OR 2.05 [1.50–2.80], $p=4\times10^{-6}$). Adjustment for age, sex and BMI did not alter the results.

We also noted a significant correlation with the age at diagnosis of type 2 diabetes in our study sample. We observed a per allele effect of −0.46 (−0.80 to −0.11) years in age at diagnosis per additional risk allele (p=0.009) (Table 2). At the extremes of the distribution, i.e. 0 or 1 versus 6–8 risk alleles, there was a difference of almost 4.5 years in age at diagnosis between the two groups (p=0.002) (Table 2).

Table 2 Association of risk allele scores with type 2 diabetes

Risk alleles	Count (frequency)		Age at diagnosis[a] (years, SD)	OR for T2D (95% CI)	*p* value T2D
	Controls (*n*=2041)	Cases (*n*=2628)			
0 or 1	76 (4.2)	115 (4.9)	57.5±1.1	0.75 (0.55–1.02)	0.07
2	243 (13.5)	352 (15.0)	57.3±0.6	0.78 (0.64–0.95)	0.02
3	522 (29.0)	667 (28.4)	56.1±0.5	0.89 (0.76–1.04)	0.14
4	605 (33.6)	685 (29.1)	55.6±0.4	1.00	ref
5	288 (16.0)	381 (16.2)	56.1±0.6	1.17 (0.97–1.41)	0.11
6-8	65 (3.6)	151 (6.4)	52.9±0.9	2.05 (1.50–2.80)	4×10^{-6}

[a] Age at diagnosis was available for 2132 participants with type 2 diabetes
$Beta_{age\ at\ diagnosis}$=–0.46 (–0.80 to –0.11) years, *p*=0.009 adjusted for sex
OR for type 2 diabetes for <3 versus 4 risk alleles was 0.77 (0.65–0.93), *p*=0.005
OR for type 2 diabetes for >4 versus 4 risk alleles was 1.33 (1.12–1.58), *p*=0.001
T2D, type 2 diabetes

Discussion

Several studies have shown that SNPs in *GCK*, *GCKR*, *G6PC2* and *MTNR1B* are associated with FPG levels (4-7;9;11-13;20). In this study we replicated these findings in a Dutch population, with the exception of the association of *GCKR* with FPG. However, our results for *GCKR* are in the same direction as those of most other studies and it should be noted that some other recent publications reported considerable variability in effect size between different samples (8) or failed to replicate this observation (11). *GCK* and *G6PC2* were associated with HbA_{1c} in our study, which confirms previous observations (13;21).

We observed a significant combined effect of all variants on FPG levels. This confirms a recent observation in a French study (13). The association of FPG levels with the risk allele count was also reflected in increased HbA_{1c} levels, arguing against previous findings in which it was suggested that FPG and HbA_{1c} have independent underlying risk loci (22;23). Our cross-sectional data suggest that these loci cause a physiological disturbance of glucose homeostasis by raising the set point of insulin secretion, leading to an elevation of FPG depending on the number of risk alleles present, which is not further affected by ageing. However, longitudinal studies and a wider age span would be needed to confirm this observation.

To our knowledge, this is the first report showing that the analysed loci have a combined effect on type 2 diabetes susceptibility, although the contribution of each individual variant to the risk of type 2 diabetes is very low or undetectable (Table

1). Our data show that carriers of fewer than three risk alleles are at decreased risk of type 2 diabetes whereas those with more than five risk alleles have increased susceptibility to type 2 diabetes compared with the most common risk allele group of four risk alleles. We also noted a significantly different age at diagnosis between the different groups, indicating that the number of risk alleles also influences the age at which the disease becomes manifest. This might also have implications for the development of complications. If replicated, our results imply that these loci not only influence FPG levels, probably through an altered set point for glucose at which an insulin response is elicited, but also jointly increase the risk of type 2 diabetes and the age at diagnosis.

In conclusion, we replicated the combined effect of *GCK*, *GCKR*, *G6PC2* and *MTNR1B* risk alleles with FPG. Furthermore, we showed that the risk allele score is also associated with HbA_{1c} and that carriers of a low or high number of risk alleles have significantly different susceptibilities to the development of type 2 diabetes and age at diagnosis of the disease.

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Supplementary table s1. Association of GCK, GCKR, G6PC2 and MTNR1B with clinical variables in

Variant	FPG (mmol / L)	2hrG (mmol / L)	HbA_{1C} (%)	TG (mmol / L)
***GCK* (rs1799884)**				
GG	5.39 (0.01)	5.37 (0.04)	5.34 (0.01)	1.39 (0.02)
GA	5.45 (0.02)	5.49 (0.06)	5.40 (0.01)	1.39 (0.03)
AA	5.47 (0.05)	5.68 (0.18)	5.44 (0.03)	1.31 (0.09)
p-value	0.001	0.06	$5 \cdot 10^{-8}$	0.86[a]
***GCKR* (rs1260326)**				
TT	5.35 (0.03)	5.40 (0.08)	5.33 (0.02)	1.56 (0.05)
TC	5.39 (0.01)	5.36 (0.04)	5.35 (0.01)	1.39 (0.02)
CC	5.38 (0.01)	5.19 (0.04)	5.35 (0.01)	1.30 (0.02)
p-value	0.23	0.008	0.50	$9 \cdot 10^{-7}$[a]
***G6PC2* (rs560887)**				
AA	5.32 (0.03)	5.42 (0.09)	5.30 (0.02)	1.33 (0.05)
AG	5.36 (0.01)	5.34 (0.05)	5.34 (0.01)	1.37 (0.03)
GG	5.43 (0.01)	5.33 (0.04)	5.37 (0.01)	1.39 (0.02)
p-value	$5 \cdot 10^{-6}$	0.67	$3 \cdot 10^{-5}$	0.30[a]
***MTNR1B* (rs10830963)**				
CC	5.37 (0.01)	5.40 (0.04)	5.35 (0.01)	1.38 (0.02)
CG	5.44 (0.01)	5.38 (0.05)	5.37 (0.01)	1.38 (0.03)
GG	5.52 (0.04)	5.50 (0.12)	5.40 (0.02)	1.45 (0.07)
p-value	$7 \cdot 10^{-8}$	0.62	0.01	0.26[a]

Association of Fasting Plasma Glucose (FPG), 2 hours glucose (2hrG), HbA_{1C}, triglycerides (TG), LDL, HDL,
Data represent estimated means, adjusted for age, gender and BMI and standard deviations are given.
a: Non transformed values are shown. P-values are calculated with 10Log transformed triglyceride values.

non diabetic subjects from the NHS (n = 2361).

LDL (mmol / L)	HDL (mmol / L)	TC (mmol / L)	BMI (kg / m^2)	WHR
3.31 (0.02)	1.53 (0.01)	5.45 (0.03)	25.97 (0.10)	0.89 (0.001)
3.36 (0.04)	1.51 (0.02)	5.50 (0.04)	25.91 (0.15)	0.89 (0.002)
3.29 (0.11)	1.48 (0.05)	5.36 (0.12)	25.89 (0.47)	0.89 (0.006)
0.39	0.46	0.40	0.94	0.79
3.33 (0.05)	1.52 (0.02)	5.54(0.06)	25.85 (0.23)	0.89 (0.003)
3.32 (0.03)	1.52 (0.01)	5.46 (0.03)	25.78 (0.12)	0.89 (0.002)
3.35 (0.03)	1.54 (0.01)	5.47 (0.03)	25.94 (0.12)	0.89 (0.002)
0.76	0.37	0.53	0.66	0.63
3.23 (0.06)	1.57 (0.03)	5.39 (0.07)	25.87 (0.26)	0.89 (0.004)
3.31 (0.03)	1.51 (0.01)	5.44 (0.03)	25.93 (0.12)	0.89 (0.002)
3.36 (0.03)	1.52 (0.01)	5.50 (0.03)	25.87 (0.12)	0.89 (0.002)
0.12	0.18	0.18	0.93	0.99
3.34 (0.03)	1.52 (0.01)	5.48 (0.03)	25.88 (0.11)	0.89 (0.001)
3.28 (0.03)	1.54 (0.01)	5.43 (0.03)	25.93 (0.13)	0.89 (0.002)
3.34 (0.08)	1.48 (0.03)	5.47 (0.08)	26.63 (0.33)	0.89 (0.004)
0.29	0.27	0.54	0.09	0.82

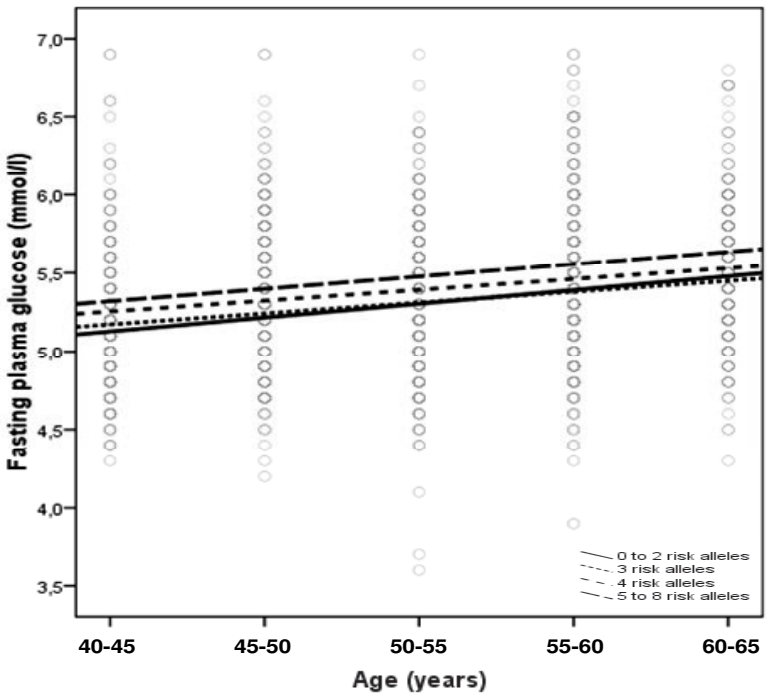
total cholesterol (TC), BMI and waist-hip ratio (WHR) with SNPs in *GCK*, *GCKR*, *G6PC2* and *MTNR1B*.

Supplementary table s2. Genotyping results of the independent SNPs in 2041 controls and 2628 cases.

SNP	Gene	risk allele	Allele frequency risk allele (AA, AB, BB)		OR (95% CI)	P-value
			controls (n)	cases (n)		
rs1799884	*GCK*	A	16.8 (641) 1326, 531, 55	18.4 (917) 1668, 743, 87	1.12 1.00 – 1.25	0.06
rs1260326	*GCKR*	C	65.8 (2562) 235, 864, 848	64.2 (3232) 313, 1174, 1029	0.94 0.86 – 1.02	0.13
rs560887	*G6PC2*	G	69.6 (2750) 192, 816, 967	68.6 (3502) 263, 1074, 1214	0.96 0.87 – 1.05	0.32
rs10830963	*MTNR1B*	G	25.0 (994) 1111, 764, 115	27.1 (1377) 1343, 1011, 183	1.12 1.02 – 1.23	0.02

Allele frequencies (counts) and genotype counts (AA, AB, BB) are shown. B represents the risk allele.

Supplementary Figure 1: Age related increase in FPG in non-diabetic subjects.



For ease of interpretation we have divided the non-diabetic subjects into four groups depending on the number of risk alleles. Group 1, 0 to 2 risk alleles (n=359); group 2, 3 risk alleles (n=580); group 3, 4 risk alleles (n=652) and group 4, >5 risk alleles (n=393). Unadjusted trend lines for each group are shown. Beta's with (95% CI) are calculated with linear regression adjusted for gender and BMI. Group 1: $\beta = 0.013$ (0.007-0.019); Group 2; $\beta = 0.011$ (0.006-0.016); Group 3; $\beta = 0.011$ (0.007-0.016); Group 4; $\beta = 0.013$ (0.007-0.019); all $P<1.0*10^{-4}$)

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