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ORIGINAL ARTICLE

Haplotypes of the genes (*GCK* and *G6PC2*) underlying the glucose/glucose-6-phosphate cycle are associated with pancreatic beta cell glucose sensitivity in patients with newly diagnosed type 2 diabetes from the VNDS study (VNDS 11)

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Abstract

Background Elevated fasting plasma glucose has been associated with increased risk for development of type 2 diabetes (T2D). The balance between glucokinase (GCK) and glucose-6-phosphate catalytic subunit 2 (G6PC2) activity are involved in glucose homeostasis through glycolytic flux, and subsequent insulin secretion.

Aim In this study, we evaluated the association between the genetic variability of *G6PC2* and *GCK* genes and T2D-related quantitative traits.

Methods In 794 drug-naïve, GADA-negative, newly diagnosed T2D patients (VNDS; NTC01526720) we performed: genotyping of 6 independent tag-SNPs within *GCK* gene and 5 tag-SNPs within *G6PC2* gene; euglycaemic insulin clamp to assess insulin sensitivity; OGTT to estimate beta-cell function (derivative and proportional control; DC, PC) by mathematical modeling. Genetic association analysis has been conducted using Plink software.

Results Two SNPs within *GCK* gene (rs882019 and rs1303722) were associated to DC in opposite way (both $p < 0.004$). Two *G6PC2* variants (rs13387347 and rs560887) were associated to both parameters of insulin secretion (DC and PC) and to fasting C-peptide levels (all $p < 0.038$). Moreover, subjects carrying the A allele of rs560887 showed higher values of 2h-plasma glucose (2hPG) ($p = 0.033$). Haplotype analysis revealed that *GCK* (AACAAA) haplotype was associated to decreased fasting C-peptide levels, whereas, the most frequent haplotype of *G6PC2* (GGAAG) was associated with higher fasting C-peptide levels ($p = 0.001$), higher PC ($\beta = 6.87$, $p = 0.022$) and the lower 2hPG ($p = 0.012$).

Conclusion Our findings confirmed the role of GCK and G6PC2 in regulating the pulsatility in insulin secretion thereby influencing insulin-signaling and leading to a gradual modulation in glucose levels in Italian patients with newly diagnosed T2D.

Keywords G6PC2 · GCK · Fasting plasma glucose · Beta-cell function · Haplotypes · SNP

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Introduction

Homeostatic control of glucose levels involves complex interactions between molecules that act in concert to maintain a normal fasting glucose concentration. High, but still normal, fasting plasma glucose (FPG) levels are associated with type 2 diabetes (T2D) increased risk [1–3], are inversely correlated to beta cell function (first phase insulin release) [4] and, in several genome wide association studies (GWAS), are reported to be influenced by a broad number of *loci* influencing T2D-related quantitative traits and/or T2D development [5–9]. Deep phenotyping of beta cell function in vivo by mathematical modeling can be summarized with

two distinct beta cell sensitivities, one to the rate of increase of plasma glucose (derivative or dynamic control, DC) and the other to glucose concentration itself (proportional or static control, PC). In the experimental setting of the IVGTT and the hyperglycemic clamp, DC and PC are responsible of classical first phase and second phase insulin release, respectively. PC, which to many researchers is beta cell glucose sensitivity by antonomasia, shows a progressive fall from normal glucose tolerance to pre-diabetes [10] and to type 2 diabetes [11, 12] and, importantly, predicts the development of glucose intolerance [13] and type 2 diabetes [14].

In the liver, the glucoseglucose-6-phosphate futile cycle can work as an ATP-consuming sliding door, the setting level of which directly affects glucose levels. Glucokinase and glucose-6-phosphatase are the molecular bases of the glucoseglucose-6-phosphate cycle. Glucokinase is a protein encoded by the *GCK* gene and phosphorylates glucose to glucose-6-phosphate, regulating the first step of glucose pathways [15] in liver, pancreatic beta cells and some glucose-sensing neurons in the central nervous system. Isozymes of glucokinase and glucose-6-phosphatase are expressed also in pancreatic beta cells and can sustain the operation of the glucoseglucose-6-phosphate cycle [16]. As in the liver, an unbalance in favor of glucose-6-phosphatase would lead to hyperglycemia because it would impair the sensitivity of one major beta cell glucose sensor to plasma glucose on one side, and it would consume ATP on the other side, thereby decreasing the net ATP yield achieved through the glucose molecules channeled into glycolysis. Both mechanisms would result into reduced glucose stimulated insulin secretion. Strictly speaking, indeed, beta cells are sensors of glucose-6-phosphate, not glucose, bioavailability through its metabolic transduction in ATP bioavailability.

Genome wide association studies have reported that both *GCK* and the beta cell specific glucose-6-phosphatase catalytic subunit 2 gene (*G6PC2*) harbor common variants firmly associated to fasting plasma glucose and type 2 diabetes mellitus. Mutations of *GCK* are the cause of MODY2, and glucokinase, in liver and beta cell or in liver only, has been considered for a while a viable molecular target for novel treatments of type 2 diabetes, leading to the development of glucokinase stimulators. Both germline [17, 18] and beta cell selective [19] deletion of *G6PC2* result into lower glucose levels in the mouse. The collective body of evidence, therefore, has led to include G6PC2 among the potential molecular targets of type 2 diabetes treatment [20].

Subtle, gene-based changes in the operation of the glucoseglucose-6-phosphate cycle may be anticipated to impair or to improve the glucose sensing mechanism(s) of beta cell. No previous studies, however, have addressed simultaneously the potential role of common genetic variability at *GCK* and *G6PC2*, i.e., the genes underpinning the glucoseglucose-6-phosphate cycle, on beta cell glucose sensitivity. The present investigation, therefore, was undertaken to assess the potential role of common *GCK* and *G6PC2* variants, either isolated or as haplotypes [21, 22], in modulating beta cell glucose sensitivity in a cohort of patients with newly diagnosed type 2 diabetes belonging to the Verona Newly Diagnosed T2D Study (VNDS).

Material and methods

Study population

The VNDS is a cohort consisting of Caucasian patients with newly diagnosed type 2 diabetes, drug-naïve and glutamic acid decarboxylase antibodies negative (GAD65 < 1 KU/L). As of January 1, 2002, all patients with T2D, defined on the basis of the American Diabetes Association [23], referred to the Diabetes Clinic embedded into the Division of Endocrinology, Diabetes and Metabolic Diseases of the University and Hospital Trust of Verona and whose disease was diagnosed in the past 6 months were offered to participate in this study. Recruitment was ended on December 31, 2015 and a follow-up was then planned and is ongoing. A detailed description of the experimental design has been previously published [24, 25]. In this study, that represent the 11th research performed on this cohort, we report the data collected in 794 consecutive patients, whose characteristics are summarized in Table 1. This research was approved by the Human Investigation Committee of the Verona City Hospital and the study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all study participants after a full explanation of the study.

Metabolic phenotypes

Standard clinical parameters were assessed in all patients. Metabolic tests were performed on two separate days in random order. On 1 day, a euglycaemic insulin clamp was performed to assess insulin sensitivity [26]. The amount of glucose metabolized during the last 60 min of the clamp [M-value, reference insulin sensitivity; units: μmol/min/m^2 body surface area (BSA)] was computed with standard formulas [27].

On a separate day, a frequently sampled, prolonged (240 or 300 min) OGTT (75 g) was carried out and beta-cell function (BF) was reconstructed by mathematical modelling, as previously described [27]. By this method, BF is described by two parameters of beta cell glucose sensitivity:

1. Derivative (or dynamic) control (DC): the response of the beta cell to the rate of increase of plasma glucose;

Table 1 Anthropometric and biochemical features of the total sample

Variable	All
N (M/F)	794 (542/252)
Age (years)	59 (52–66)
BMI ($Kg{\cdot}m^{-2}$)	29.3 (26.6–32.9)
Waist (cm)	100 (94–109)
Current smokers (%)*	18.3
$HbA_{1c\ DCCT}$ (%)	6.6 (6.1–7.3)
$HbA_{1c\ IFCC}$ (mmol/mol)	48.7 (44.2–56.3)
SBP (mmHg)	134 (120–145)
DBP (mmHg)	80 (80–90)
FPG (mmol/L)	7.0 (6.2–7.9)
2hPG (mmol/L)	12.9 (10.4–16.0)
Fasting c-peptide (nmol/L)	0.98 (0.76–1.26)
Insulin Sensitivity ($N=773$)	
M-clamp (μmol/min/m^2 BSA)	605 (380–874)
Beta-cell glucose sensitivity ($N=735$)	
Derivative control ($\sigma 1$) (pmol/m^2 BSA)·$(mmol{\cdot}L^{-1}{\cdot}min^{-1})^{-1}$	444 (68–938)
Proportional control ($\sigma 2$) [(pmol/min/m^2 BSA)/(mmol/L)]	46.8 (25.3–76.1)

Data expressed as median and interquartile range (IQR) *Data are expressed as percentage

BMI Body Mass Index; *SBP* systolic blood pressure; *DBP* diastolic blood pressure; *HbA1c DCCT* diabetes control and complication trial-aligned hemoglobin A1c; *HbA1c IFCC* international federation of clinical chemistry-aligned hemoglobin A1c; *FPG* fasting plasma glucose; *2hPG* 2-h plasma glucose

2. Proportional (or static) control (PC): the response of the beta cell to glucose concentration per se, herein presented as the stimulus–response curve relating insulin secretion rate (ISR, pmoles per min) to glucose concentration (mmol/l).

Laboratory data

Plasma glucose was assessed in duplicate with a Beckman Glucose Analyzer II (Beckman Instruments, Fullerton, CA, USA) or with an YSI 2300 Stat Plus Glucose & Lactate Analyzer (YSI Inc., Yellow Springs, OH, USA). Chemiluminescence based immunometric methods were used to measure serum C-peptide and insulin [24]. Glycosylated haemoglobin and serum lipids by standard in-house methods.

Genotyping

Two genomic area based on the International HapMap Project Phase III data on the CEU population were screened. *GCK* gene, on chromosome 7p13, and 17 kb on its 3′ flanking region and the human chromosome 2q31.1 encompassing the *G6PC2* gene, as well as 7 kb of its 5′ flanking region. Based on Tagger analysis using GEVALT (Genotype Visualization and Algorithmic Tool) [21] software, five SNPs (rs853770, rs483109, rs12475700, rs13387347 and rs560887) on *G6PC2* gene and six SNPs (rs11768607, rs882019, rs17832252, rs1476891, rs1303722 and rs4607517) on *GCK* region were selected as tagging SNPs covering at least 97% of the common genetic variability. Peripheral blood samples were collected from the participants and DNA was extracted by standard salting-out method. Genotypes were assessed by Veracode technique (Illumina Inc, CA), applying the GoldenGate Genotyping Assay according to manufacturer's instructions [28]. Plink (http://pngu.mgh.harvard.edu/purcell/plink/) [29] was used to obtain the posterior distribution of haplotypes consistent with the observed genotypes. Haplotypes' associations were tested in a linear regression model, as a function of haplotype dosage from posterior distribution, and including age, sex, and BMI as covariates. The selected SNPs were in low linkage disequilibrium (LD), with r^2 between the SNPs at each locus comprised between 0.0 and 0.54 (Figure S1 and S2).

Statistical analysis

Data are summarized as medians and interquartile range. Generalized Linear Models, adjusted for age, sex and BMI, with or without repeated measures as appropriate, were applied to test the associations between metabolic traits and SNP alleles or haplotypes. The covariates included in the multivariable regression models were selected on the basis of their biological plausibility as potential

confounding factors [30, 31]. Both SNPs and haplotype analyses were performed applying an additive genetic model. Statistical calculations were performed using SPSS 22.0 software (SPSS Inc., Chicago, IL, USA). Statistical significance was declared at $p < 0.05$ (95% confidence interval).

Results

We studied a cohort of 794 Italian newly diagnosed type 2 diabetes subjects whose anthropometric, clinical, and metabolic features are shown in Table 1. Among the 794 patients included in the study, 542 (68.3%) were male with a median age of 59 years [interquartile range: (52–66)]. Patients were more likely to be overweight or obese, with less than 50% having obesity. Glucose control, as measured by HbA1c, was fairly good with 25% patients having HbA1c > 7.3%. The frequency of subjects with antihypertensive therapy was 55.8% ($n = 443$) and the prevalence of current smoker subjects was of about 20%.

Effects of single GCK and G6PC2 variants on beta cell glucose sensitivity

The distributions of all genotypes were compatible with Hardy–Weinberg equilibrium (all $p > 0.161$).

Two (rs882019 and rs1303722) out of six SNPs located in the *GCK* region showed a significant association with DC of beta cell function. Carriers of the minor (G) rs882019 allele had significantly higher DC ($p < 0.001$), whereas carriers of of the minor (A) rs1303722 allele had significantly lower DC ($p = 0.004$) (Table 2). Likewise, two out of five SNPs located in the *G6PC2* region were associated to beta cell glucose sensitivity. The minor allele of rs560887 (A) was associated with a significant reduction in both DC ($p = 0.018$) and PC, i.e., the curve relating glucose (stimulus) to ISR (response) ($p = 0.03$). The minor allele of rs13387347 (A) was associated with a significant enhancement of both DC ($p = 0.03$) and PC ($p = 0.04$) (Table 2). Moreover, subjects carrying the minor allele of rs560887 (A), i.e., the ones with reduced beta cell glucose sensitivity, had also higher 2-h plasma glucose ($p = 0.033$). None of the SNPs of either gene was associated to fasting plasma glucose (Table 2).

Effects of GCK and G6PC2 haplotypes on beta cell glucose sensitivity

We detected 24 and 13 haplotypes at *GCK* and *G6PC2* regions, respectively. Their frequencies are presented in detail in Table S1 and Table S2. The highest frequencies of *GCK* (AACAAA) and *G6PC2* (GGAAG) haplotypes were about 19.5% and 22%, respectively. The latter haplotype was associated with higher beta cell glucose sensitivity (PC) ($\beta = 6.87$, $p = 0.022$) and lower 2-h plasma glucose ($\beta = -0.678$, $p = 0.012$) (Fig. 1). No association was found between any haplotype and fasting plasma glucose.

Discussion

In this single center cross-sectional study, we assessed the distribution of common variants within *GCK* and *G6PC2*, i.e., the genes underlying the glucoseglucose-6-phosphate cycle, in patients with newly diagnosed type 2 diabetic subjects who had a state-of-the-art assessment of two parameters (DC and PC) of beta cell glucose sensitivity.

Two SNPs of *GCK* (rs882019 and rs1303722) and two of *G6PC2* (rs560887 and rs13387347), as well as the most frequent haplotype of *G6PC2*, showed significant associations with beta cell glucose sensitivity. These findings are novel and relevant, in that they suggest a potential mechanistic link between genetic variation of these *loci* and beta cell glucose sensitivity, a parameter measurable only with deep phenotyping, which is associated with and predicts changes in glucose regulation. These findings may be mechanistically underlined by changes in the rate of the glucose glucose-6-phosphate cycle in the pancreatic beta cells, which result into concomitant changes of beta cell glucose sensitivity of opposite sign. Thus, our study suggests a scenario in which the common genetic variability of *GCK* and *G6PC2*, by affecting the rate of the glucose glucose-6-phosphate cycle, modulates beta cell glucose sensitivity and glucose stimulated insulin secretion, which in turn exerts a measurable impact on glucose regulation and, eventually, on the risk of developing type 2 diabetes.

Previous studies identified *GCK* and *G6PC2* as risk genes for T2D and for elevated fasting plasma glucose in people without diabetes mellitus [3, 32, 33]. The SNP rs1799884, located in the pancreatic β-cell-specific promoter of *GCK* gene, harbours a variant reported to be associated with fasting glucose and insulin secretion [34, 35]. It is in strong LD relationship ($D' = 1$ and $r^2 = 0.986$) with one tag-SNPs (rs4607517) selected in our study. However, we failed to find any association between rs4607517 and fasting glucose or beta cell glucose sensitivity. This data are in agreement with other studies which found no associations with fasting glucose or insulin secretion or other T2D-related quantitative trait [36]. Our findings that the genetic variability of two SNPs of GCK affect DC f beta cell function are in agreement with an early study by Hu et al. [37].

Table 2 Associations of tag-SNPs within *GCK* and *G6PC2* with beta cell glucose sensitivity and glucose levels in the VNDS

Gene	SNP	Chr	Chr position	Minor allele	MAF	Derivative control (σ1)		Proportional control (σ2)		FPG		2hPG	
						Per-allele effect β coefficient (95% Cis)	p value	Per-allele effect β coefficient (95% Cis)	p value	Per-allele effect β coefficient (95% Cis)	p value	Per-allele effect β coefficient (95% Cis)	p value
GCK	rs11768607	7	44,095,976	G	0.31	24.4 (−60.9 to 109.8)	0.574	2.0 (−3.1 to 7.0)	0.940	−0.09 (−0.28 to 0.10)	0.365	−0.19 (−0.64 to 0.26)	0.311
	rs882019		44,139,230	G	0.45	146.4 (67.1–225.8)	**< 0.001**	4.0 (−0.7 to 8.6)	0.815	0.06 (−0.13 to 0.24)	0.539	−0.02 (−0.44 to 0.41)	0.872
	rs17832252		44,160,500	A	0.16	−30.9 (−137.3 to 75.5)	0.569	0.6 (−5.6 to 6.9)	0.998	0.02 (−0.23 to 0.26)	0.892	0.11 (−0.45 to 0.66)	0.685
	rs1476891		44,190,369	G	0.35	−25.4 (−106.9 to 56.1)	0.542	0.1 (−4.7 to 4.8)	0.902	−0.02 (−0.20 to 0.17)	0.849	−0.15 (−0.58 to 0.28)	0.459
	rs1303722		44,179,475	A	0.49	−117.9 (−198.4 to −37.3)	**0.004**	−3.7 (−8.4 to 1.0)	0.705	−0.08 (−0.26 to 0.11)	0.405	−0.01 (−0.43 to 0.42)	0.968
	rs4607517		44,196,069	A	0.20	41.6 (−61.7 to 144.9)	0.429	−3.2 (−9.2 to 2.9)	0.168	0.14 (−0.10 to 0.37)	0.249	0.27 (−0.27 to 0.81)	0.268
G6PC2	rs853770	2	168,893,331	A	0.32	67.0 (−19.2 to 153.2)	0.411	−0.6 (−5.7 to 4.5)	0.831	0.14 (−0.06 to 0.33)	0.171	0.41 (−0.04 to 0.86)	0.055
	rs483109		168,895,046	G	0.33	11.9 (−72.2 to 95.9)	0.337	5.0 (−0.1 to 9.9)	0.118	−0.13 (−0.32 to 0.06)	0.180	−0.41 (−0.85 to 0.03)	0.061
	rs12475700		168,897,166	G	0.42	−79.9 (−162.2 to 2.24)	0.484	−0.1 (−10.8 to −1.1)	**0.036**	0.15 (−0.03 to 0.34)	0.105	0.50 (0.07–0.93)	**0.023**
	rs13387347		168,898,336	A	0.45	89.4 (8.7–170.1)	**0.030**	2.4 (0.8–8.7)	**0.038**	−0.01 (−0.19 to 0.18)	0.938	−0.37 (−0.80 to 0.05)	0.087
	rs560887		168,906,638	A	0.29	−104.0 (−189.9 to −18.2)	**0.018**	−5.4 (−10.5 to −0.4)	**0.018**	0.07 (−0.13 to 0.27)	0.482	0.49 (0.04–0.94)	**0.033**

Statistically significant associations are reported in bold

Allele frequency distributions of *GCK* and *G6PC2* gene polymorphisms and their associations with beta cell glucose sensitivity and glucose levels. Associations were sought using generalized linear model with adjustment for age, sex and BMI

Chr chromosome, chromosome position according to GRCh38.p7; *MAF* minor allele frequency; *FPG* fasting plasma glucose; *2hPG* 2-h plasma glucose

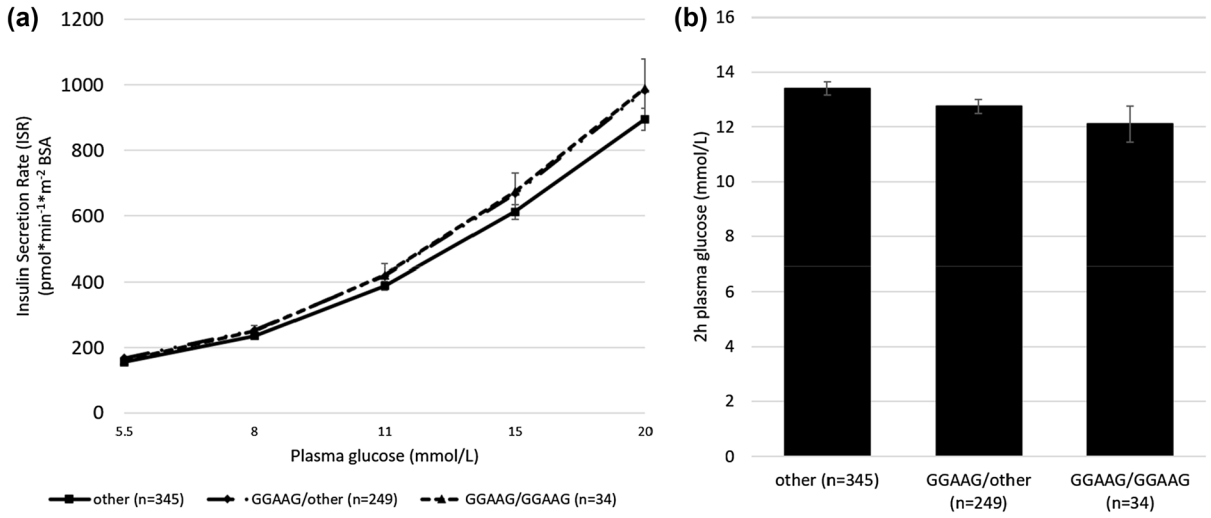

Fig. 1 Association of the GGAAG haplotype of *G6PC2* with beta cell glucose sensitivity (proportional control of β-cell function) and with 2h-plasma glucose levels. **a** The GGAAG haplotype is associated to higher beta cell glucose sensitivity (proportional control of beta cell function presented as the stimulus–response curve relating insulin secretion rate (ISR) to glucose concentration) in patients with newly diagnosed type 2 diabetes ($p=0.022$); **b** the GGAAG haplotype is associated to lower 2hPG in patients with newly diagnosed type 2 diabetes ($p=0.012$)

Previous studies have highlighted the potential role of rs560887 variants of *G6PC2* in affecting glucose regulation [38], fasting glucose and 2-h glucose levels [39]. Importantly, the association between *G6PC2* SNPs and FPG was reported in non-diabetic population, but not in T2D cohorts [6, 37, 39]. Similarly, in our work, the rs560887 variants had no statistically significant association with fasting glucose.

In our study, rs560887 seems to affect both parameters of beta cell glucose sensitivity, i.e., DC and PC, which may be causally related to the significant association with 2-h glucose after the OGTT. Similar results were reported in previous papers, but those studies were performed in non-diabetic subjects or in pooled T2D/control cohort [37, 40, 41]. Functional studies performed on G6PC2 knockout mice identified that the complete isoform of G6pc2 may be critical for hydrolytic activity and, consequently, may have implications for activity of G6pc2 and its potential role in glucose regulation [32, 42]. Moreover, previous studies have reported that G6PC2 can modulate islet calcium metabolism in the endoplasmatic reticulum and affect the pulsatility of insulin secretion [40]. These data were not confirmed in humans [43]. To the best of our knowledge, this is the first study in which *GCK* and *G6PC2* haplotypes are assessed together with deep phenotyping of beta cell glucose sensitivity in a large cohort of drug naïve T2D subjects. Furthermore, a number of reports [21, 22] have emphasized that the haplotype analysis is the optimal approach to capture the global genetic variability, since it takes into account the allelic heterogeneity of the whole genetic regions. However, our data cannot prove a cause-effect relationship and they need be confirmed by other studies. Finally, we cannot exclude the possibility that other complex interactions among *GCK*, *G6PC2* and glucokinase regulatory protein (*GCKR*) might have an influence on glucose levels [44]. In a subset of this same cohort, we previously reported that two (rs6717980 and rs2384628) out of six *GCKR* SNPs exerted a strong, independent influence on β-cell function [26].

Strengths of our study are: (a) large sample of subjects with newly diagnosed drug naïve T2D, with moderately high glucose levels and with limited confounding effects of glucose toxicity or diabetes therapy; (b) deep phenotyping of beta cell glucose sensitivity; (c) investigation extended from SNPs to haplotypes, thereby providing improved mapping of causal genetic regions, new insights on single variants influencing clinical traits, and additional statistical and biologic power in comparison to single variants studies.

Limitations of this study are: (a) the sample is not population based, although, on the basis of its phenotypic profile, the VNDS cohort is fairly representative of people with newly diagnosed T2D; (b) very cautious extrapolation of our results to other ethnic groups; (c) lack of replication of our findings in an independent sample of patients.

In conclusion, we have reported that the common genetic variability of the two loci underlying the glucoseglucose-6-phosphate cycle is related to beta cell glucose sensitivity in T2D. These findings may be relevant for the natural history of the disease and may support the rationale to develop novel treatments which target beta cell glucoseglucose-6-phosphate cycle through G6PC2.

Supplementary material The online version of this article (https://doi.org/10.1007/s40618-020-01483-3) contains supplementary material, which is available to authorized users.

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Author contributions CZ and ER researched and analysed data and cowrote the manuscript. SB genotyped all samples and discussed the article. MLB carried out mathematical modelling of the data. ET and GM discussed the article. RCB developed the mathematical models and designed the study. EB edited the manuscript and provided substantial contribution to the overall manuscript. MT is the guarantor of this work and, as such, had full access to all the data in the study and take responsibility for the integrity and the accuracy of the data analysis.

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Compliance with ethical standards

Conflict of interest The authors have no potential conflicts of interest to disclose.

Research involving human participants and/or animals We studied the genetics of a cohort and in the main text is reported that the research was approved by the Human Investigation Committee of the Verona City Hospital and the study was conducted in accordance with the Declaration of Helsinki.

Informed consent Written consent was obtained from all study participants after a full explanation of the study.

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