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ARTICLE

Glucose tolerance, insulin sensitivity and insulin release in European non-diabetic carriers of a polymorphism upstream of *CDKN2A* and *CDKN2B*

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Abstract
Aims/hypothesis The aim of this study was to investigate the association of the rs10811661 polymorphism near the *CDKN2B*/*CDKN2A* genes with glucose tolerance, insulin sensitivity and insulin release in three samples of white people with European ancestry.
Methods Sample 1 comprised 845 non-diabetic offspring of type 2 diabetes patients recruited in five European centres participating in the EUGENE2 study. Samples 2 and 3 comprised, respectively, 864 and 524 Italian non-diabetic participants. All individuals underwent an OGTT. Screening for the rs10811661 polymorphism was performed using a TaqMan allelic discrimination assay.
Results The rs10811661 polymorphism did not show a significant association with age, BMI and insulin sensitivity. Participants carrying the TT genotype showed a significant reduction in insulin release, measured by an OGTT-derived index, compared with carriers of the C allele, in the three samples. When these results were pooled with those of three published studies, and meta-analysed with a random-effects model, the T allele was significantly associated with reduced insulin secretion (−35.09 [95% CI 14.68–55.52], p=0.0008 for CC+CT vs TT; and −29.45 [95% CI 9.51–49.38], p=0.0038, for the additive model). In addition, in our three samples, participants carrying the TT genotype exhibited an increased risk

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for impaired glucose tolerance (IGT) compared with carriers of the C allele (OR 1.55 [95% CI 1.20–1.95] for the meta-analysis of the three samples).
Conclusions/interpretation Our data, together with the meta-analysis of previously published studies, show that the rs10811661 polymorphism is associated with impaired insulin release and IGT, suggesting that this variant may contribute to type 2 diabetes by affecting beta cell function.

Keywords Beta cell dysfunction · *CDKN2A/CDKN2B* · Genetics · Insulin · Meta-analysis · Offspring · Type 2 diabetes

Abbreviations

CATAMERIS	CAtanzaro MEtabolic RIsk factors Study
CDK4	Cyclin-dependent kinase 4
CDK6	Cyclin-dependent kinase 6
DPP	Diabetes Prevention Program
GWA	Genome-wide association
IGT	Impaired glucose tolerance
ISI	Matsuda index of insulin sensitivity
$p15^{INK4a}$	Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)
$p15^{INK4b}$	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)

Introduction

The pathophysiology of type 2 diabetes is characterised by a combination of impaired insulin action at the level of skeletal muscle, fat and liver, and failure of pancreatic beta cells to compensate for the enhanced insulin demand, ultimately resulting in hyperglycaemia [1, 2]. The pathogenesis of these two components is generally thought to be multifactorial, involving both genetic susceptibility and environmental factors [3]. However, identifying genes that confer susceptibility to type 2 diabetes has proven problematic. The availability of high-density genotyping arrays has enabled a systematic search for type 2 diabetes-associated common variants on a genome-wide scale [4–9]. Results of these studies have led to the identification of novel risk loci for type 2 diabetes. Among these loci, a polymorphism on chromosome 9p (rs10811661), located 125 kb upstream of the *CDKN2B* and *CDKN2A* genes (encoding cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4) [$p15^{INK4b}$] and cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4) [$p16^{INK4a}$], respectively), has been associated with type 2 diabetes in three of the genome-wide association (GWA) studies (OR for pooled studies 1.20 [95% CI 1.14–1.25], $p=5\times10^{-15}$) [6–8]. This association was replicated in several populations including Danish, Norwegian, French, Korean, Japanese and Chinese participants [10–15], but not confirmed in African-Americans and Pima Indians [16, 17]. Furthermore, the polymorphism rs10811661 was not associated with incident diabetes in the Diabetes Prevention Program (DPP) [18].

$p15^{INK4b}$ and $p16^{INK4a}$, the proteins encoded by the *CDKN2B* and *CDKN2A* genes, are tumour suppressors that inhibit cyclin-dependent kinase 6 (CDK6) and CDK4, respectively, two regulators of pancreatic beta cell replication [19–21]. Both *CDKN2B* and *CDKN2A* are expressed in pancreatic islets and adipocytes [6–8]. In murine models, it has been demonstrated that increased production of $p15^{INK4b}$ is associated with pancreatic islet hypoplasia and impaired glucose-induced insulin secretion [19]. On the other hand, mice lacking CDK4 exhibit insulin-deficient diabetes due to a reduction in pancreatic beta cells, and mice expressing a mutant CDK4 that cannot bind the cell-cycle inhibitor $p16^{INK4a}$ display pancreatic hyperplasia due to proliferation of beta cells [20, 21]. These data suggest that the rs10811661 polymorphism located upstream of the *CDKN2B* and *CDKN2A* genes may confer increased risk for type 2 diabetes by affecting beta cell function. However, studies aimed at evaluating the potential role of the rs10811661 polymorphism in affecting quantitative metabolic traits associated with the risk of diabetes, such as measures of beta cell function and insulin sensitivity, have led to discordant results. Two studies have reported that non-diabetic carriers of the type 2 diabetes risk allele (T), the rs10811661 polymorphism, had lower insulin release during an OGTT [10, 22], whereas other studies carried out in participants of European ancestry and in Pima Indians did not find an association between this polymorphism and insulin secretion [17, 23, 24]. Thus, in order to obtain additional data on the association of the rs10811661 polymorphism with impaired glucose tolerance and pathophysiological quantitative traits, i.e. measures of beta cell function and insulin sensitivity, we studied three well-characterised samples of white Europeans.

Methods

Three different samples of adult (≥18 years of age) non-diabetic white people of European ancestry were studied.

Sample 1 comprised 845 non-diabetic offspring who had one parent with type 2 diabetes and the other parent with no family history of type 2 diabetes and/or a normal response to an OGTT. The offspring of type 2 diabetic patients were recruited in five different European centres participating in the EUGENE2 project (www.eugene2.com) [25] as follows: Copenhagen, Denmark ($n=268$), Kuopio, Finland ($n=217$), Tübingen, Germany ($n=152$), Catanzaro, Italy ($n=109$) and

Gothenburg, Sweden (n=99). Of these, 136 individuals (16.1%) had impaired glucose tolerance (IGT).

All study centres followed the same protocol, as previously reported [25]. Briefly, all participants underwent anthropometrical evaluation, and a 75 g OGTT was performed with 0, 30, 60, 90 and 120 min sampling for plasma glucose and insulin. On the second visit after a 12 h fast, the participants underwent a euglycaemic hyperinsulinaemic clamp study. The rate of total insulin-stimulated glucose disposal (M) was calculated for the last 60 min of the insulin infusion. In the Copenhagen centre, the euglycaemic clamp was not performed. Glucose levels were measured by the glucose oxidase method. Because plasma insulin was measured by different methods (except for the Gothenburg centre, for which insulin was measured in Tübingen), the assay applied in Tübingen (micro-particle enzyme immunoassay; Abbott Laboratories, Tokyo, Japan) was selected as a reference assay. The Catanzaro, Copenhagen and Kuopio centres each sent between 40 and 100 fasting and post-glucose challenge plasma insulin samples for parallel analysis in Tübingen. Plasma insulin levels from these three centres were converted by linear regression analysis to plasma insulin levels corresponding to the Tübingen assay.

Sample 2 comprised 864 unrelated non-diabetic individuals participating in the CAtanzaro MEtabolic RIsk factors Study (CATAMERIS), an observational study focused on assessment of cardio-metabolic risk factors [26]. Of these, 237 individuals (27.5%) had IGT.

Sample 3 comprised 524 unrelated non-diabetic participants consecutively recruited at the Department of Internal Medicine of the University of Rome-Tor Vergata [27]. Of these, 125 individuals (23.9%) had IGT. All participants included in samples 2 and 3 underwent anthropometrical evaluation and a venous blood sample was drawn for laboratory determination. After a 12 h overnight fast, a 75 g OGTT was performed with 0, 30, 60 and 120 min sampling for plasma glucose and insulin. The Matsuda index of insulin sensitivity (ISI) was calculated as described by Matsuda and DeFronzo [28]. Glucose-stimulated insulin secretion was estimated using the Stumvoll index for first-phase insulin secretion and was calculated as: $2{,}503+6.476 \times \text{insulin}_0$ (pmol/l) $- 126.5 \times \text{glucose}_{120}$ (mmol/l) $+ 0.954 \times \text{insulin}_{120}$ (pmol/l) $- 239.3 \times \text{glucose}_0$ (mmol/l), as previously described [29].

The study protocol was approved by appropriate institutional review boards and was in accordance with the Helsinki Declaration II. All study participants gave informed consent.

DNA analysis DNA was isolated from whole blood using commercial DNA isolation kits. Samples from the EUGENE2 Consortium were genotyped in the Kuopio centre, whereas the two Italian samples were genotyped in the Catanzaro centre. Screening of the rs10811661 polymorphism was performed using a TaqMan allelic discrimination assay (Applied Biosystems, Foster City, CA, USA). The TaqMan genotyping reaction was amplified on a GeneAmp PCR system 2700 (Applied Biosystems) and fluorescence was detected using an ABI Prism 7000 sequence detector (Applied Biosystems). The overall genotyping success rate was 99.5%; genotype distributions obeyed Hardy–Weinberg equilibrium in the three different samples.

Statistical analysis Data analyses were performed using Statistical Package for Social Science (SPSS Inc., Chicago, IL, USA) version 14.0. The results for continuous variables are given as means ± SD. Because of the low number of CC homozygotes in the study samples, the rs10811661 polymorphism was tested using both the additive and the dominant inheritance model. Unpaired Student's t tests or ANOVAs were used to compare differences among groups for continuous variables, as appropriate, and the χ^2 test for non-continuous variables. A general linear model was used to compare phenotypic differences in the three samples. A multivariable logistic regression analysis, adjusted for age, sex and centre, was used to determine the association between the genotype frequencies and IGT. We report a nominal p value of <0.05 without adjustment for multiple testing given the high prior probabilities for association of the rs10811661 polymorphism with the examined phenotypes. A fixed-effect meta-analysis was performed using a comprehensive meta-analysis programme to assess the global OR for IGT in samples 1–3. To meta-analyse the effect of rs10811661 on insulin secretion in the three samples analysed in this study, together with three previously published studies [10, 22, 23], effect size estimates and standard errors from the six samples were pooled by RGui version 2.10.0 (www.r-project.org) applying the meta package and the combined effect was derived from the random-effects method (weight of studies estimated using the DerSimonian–Laird method [30]).

Results

The clinical characteristics of sample 1 according to the rs10811661 polymorphism are shown in Table 1. The rs10811661 polymorphism did not show any significant association with age, BMI or fasting plasma glucose levels. We also found no association between the rs10811661 polymorphism and whole-body insulin sensitivity as measured by a hyperinsulinaemic, euglycaemic clamp (M value; Table 1). By contrast, the rs10811661 polymor-

Table 1 Clinical and metabolic characteristics of 845 non-diabetic offspring of patients with type 2 diabetes according to the rs10811661 polymorphism at *CDKN2A/B*

Variable	rs10811661 genotype			*p* value	*p* value[a]
	TT	TC	CC	TT vs TC vs CC	TT vs TC+CC
Men/women (*n*=845)	261/345	83/140	7/9	0.31	0.15
Age (years)	40±10	40±10	40±9	0.90[b]	0.99[b]
BMI (kg/m^2)	26.6±5.0	26.9±4.9	25.9±5.2	0.64[c]	0.92[c]
Fasting glucose (mmol/l)	5.05±0.55	5.1±0.5	4.77±0.5	0.11	0.46
2 h post-load glucose (mmol/l)	6.38±1.6	6.05±1.5	6.22±1.6	0.03	0.03
Stumvoll first-phase insulin secretion index	1,114±412	1,200±628	1,212±340	0.05	0.03
Glucose disposal (μmol kg^{-1} min^{-1}) (*n*=577)	41.5±16.3 (*n*=413)	41.7±17.2 (*n*=150)	37.6±16.6 (*n*=14)	0.15	0.20
Disposition index (Stumvoll first-phase insulin secretion index × glucose disposal) (*n*=577)	45,335±19,301	48,526±26,391	45,768±20,170	0.31	0.14
NGT/IGT, *n*/*n* (%)	497/109 (18)	199/24 (10.8)	13/3 (18.8)	0.04	0.01

Data are mean ± SD

Comparisons between the three groups were performed using a general linear model. Categorical variables were compared by χ^2 test. *p* values are after adjustment for centre, age, sex and BMI

NGT, normal glucose tolerance

[a] *p* values for comparisons of differences in continuous variables in a dominant model while adjusting for age, sex and BMI

[b] *p* values after adjustment for centre and sex

[c] *p* values after adjustment for centre, age and sex

phism was significantly associated with 2 h post-load glucose levels, with carriers of the C allele having significantly lower levels compared with participants carrying the TT genotype (p=0.03 according to a dominant model; Table 1). In addition, the rs10811661 polymorphism was significantly associated with the OGTT-derived index of insulin secretion, with participants carrying the TT genotype showing a significant reduction in insulin release compared with carriers of the C allele (p=0.03). In a logistic regression analysis with adjustment for age, sex and centre, participants carrying the TT genotype exhibited an increased risk for IGT compared with carriers of the C allele (OR 1.78 [95% CI 1.12–2.82], p=0.014).

To replicate these data, two independent samples of non-diabetic Italian individuals were studied. The clinical characteristics of sample 2 according to the rs10811661 polymorphism are shown in Table 2. The rs10811661 polymorphism did not show any significant association with age, BMI, fasting plasma glucose levels or insulin sensitivity as measured by the ISI index. Carriers of the C allele have significantly lower levels of 2 h post-load glucose levels compared with participants carrying the TT genotype (p=0.04 according to a dominant model; Table 2). Furthermore, the rs10811661 polymorphism was significantly associated with the OGTT-derived index of insulin secretion, with participants carrying the TT genotype showing a significant reduction in insulin release compared with carriers of the C allele (p=0.05). In a logistic regression analysis with adjustment for age and sex, participants carrying the TT genotype exhibited an increased risk for IGT, compared with carriers of the C allele (OR 1.44 [95% CI 1.02–2.03], p=0.04).

Clinical characteristics of sample 3 according to the rs10811661 polymorphism are shown in Table 3. The rs10811661 polymorphism was not associated with age, BMI or insulin sensitivity as measured by the ISI index. Carriers of the C allele showed significantly lower fasting and 2 h post-load glucose levels compared with participants carrying the TT genotype (p=0.01 and p=0.04 according to the dominant model, for fasting and 2 h post-load glucose, respectively). In addition, the rs10811661 polymorphism was significantly associated with the OGTT-derived index of insulin secretion, with participants carrying the TT genotype showing a significant reduction in insulin release compared with carriers of the C allele (p=0.03). In a logistic regression analysis with adjustment for age and sex, participants carrying the TT genotype exhibited an increased risk for IGT compared with carriers of the C allele (OR 1.61 [95% CI 1.01–2.59], p=0.04).

As no genetic heterogeneity across the three samples was evident (p value for heterogeneity=0.76), data from the three samples were meta-analysed with a fixed-effect model. The results of this meta-analysis showed that participants carrying the TT genotype have an OR for

Table 2 Clinical and metabolic characteristics of 864 non-diabetic unrelated participants according to the rs10811661 polymorphism at *CDKN2A/B*

Variable	rs10811661 genotype			*p* value	*p* value[a]
	TT	TC	CC	TT vs TC vs CC	TT vs TC+CC
Men/women	301/281	121/121	27/13	0.11	0.33
Age (years)	50±13	50±12	50±13	0.92[b]	0.70[b]
BMI (kg/m^2)	30.3±5.6	30.0±5.4	30.9±6.1	0.37[c]	0.46[c]
Fasting glucose (mmol/l)	5.27±0.66	5.22±0.61	5.27±0.61	0.25	0.10
2 h post-load glucose (mmol/l)	6.94±2.33	6.78±1.94	5.89±1.27	0.003	0.04
Stumvoll first-phase insulin secretion index	1,325±520	1,389±621	1,576±569	0.03	0.03
Matsuda index/ISI	69±42	75±41	60±34	0.52	0.12
Disposition index (Stumvoll first-phase index insulin secretion × Matsuda index)	84,716±47,196	89,437±40,994	80,371±30,548	0.24	0.11
NGT/IGT, *n*/*n* (%)	409/172 (29.6)	183/59 (24.4)	35/5 (12.5)	0.03	0.03

Data are mean ± SD

Comparisons between the three groups were performed using a general linear model. Categorical variables were compared by χ^2 test. *p* values are after adjustment for age, sex and BMI

NGT, normal glucose tolerance

[a] *p* values for comparisons of differences in continuous variables in a dominant model while adjusting for age, sex and BMI

[b] *p* values after adjustment for sex

[c] *p* values after adjustment for age and sex

IGT of 1.55 (95% CI 1.20–1.95, p=0.0001) compared with carriers of the C allele.

Finally, in order to provide a more confident estimate of the effect of rs10811661 on insulin secretion, the results for the three samples analysed in this study were pooled with those of three available studies on European non-diabetic participants [10, 22, 23]. Borderline significant heterogeneity indicating differential effects between the studies was observed in the meta-analysis (p=0.068 for the dominant model and p=0.053 for the additive model). When data for

Table 3 Clinical and metabolic characteristics of 524 non-diabetic unrelated participants according to the rs10811661 polymorphism at *CDKN2A/B*

Variable	rs10811661 genotype			*p* value	*p* value[a]
	TT	TC	CC	TT vs TC vs CC	TT vs TC+CC
Men/women	122/232	47/96	9/18	0.94	0.73
Age (years)	42±13	42±13	38±11	0.28[b]	0.66[b]
BMI (kg/m^2)	30.0±7.2	29.9±7.8	27.5±6.7	0.27[c]	0.46[c]
Fasting glucose (mmol/l)	5.05±0.67	4.89±0.55	4.78±0.55	0.07	0.01
2 h post-load glucose (mmol/l)	6.61±1.94	6.33±1.83	5.67±1.78	0.02	0.04
Stumvoll first-phase insulin secretion index	1,302±513	1,430±677	1,471±697	0.02	0.007
Matsuda index/ISI	88±52	89±55	99±60	0.52	0.57
Disposition index (Stumvoll first-phase insulin secretion index × Matsuda index)	104,173±56,150	110,106±58,113	123,465±55,368	0.34	0.15
NGT/IGT (%)	260/94 (26.6)	115/28 (19.6)	24/3 (11.1)	0.07	0.03

Data are mean ± SD

Comparisons between the three groups were performed using a general linear model. Categorical variables were compared by χ^2 test. *p* values are after adjustment for age, sex and BMI.

NGT, normal glucose tolerance

[a] *p* values for comparisons of differences in continuous variables in a dominant model while adjusting for age, sex and BMI

[b] *p* values after adjustment for sex

[c] *p* values after adjustment for age and sex

the 13,896 individuals were meta-analysed with a random-effects model, the T allele was significantly associated with reduced insulin secretion (−35.09 [95% CI 14.68–55.52], p= 0.0008 for CC+CT vs TT; and −29.45 [95% CI 9.51–49.38], p=0.0038, for the additive model; Fig. 1).

Discussion

The present study aimed to elucidate the metabolic effects of the rs10811661 polymorphism, which has been recently identified as a type 2 diabetes susceptibility locus by GWA studies [6–8]. Using three samples of non-diabetic white people of European ancestry, we found that the rs10811661 polymorphism of *CDKN2B/CDKN2A* was significantly associated with impaired glucose-stimulated insulin release, but not with insulin sensitivity as measured by hyperinsulinaemic, euglycaemic clamp (sample 1) or by an OGTT-derived index of insulin sensitivity (samples 2 and 3). Association of the rs10811661 polymorphism of *CDKN2B/CDKN2A* with estimates of insulin release derived from an OGTT has been evaluated in previous studies, with divergent results [10, 17, 22–24]. In the Danish population-based Inter99 study, comprising 5,970 middle-aged participants, it was reported that carriers of the C allele had an estimated higher level of insulin release in response to an oral glucose load compared with participants carrying the TT genotype [10]; similar results were observed in a study of 5,327 non-diabetic Finnish men [22]. Interestingly, when we performed a meta-analysis including our samples and data from three previously published studies [10, 22, 23], we observed that the T allele was significantly associated with reduced insulin secretion. Of note, in Pima Indians, the rs10811661 polymorphism was not associated with either type 2 diabetes or insulin release [17]. The reasons for these discrepancies are unclear. Difference in ethnicity is the most obvious explanation for these divergent results. In support of this hypothesis, several type 2 diabetes risk variants found in Europid participants, such as polymorphisms in *CDKAL1*, *SLC30A8*, *HHEX*, *EXT2*, *IGF2BP2* and *LOC387761*, were not significantly associated with type 2 diabetes in Pima Indians [17]; furthermore it is possible that the lack of data on the causative variant associated with the variant under study may play a role in the discrepancies between the different ethnicities.

We also found that participants carrying the TT genotype have higher 2 h post-load glucose levels and increased risk of IGT (OR 1.55 [95% CI 1.20–1.95] in the pooled data from the three samples) compared with carriers of the C allele. Interestingly, participants carrying the low-risk CC genotype at the *CDKN2A/B* locus enrolled in the DPP exhibited a greater improvement in beta cell function than those with the high-risk TT genotype after treatment with troglitazone and lifestyle modification for 1 year, suggesting that they may have benefited more from these interventions [18].

Mechanisms underlying the association of the rs10811661 polymorphism with impaired insulin secretion and the risk of IGT and type 2 diabetes are not fully understood. The nearest annotated genes *CDKN2B* and *CDKN2A,* encoding $p15^{INK4b}$ and $p16^{INK4a}$, respectively, have been implicated in pancreatic islet regenerative capacity [19, 31]. $p16^{INK4a}$ has been shown to accumulate in many tissues, including pancreatic islets, as a function of ageing [32, 33]. Transgenic mice overproducing $p16^{INK4a}$ showed decreased islet proliferation with ageing, and aged mice lacking $p16^{INK4a}$ demonstrated enhanced islet proliferation. These observations support the idea that $p16^{INK4a}$ mediates a decline in the replicative capacity of beta cells associated with ageing. Because type 2 diabetes mellitus patients exhibit a decrease in beta cell mass [34], it may be tempting to speculate that increased $p16^{INK4a}$ levels with ageing may contribute to the relative failure of islet proliferation associated with type 2 diabetes mellitus. However, a recent study, evaluating whether genetic variants robustly associated with type 2 diabetes also modulate expression levels of nearby candidate genes, has

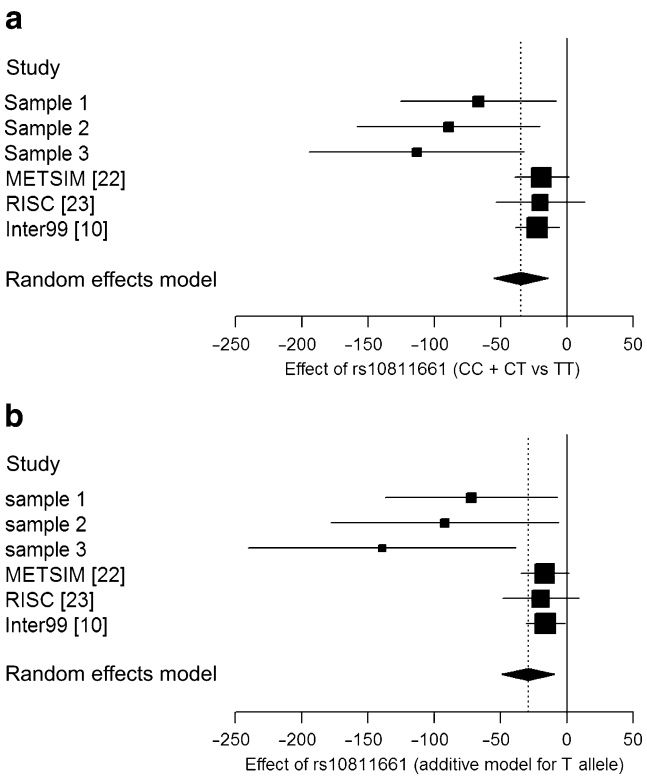

Fig. 1 Results of the meta-analysis of the association of the rs10811661polymorphism at *CDKN2A/B* and insulin secretion in 13,986 individuals. **a** CC+CT vs TT: −35.09 (95% CI 14.68–55.52), p=0.0008 (diamond) and (**b**) additive model: −29.45 (95% CI 9.51–49.38), p=0.0038 (diamond)

reported no evidence of an association between the rs10811661 polymorphism and *CDKN2B* and *CDKN2A* gene expression in human tissues [35].

This study has some limitations. The present findings obtained in a cross-sectional study of cohorts of Europids are explorative in nature and replication in independent prospective population-based studies with different ethnicities is needed to firmly determine whether the rs10811661 polymorphism affects insulin secretion and whether pancreatic beta cell dysfunction is truly implicated in the pathogenesis of type 2 diabetes.

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