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## Association analysis of 29,956 individuals confirms that a low-frequency variant at CCND2 halves the risk of type 2 diabetes by enhancing insulin secretion

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## Association Analysis of 29,956 Individuals Confirms That a Low-Frequency Variant at *CCND2* Halves the Risk of Type 2 Diabetes by Enhancing Insulin Secretion



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A recent study identified a low-frequency variant at *CCND2* associated with lower risk of type 2 diabetes, enhanced insulin response to a glucose challenge, higher height, and, paradoxically, higher BMI. We aimed to replicate the strength and effect size of these associations in independent samples and to assess the underlying mechanism. We genotyped the variant in 29,956 individuals and tested its association with type 2 diabetes and related traits. The low-frequency allele was associated with a lower risk of type 2 diabetes (OR 0.53;  $P = 2 \times 10^{-13}$ ; 6,647 case vs. 12,645 control subjects), higher disposition index ( $\beta = 0.07 \log_{10}$ ;  $P = 2 \times 10^{-11}$ ;  $n = 13,028$ ), and higher

Matsuda index of insulin sensitivity ( $\beta = 0.02 \log_{10}$ ;  $P = 5 \times 10^{-3}$ ;  $n = 13,118$ ) but not fasting proinsulin ( $\beta = 0.01 \log_{10}$ ;  $P = 0.5$ ;  $n = 6,985$ ). The low frequency allele was associated with higher adult height ( $\beta = 1.38 \text{ cm}$ ;  $P = 6 \times 10^{-9}$ ;  $n = 13,927$ ), but the association of the variant with BMI ( $\beta = 0.36 \text{ kg/m}^2$ ;  $P = 0.02$ ;  $n = 24,807$ ), estimated in four population-based samples, was less than in the original publication where the effect estimate was biased by analyzing case subjects with type 2 diabetes and control subjects without diabetes separately. Our study establishes that a low-frequency allele in *CCND2* halves the risk of type 2 diabetes primarily through enhanced insulin secretion.

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A recent study used whole-genome sequencing and imputation techniques to identify one of the first robust associations between a low-frequency variant (1.47% in Icelandic population) and type 2 diabetes (1). The effect of the G minor allele at rs76895963 was appreciably larger than that of known common variants (odds ratio [OR] 0.53) (1). The G allele was associated with lower fasting glucose levels and higher insulinogenic index, suggesting an effect on insulin secretion, but, paradoxically, was associated with higher BMI (0.56 kg/m<sup>2</sup>) (1).

Genetic associations need testing in independent studies to ensure associations are not false positive results and to establish an effect size less biased by winner's curse (regression to the mean). Once replicated, it is then important to test the underlying physiological mechanisms.

The apparently paradoxical association between the diabetes protective allele and higher BMI needs further explanation. Genetic associations that are paradoxical to epidemiological correlations have been described before and provide excellent targets for further investigation of biological mechanisms (2,3). However, associations between known type 2 diabetes alleles and BMI can be biased by a form of "index event bias," sometimes referred to as "truncation bias," if data sets are restricted to case or control subjects. This form of bias has likely led to associations between the risk allele at *TCF7L2* and lower BMI in case subjects because carriers of the risk allele do not need to be as overweight to develop diabetes (4).

We aimed to assess whether independent samples provide robust replication of the strength and effect sizes of the *CCND2* associations and to investigate further the underlying mechanisms that result in a low-frequency allele reducing the risk of type 2 diabetes but increasing height and BMI. We genotyped the *CCND2* variant in 29,956 individuals and tested its association with risk of type 2 diabetes and with measures of insulin sensitivity and insulin secretion.

## RESEARCH DESIGN AND METHODS

We genotyped the low-frequency *CCND2* variant (rs76895963) in 29,496 individuals of European origin. Study characteristics and genotyping details are in Table 1. Call rates in all samples exceeded 95%, with no evidence of departure from Hardy-Weinberg equilibrium ( $P > 0.05$ ).

We tested the association of the low-frequency variant with risk of type 2 diabetes, diabetes-related intermediate traits (fasting glucose, 2-h oral glucose tolerance test [OGTT] glucose, Matsuda index of insulin sensitivity, insulinogenic index, disposition index of  $\beta$ -cell function, and proinsulin levels), BMI, fat percentage, and height.

We used Matsuda index as a surrogate index of peripheral insulin sensitivity, which is highly correlated ( $\rho = 0.7$ ) with the gold standard measure of insulin resistance (euglycemic-hyperinsulinemic clamp [*M* value]) (5).

We calculated the following:  
Matsuda index of insulin sensitivity

$$\frac{1,000}{\sqrt{\text{Ins0} \times \text{Gluc0} \times \frac{\text{Ins0} + \text{Ins30} + \text{Ins120}}{3} \times \frac{\text{Gluc0} + \text{Gluc30} + \text{Gluc120}}{3}}}$$

Insulinogenic index

$$\frac{\text{Ins30} - \text{Ins0}}{\text{Gluc30} - \text{Gluc0}}$$

where Ins0, Ins30, Ins120, Gluc0, Gluc30, and Gluc120 are insulin and glucose levels at 0, 30, and 120 min of the OGTT, respectively. We also calculated insulin disposition index as follows: Matsuda index of insulin sensitivity  $\times$  insulinogenic index.

To provide additional statistical power to estimate the effect of the variant on BMI, we genotyped the variant in 6,597 female participants with prepregnancy BMI data from the Avon Longitudinal Study of Parents and Children (ALSPAC) (6). Ethics approval for the study was obtained from the ALSPAC Ethics and Law Committee and the local research ethics committees. The ALSPAC Web site contains details of all the data that are available through a fully searchable data dictionary (7).

To increase our statistical power to estimate the effect of the low-frequency variant on Matsuda index, insulinogenic index, and disposition index, we included 5,114 samples from the Inter99 study that was part of the original discovery (1,8). The Danish study was approved by the ethics committee of the Capital Region of Denmark.

Diabetes-related intermediate traits were log<sub>10</sub> transformed. We used age, sex (and age squared for height and BMI), and, if applicable, measures required to correct for genetic background, as covariates. We assumed an additive genetic model.

Analyses of glycemic traits, Matsuda index, insulinogenic index, and disposition index were performed in individuals without diabetes. For BMI, we limited analyses to studies most representative of the general population, with no or limited enrichment for or against type 2 diabetes. For the Genetics of Diabetes Audit and Research in Tayside Scotland (GoDARTS), a diabetes case-control study, we randomly selected a subset of case subjects to include with all the control subjects such that the "population" consisted of 5% with type 2 diabetes and 95% control subjects. We also reanalyzed data from the population-based studies from the original study but without separating individuals with diabetes from individuals without and assessed the extent of enrichment for diabetes in the deCODE population-based study.

We performed fixed-effects inverse variance-weighted meta-analysis in R (9). Evidence of between-study heterogeneity was assessed using the Cochran Q test and the  $I^2$  statistic (10). The study complies with the Declaration of Helsinki.

## RESULTS

### The *CCND2* Low-Frequency Allele Is Associated With a Lower Risk of Type 2 Diabetes

The frequency of rs76895963[G] was 1.97% in GoDARTS (Scottish), 2.15% in Metabolic Syndrome In Men (METSIM)

**Table 1—Summary details and relevant characteristics of the studies**

Study	N (N males/N females)	Age (years)	Type 2 diabetes case/control subjects (n/n)	Fasting glucose (mmol/L)	2-h OGTT (mmol/L)	Matsuda index	Insulinogenic index (pmol/mmol)	Disposition index	BMI (kg/m <sup>2</sup> )	Height (cm)	Genotyping
Avon Longitudinal Study of Parents and Children (ALSPAC) (6)	6,597 (0/6,597)	28.1 (4.8)	NA	NA	NA	NA	NA	NA	22.9 (3.8)	NA	Fluorescence-based competitive allele-specific assay (KASPar) at LGC Genomics (Hoddesdon, U.K.)
Genetics of Diabetes Audit and Research in Tayside Scotland (GODARTS) (12)	13,512 (7,078/6,434)	61.1 (10.6)	6,145/5,045	4.9 (0.7)	NA	NA	NA	NA	29.3 (5.8)	167.9 (9.7)	Fluorescence-based competitive allele-specific assay (KASPar) at LGC Genomics
Metabolic Syndrome In Men (METSIM) (5)	8,102 (8,102/0)	57.2 (7.1)	1,602/6,500	5.7 (0.5)	6.1 (1.7)	6.9 (4.2)	131.3 (220.3)	739.2 (1,566)	26.8 (3.8)	176.0 (6.4)	TaqMan Allelic Discrimination Assays (Applied Biosystems)
Relationship between Insulin Sensitivity and Cardiovascular disease (RISC) (13)	1,285 (574/711)	43.8 (8.3)	NA	5.1 (0.6)	5.7 (1.5)	11.6 (6.1)	96.9 (82.6)	996.7 (990)	25.5 (4.1)	170.7 (9.4)	Fluorescence-based competitive allele-specific assay (KASPar) at LGC Genomics

Data are mean (SD) unless otherwise indicated. NA, not available.

(Finnish), 1.51% in Relationship between Insulin Sensitivity and Cardiovascular disease (RISC) (European wide), and 2.04% in ALSPAC (southwest U.K.). The rs76895963[G] allele was associated with a lower risk of type 2 diabetes with an effect size very similar to that described in the initial discovery study (OR unadjusted for BMI 0.53 [95% CI 0.45, 0.63],  $P = 2 \times 10^{-13}$ , OR adjusted for BMI 0.49 [0.40, 0.58],  $P = 2 \times 10^{-14}$ ; 6,647 case vs. 12,645 control subjects) (Table 2 and Fig. 1). Meta-analysis with 12,939 case and 70,909 control subjects from the discovery studies revealed no evidence of heterogeneity of effect size across five studies (OR unadjusted for BMI 0.53 [0.48, 0.59],  $P = 1 \times 10^{-30}$ , OR adjusted for BMI 0.47 [0.42, 0.53],  $P = 1 \times 10^{-35}$ ; 19,586 case vs. 83,554 control subjects; heterogeneity  $P = 0.9$  for both unadjusted and adjusted model) (Table 2 and Fig. 1).

**The CCND2 Low-Frequency Allele Is Associated With Higher Insulin Secretion**

The G minor allele was associated with lower fasting glucose ( $\beta = -0.02 \log$  [95% CI  $-0.02, -0.01$ ],  $P = 5 \times 10^{-5}$ ;  $n = 11,739$ ) and lower 2-h OGTT glucose ( $\beta = -0.05 \log$  [ $-0.08, -0.03$ ],  $P = 6 \times 10^{-5}$ ;  $n = 8,261$ ). The combined meta-analysis estimated  $-0.01 \log$  [ $-0.02, -0.01$ ] lower fasting glucose levels ( $P = 9 \times 10^{-8}$ ;  $n = 23,503$ ) and  $-0.04 \log$  [ $-0.05, -0.02$ ] lower 2-h OGTT glucose levels ( $P = 1 \times 10^{-4}$ ;  $n = 13,161$ ) per copy of type 2 diabetes protective allele (Table 2).

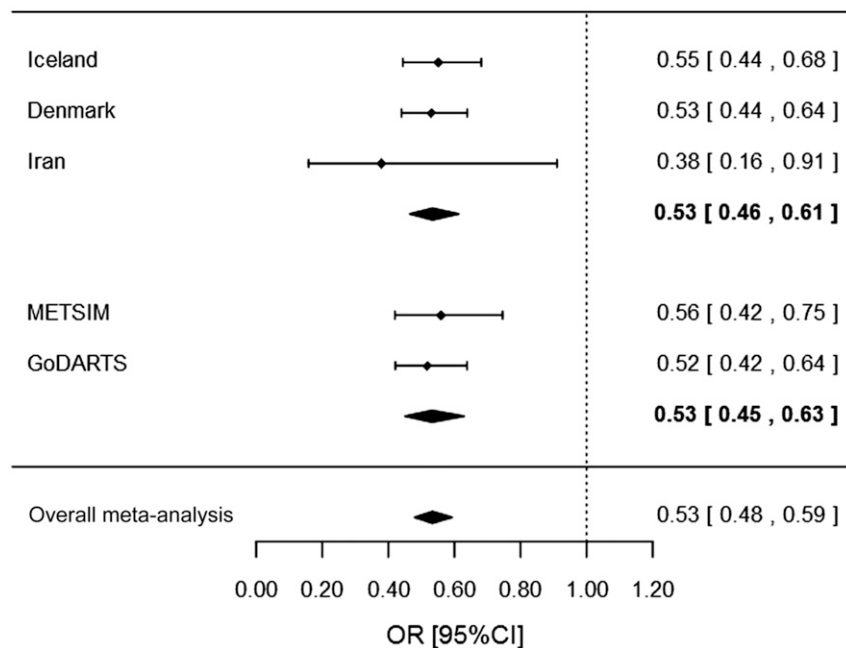
The type 2 diabetes protective allele was associated with improved ability to secrete insulin in response to a glucose challenge test: higher insulinogenic index ( $\beta = 0.06 \log_{10}$  [95% CI 0.03, 0.09],  $P = 1 \times 10^{-4}$ ,  $n = 8,067$ ; and  $\beta = 0.05 \log_{10}$  [0.03, 0.07],  $P = 8 \times 10^{-6}$ ,  $n = 13,181$

**Table 2—Association of rs76895963 in CCND2 with type 2 diabetes, diabetes-related intermediate traits, and anthropometric traits**

Trait/disease	Study	Effect size	95% CI	P	N	I <sup>2</sup> (%)	P <sub>het</sub>
Type 2 diabetes (BMI adjusted) (OR)	Original study	0.46	0.40, 0.54	$6 \times 10^{-23}$	12,939 vs. 70,909††	0	0.7
	Current study	0.49	0.40, 0.58	$2 \times 10^{-14}$	6,647 vs. 12,645††	0	0.6
	Combined	0.47	0.42, 0.53	$1 \times 10^{-35}$	19,586 vs. 83,554††	0	0.9
Type 2 diabetes (BMI unadjusted) (OR)	Original study	0.53	0.46, 0.61	$8 \times 10^{-19}$	12,939 vs. 70,909††	0	0.7
	Current study	0.53	0.45, 0.63	$2 \times 10^{-13}$	6,647 vs. 12,645††	0	0.7
	Combined	0.53	0.48, 0.59	$1 \times 10^{-30}$	19,586 vs. 83,554††	0	0.9
Fasting glucose (log)	Original study	-0.01	-0.02, -0.01	$3 \times 10^{-4}$	11,764	NA	NA
	Current study	-0.02	-0.02, -0.01	$5 \times 10^{-5}$	11,739	0	0.9
	Combined	-0.01	-0.02, -0.01	$9 \times 10^{-8}$	23,503	0	0.8
2-h OGTT (log)	Original study	-0.02	-0.04, 0.01	0.15	4,900	NA	NA
	Current study	-0.05	-0.08, -0.03	$6 \times 10^{-5}$	8,261	0	0.6
	Combined	-0.04	-0.05, -0.02	$1 \times 10^{-4}$	13,161	44	0.2
Matsuda index (log <sub>10</sub> )†	Current study	0.02	0.01, 0.03	$5 \times 10^{-3}$	13,118	0	0.8
Disposition index (log <sub>10</sub> )†	Current study	0.07	0.05, 0.09	$2 \times 10^{-11}$	13,028	0	0.7
Insulinogenic index (log <sub>10</sub> )†	Current and original study	0.05	0.03, 0.07	$8 \times 10^{-6}$	13,181	0	0.5
Fasting proinsulin (log <sub>10</sub> )*	Current study	0.01	-0.01, 0.02	0.5	6,985	NA	NA
30-min proinsulin (log <sub>10</sub> )*	Current study	-0.01	-0.02, 0.01	0.3	6,947	NA	NA
120-min proinsulin (log <sub>10</sub> )*	Current study	-0.01	-0.02, 0.00	0.1	6,978	NA	NA
BMI (kg/m <sup>2</sup> )	Population-based studies**	0.36	0.06, 0.65	0.02	24,807	0	0.8
	Current study***	0.05	-0.21, 0.30	0.7	22,464	0	0.7
	Combined	0.25	0.08, 0.43	$4 \times 10^{-3}$	109,492	2	0.4
Fat mass % (log <sub>10</sub> )	Current study	0.00	-0.01, 0.01	0.5	6,979	NA	NA
Height (cm)	Original study	1.16	0.83, 1.50	$6 \times 10^{-12}$	78,236	0	0.7
	Current study	1.38	0.92, 1.84	$6 \times 10^{-9}$	13,927	0	0.6
	Combined	1.24	0.97, 1.51	$2 \times 10^{-19}$	92,163	0	0.8

Analysis of diabetes-related intermediate traits and height reported in the table were performed in individuals without diabetes. NA, not applicable because data from only one study were available; P<sub>het</sub>, heterogeneity P value. \*Values were adjusted for corresponding insulin measurements at the same time points during OGTT. \*\*Results from population studies with no apparent enrichment for or against type 2 diabetes, including three studies from the original publication (Iranian TLGS study, Danish Inter99 study, and Danish Health2006 study) and ALSPAC. \*\*\*To avoid index event bias or truncation bias, we used our population-based studies. (See RESEARCH DESIGN AND METHODS AND DISCUSSION.) †For insulinogenic index, we give the meta-analysis results including data presented in the original article from the Inter99 study. For Matsuda index and disposition index, we give the meta-analysis results including a new analysis of the Inter99 study not previously presented. ††Number of cases vs. controls.

## Type 2 diabetes (BMI unadjusted)



**Figure 1**—Forest plot of the association between the *CCND2* rs76895963 low-frequency allele and type 2 diabetes (unadjusted for BMI) in discovery and replication studies. The dashed line indicates null effect. The top, middle, and bottom diamonds represent the effect size (center of diamond) and 95% CIs (horizontal ends) from the discovery studies, replication studies, and overall meta-analysis, respectively.

including the original study) (Table 2). The low-frequency allele was associated with higher disposition index ( $\beta = 0.08 \log_{10}$  [0.05, 0.11],  $P = 1 \times 10^{-7}$ ;  $n = 8,050$ ). Disposition index was not presented in the original study, but we analyzed the Danish Inter99 study and meta-analyzed with METSIM and RISC, which provided an effect of  $0.07 \log_{10}$  [0.05, 0.09] with higher disposition index ( $P = 2 \times 10^{-11}$ ;  $n = 13,028$ ) (Table 2).

The G allele was not associated with any measures of proinsulin levels adjusted for corresponding insulin levels at the same time points during OGTT (Table 2).

The analysis of the *CCND2* low-frequency allele and the Matsuda index in METSIM and RISC produced a borderline result ( $\beta = 0.03 \log_{10}$  [95% CI 0, 0.05],  $P = 0.05$ ;  $n = 8,134$ ). A meta-analysis of all 13,118 individuals without diabetes from METSIM, RISC, and Danish Inter99 resulted in a small association with Matsuda index ( $\beta = 0.02 \log_{10}$  [0.01, 0.03],  $P = 5 \times 10^{-3}$ ) (Table 2).

### The Effect Size of the *CCND2* Low-Frequency Allele With Height Is Consistent With the Original Study

The G minor allele was associated with higher adult height ( $\beta = 1.38 \text{ cm}$  [95% CI 0.92, 1.84],  $P = 6 \times 10^{-9}$ ;  $n = 13,927$ ) (Table 2). The combined meta-analysis including data from the original study estimated 1.24 cm [0.97,

1.51] higher height per copy of the type 2 diabetes protective allele ( $P = 2 \times 10^{-19}$ ;  $n = 92,163$ ) (Table 2).

### The Effect Size of the *CCND2* Low-Frequency Allele With BMI Is Lower Than Reported in the Original Study

The original report found an association between the low-frequency *CCND2* allele and higher BMI ( $0.56 \text{ kg/m}^2$ ) analyzed separately in individuals with or without type 2 diabetes resulting in spurious associations resulting from index event biases. To further test the BMI association, we first showed that individuals from the deCODE study with both *CCND2* genotype and BMI available had a slight excess for diabetic cases (Supplementary Fig. 1). We showed that this type of enrichment in population studies results in a bias toward an association between the protective allele and lower BMI because the diabetes case subjects tend to be heavier and carry less protective alleles than individuals without diabetes (Supplementary Fig. 2). We thus decided to focus our analysis of the BMI association to the four population studies with no apparent enrichment for or against type 2 diabetes, i.e., three studies from the original publication (the Iranian Tehran Lipid and Glucose Study [TLGS] [ $n = 8,658$ ], the Danish Inter99 study [ $n = 6,228$ ], and the Danish Health2006 study [ $n = 3,324$ ]) and ALSPAC ( $n = 6,597$ ). This resulted in a smaller effect of the variant on BMI than reported in the original publication

( $\beta = 0.36 \text{ kg/m}^2$  [95% CI 0.06, 0.65],  $P = 0.02$ ;  $n = 24,807$ ) (Table 2). We observed no heterogeneity between the effect estimates between these four population-based studies (heterogeneity  $P = 0.8$ ) (Table 2). This analysis represented our least biased estimate of the effect size. When we combined results from the four studies included in the original publication (reanalyzed including both type 2 diabetes case and control subjects) with results from four studies added in this article (with GoDARTS individuals sampled so as to include only 5% diabetes case subjects), the association with BMI was present but with lower effect size ( $\beta = 0.25 \text{ kg/m}^2$  [0.08, 0.43],  $P = 4 \times 10^{-3}$ ;  $n = 109,492$ ) (Table 2 and Fig. 2) and with some evidence of heterogeneity ( $P = 0.03$ ).

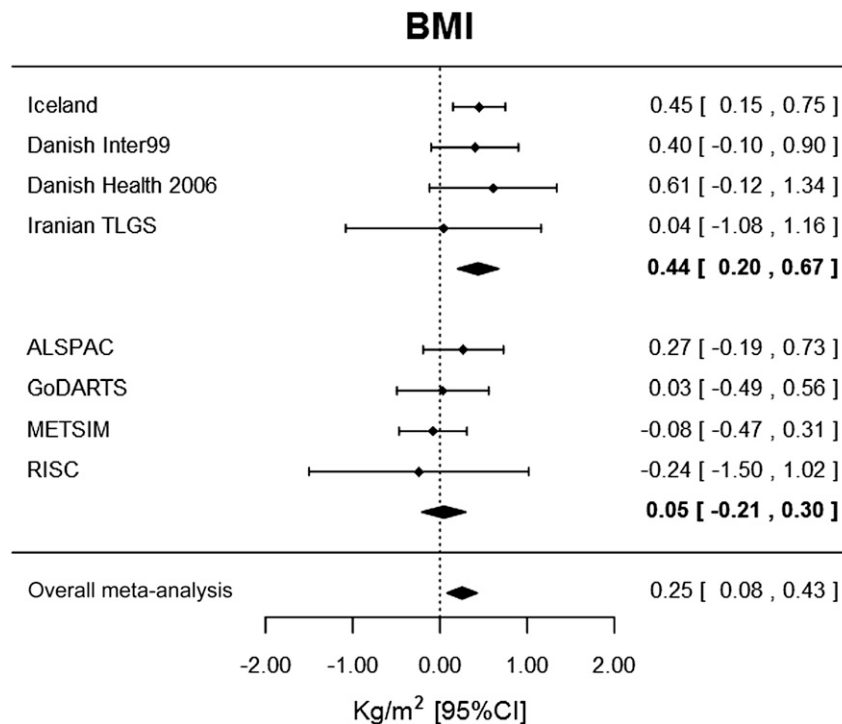
We found no association with fat mass percentage ( $\beta = 0.00$  [95% CI  $-0.01$ ,  $0.01$ ],  $P = 0.5$ ;  $n = 6,979$  individuals without diabetes) (Table 2).

## DISCUSSION

Our study provides robust replication of the relatively large protective effect of a low-frequency variant at *CCND2* against risk of type 2 diabetes and its association with improved insulin secretion and higher height. The estimate of the effect size on risk of type 2 diabetes in our study was very close to that of the discovery studies and therefore confirms an unbiased estimate of the effect size:

carriers of the low-frequency allele are at approximately half the risk of type 2 diabetes compared with noncarriers. Our results, together with data from the original study, provide very strong evidence of the mechanism of diabetes protection. The associations with improved disposition index and insulinogenic index but smaller effects with the Matsuda index, in up to 13,118 individuals, show that the protective diabetes effect operates primarily through a mechanism of relatively favorable insulin secretory response to a glucose challenge and to lower blood glucose more effectively than noncarriers. The effect is unlikely to act through improved insulin processing, as we saw no association with proinsulin levels in the METSIM study, despite previous observations of associations between the *TCF7L2* and other diabetes risk alleles in this study (2).

Our data suggest that the association between the *CCND2* protective variant and higher BMI is lower than that previously reported. A reanalysis of previous data, together with new data, provided evidence of an association between the *CCND2* protective allele and higher BMI, but we observed a smaller effect and heterogeneity between studies. Determining the true biological effect of the variant on BMI was very difficult because of “index event” bias. The “index event” in this case was a classification of normoglycemia; therefore, people carrying a type 2 diabetes protective allele remain normoglycemic at higher



**Figure 2**—Forest plot of the association between the *CCND2* rs76895963 low-frequency allele and BMI including eight studies with no, or limited, ascertainment or enrichment for or against type 2 diabetes. These eight studies included four from the original article, including deCODE individuals and a sample of GoDARTS individuals made to consist of 5% diabetes case subjects. The dashed line indicates null effect. The top, middle, and bottom diamonds represent the effect size (center of diamond) and 95% CIs (horizontal ends) from the discovery studies, replication studies, and overall meta-analysis, respectively.

BMI. Similar such likely biases have been observed between strong diabetes risk alleles and BMI where the risk allele at *TCF7L2* was associated with lower BMI in individuals with type 2 diabetes because individuals carrying a risk allele will develop diabetes at lower BMIs than non-carriers on average (4,11). Index event bias means it is extremely difficult to determine whether diabetes risk alleles have biological effects on BMI.

In summary, we replicated the diabetes and height growth effects of the low-frequency variant at *CCND2* in 29,956 individuals. Our best estimate of the effect of the variant on BMI suggests that the effect is smaller than reported in the original publication owing to index event bias. Further studies are needed to establish the size of the BMI association. Our data, together with the original finding, show a mechanism through improved insulin secretion, which results in lower fasting glucose levels, lower 2-h OGTT glucose levels, and a lower risk of type 2 diabetes. Combining all data including 19,586 type 2 diabetes case and 83,554 control subjects from the original study and our study provides evidence that carrying this variant reduces the risk of type 2 diabetes by ~50% relative to noncarriers.

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