The INFLUENCE of rare Genetic variation in *SLC30A8* on diabetes incidence and beta-cell function

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Context/Objective: The variant rs13266634 in *SLC30A8*, encoding a beta-cell specific zinc transporter, is associated with type 2 diabetes. We aimed to identify other variants in *SLC30A8* that increase diabetes risk and impair beta-cell function, and test whether zinc intake modifies this risk.

Design/Outcome: We sequenced exons in *SLC30A8* in 380 Diabetes Prevention Program (DPP) participants and identified 44 novel variants, which were genotyped in 3,445 DPP participants and tested for association with diabetes incidence and measures of insulin secretion and processing. We examined individual common variants and utilized gene burden tests to test 39 rare variants in aggregate.

Results: We detected a near nominal association between a rare-variant genotype risk score and diabetes risk. Five common variants were associated with the oral disposition index (DI_o). Various methods aggregating rare variants demonstrated associations with changes in DI_o and insulinogenic index during year-1 of follow-up. We did not find a clear interaction of zinc intake with genotype on diabetes incidence.

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Abbreviations:

Conclusions: Individual common and an aggregate of rare genetic variation in *SLC30A8* are associated with measures of beta-cell function in the DPP. Exploring rare variation may complement ongoing efforts to uncover the genetic influences that underlie complex diseases.

Individuals of European descent who carry the C vs T allele in the missense single nucleotide polymorphism (SNP) rs13266634 at *SLC30A8* (encoding ZnT8) have elevated type 2 diabetes (T2D) risk (1), impaired β-cell function, higher proinsulin levels (adjusted for fasting insulin) (2, 3) and zinc intake appears to modify glycemic effects of this locus (4). ZnT8 transports zinc molecules, essential for insulin storage and processing, into insulin granules (5). In vitro and mouse models have demonstrated that disruption of *Slc30a8* zinc transport alters insulin crystallization and results in decreased insulin secretion (6–9). The risk variant at rs13266634 was not significantly associated with diabetes incidence in the Diabetes Prevention Program (10). Therefore, we sequenced regions in *SLC30A8* in 380 DPP participants and subsequently genotyped discovered variants in the full DPP population. By examining variants individually and in aggregate, we aimed to identify variants at *SLC30A8,* beyond the index variant rs13266634, associated with diabetes incidence and impaired β-cell function, and test whether zinc intake modified these effects.

Materials and Methods

The DPP enrolled 3,819 US participants at high-risk of developing type 2 diabetes (overweight, elevated fasting glucose, and impaired glucose tolerance) (11), from which a subset, 3,445 participants, consented to genetic testing. Of these participants, we examined 2,997 who were randomized to placebo, metformin 850 mg twice daily, or lifestyle intervention with a goal weight loss of $\geq 7\%$ and ≥ 150 minutes/wk of physical activity for association with all the outcomes. Participants in a fourth troglitazone treatment arm (n = 585) were included in genotyping, but not included in the association testing due to early termination (12). Power calculation for alleles of various frequencies and effect sizes on diabetes incidence can be found in the Supplementary Table 1 in Moore et al (10). Ethical approval was obtained by local human research committees and all participants gave informed consent.

Diabetes incidence was determined by a diagnostic fasting or 2-hour glucose after OGTT, confirmed by a second test (11). We measured β-cell function including the insulinogenic index (InsIndex [U/ml]/[mg/dl] = $\Delta\text{Insulin}_{30-0\text{min}}/\Delta\text{Glucose}_{30-0\text{min}}$) (13) and the oral disposition index (DI_o [mg/dl]$^{-1}$ = InsIndex*1/fasting insulin) (14). We provided the association with fasting glucose and proinsulin (adjusted for fasting insulin) for all analyses (**Online Supplemental Tables 1–3**) to corroborate prior findings (15, 16). Baseline zinc intake was determined using a modified Block Food Frequency Questionnaire (17).

We sequenced 380 DPP participants (76 from each ethnicity group). We oversampled participants who developed diabetes to enrich our analysis for diabetes-related variants. The sequenced individuals were included in the subsequent association analyses of 2,997 participants.

We used Sanger sequencing on an ABI3730 DNA Analyzer for 2X coverage of eight exonic regions, 5′UTR with 50 base pairs (bps) around each intron/exon junction, 1,000 bps upstream and downstream of *SLC30A8*, and 1,000 bps surrounding rs13266634. We genotyped 69 SNPs discovered in sequencing and 10 SNPs annotated to *SLC30A8* but not identified during sequencing in 3,445 participants. After quality control (QC) (nonconcordance between genotyping and sequencing, failed assay design, call rate $< 95\%$, failed Hardy-Weinberg equilibrium with a $P < .001$), 61 SNPs (44 of which were novel) were further analyzed.

Twenty-two "common" SNPs (minor allele frequency [MAF] ≥ 0.01 in at least one ethnic group) were examined using Cox proportional hazard models for association with diabetes incidence and analysis of covariance (ANCOVA) for association with the quantitative traits. Results were stratified by treatment group for a genotype*treatment group interaction $P < .05$. We adjusted for sex, age at randomization, baseline BMI, self-reported ethnicity and, if applicable, treatment group and respective baseline trait. Follow-up analyses included the SNP as a class variable obtaining marginal means, and compared differences between genotypic groups

We utilized five methods to test the association between 39 "rare" genetic variants (MAF < 0.01 in all ethnicities) and the outcome.

Three genetic risk scores (GRS) were constructed by summing the number of minor alleles over the sample: 1) A GRS including all 60 SNPs, not including rs1326634, 2) a "missense GRS" included four novel missense variants (8 118228561, 8 118239185, 8 118252509, 8 118254036) and one known missense variant (rs16889462), 3) and a "rare GRS" included the 39 rare SNPs. A combined multivariate and collapsing (CMC) method, coded each participant as having a variant with a MAF $< 2\%$ as "present" or no rare variants "absent" (18). The Sequence Kernel Association Test (SKAT) allows variants to have different directions and magnitude of effects (19). The GRS and CMC were used in the models described above to test the associations with the outcomes. SKAT was used for testing associations with the β-cell function traits only. All scores were tested for interaction by treatment group and stratified if $P < .05$ except SKAT which does not allow for interaction terms and was stratified up-front by treatment group. In a follow-up analysis, we used a Wilcoxon rank sum test to examine the association between the individual rare variants and quantitative traits stratified by treatment group.

We tested whether zinc intake modified diabetes risk conferred by *SLC30A8* variants by adding additional covariates: baseline zinc intake, total caloric intake, and an interaction term for baseline total zinc intake*genotype or GRS covariate, and factors that affect intestinal zinc absorption (iron intake, log calcium intake, polyunsaturated-to-saturated fat intake and log dietary fiber) (20). For interaction $P < .05$, we stratified by ge-

notype and obtained hazard ratios (HR) per 1 mg/d difference in baseline zinc intake.

Given the high prior probability of association with T2D, we used a traditional alpha level of 0.05 for statistical significance.

Results

Sixty-one *SLC30A8* variants (44 novel) passed QC (**Online Supplemental Table 4**) and were analyzed. Five novel missense variants were "probably damaging" on bioinformatic analysis (**Online Supplemental Table 5**) and a subset of SNPs had predicted regulatory consequences (**Online Supplemental Table 6**).

Common variants were not associated with diabetes incidence (**Online Supplemental Table 7**). The minor alleles of rs2464591, rs2466296, rs2466297, and rs2466299 ($r^2>0.9$, HapMap CEU and YRI) were associated with a positive ΔDI_o ($P < .0005$), whereas the minor allele of rs2466293 was associated with a negative ΔDI_o (Table 1). rs16889462 was associated with improved InsIndex in the AG/AA vs the GG in the metformin group, but not in the other treatment groups (**Online Supplemental Table 2**). The minor allele of rs3802177 was associated with higher InsIndex, lower baseline PI(FI) levels, and greater decrease in fasting glucose during the first year (**Table 1 and Supplemental Table 1 and 2**).

The rare variant GRS showed a tentative direct relationship with diabetes incidence (HR = 1.27 [1.00–1.61] per rare variant allele; $P = .05$). (**Table 2**). One-hundred-twenty-five participants carried only one, 33 carried two, and one carried three rare variants and was grouped with the two-variant carriers.

Various rare variant methods showed an association with β-cell function (**Table 2**). SNP* treatment interactions were nonsignificant ($P > .05$) for all methods. For each additional GRS minor allele, there was a 0.001 (SE 0.0002) change in the DI_o ($P = .0003$). This association was no longer significant after removing rs2466293, rs2464591, rs2466296, rs2466297, and rs2466299 from the GRS ($P = .2$). With the CMC method, carriers of at

Table 1. Significantly associated *SLC30A8* genetic variants tested for association with glucose and insulin-related quantitative traits.

SNP	Trait	Genotype	Treatment Adjusted Means (95% CI)	*P*
Common SNPs (MAF ≥1%)				
8 118252680	ΔInsIndex	GG	0.024 (-0.037 to 0.084)	0.04
		GA/AA	−0.130 (−0.283 to 0.023)	
	ΔDI_o	AA	0.009 (0.005 to 0.013)	0.0001
		AG	0.005 (0.001 to 0.009)	
		GG	−0.003 (-0.008 to 0.003)	
rs6469675	ΔInsIndex	AA	−0.018 (-0.091 to 0.055)	0.05
		AG	0.026 (-0.048 to 0.100)	
		GG	0.100 (-0.100 to 0.209)	
rs2464591	ΔDI_o	GG	0.002 (-0.001 to 0.006)	0.0003
		GA	0.007 (0.003 to 0.011)	
		AA	0.015 (0.009 to 0.022)	
rs2466296	ΔDI_o	GG	0.002 (-0.002 to 0.006)	0.0002
		GA	0.006 (0.002 to 0.011)	
		AA	0.015 (0.009 to 0.022)	
rs2466297	ΔDI_o	CC	0.002 (-0.001 to 0.006)	0.0003
		CT	0.006 (0.002 to 0.011)	
		TT	0.016 (0.009 to 0.022)	
rs2466299	ΔDI_o	GG	0.002 (-0.002 to 0.006)	0.0002
		GA	0.007 (0.003 to 0.011)	
		AA	0.015 (0.009 to 0.022)	
rs3802177	InsIndex	GG	1.21 (1.15 to 1.27)	0.04
		GA	1.28 (1.22 to 1.35)	
		AA	1.27 (1.15 to 1.40)	
rs13266634	InsIndex	CC	1.20 (1.14 to 1.26)	0.02
		CT	1.27 (1.20 to 1.34)	
		TT	1.30 (1.17 to 1.43)	

Common SNPs rs2466293 and rs16889462 had a significant genotype*treatment interaction for ΔFG and ΔInsIndex, respectively, are found in **Online Supplemental Table 2.** Minor alleles are underlined. SNPs above did not have a significant genotype*treatment. Analysis was adjusted by age, sex, BMI, and ethnicity; and additionally adjusted for treatment group and corresponding baseline trait for the year-1 change traits. InsIndex = insulinogenic index; DI_o = oral disposition index; Δ = change in trait between year 1 and baseline. *P* values reported are from the one degree of freedom additive model. Sample size by genotype for baseline traits analysis is detailed in **Online Supplemental Table 1**.

Table 2. *P*-values for association tests using various methods to test aggregates of rare variants for association with diabetes incidence and β-cell function.

	GRS	Missense GRS	Rare GRS	CMC	SKAT*
Diabetes Incidence	0.66	0.24	**0.05**	0.31	-
Baseline InsIndex	0.96	0.41	0.46	0.44	0.25
Baseline DI_o	0.88	0.07	0.26	0.84	0.49
ΔInsIndex	0.82	0.60	0.14	**0.03**	0.98
ΔDI_o	**0.0003**	0.80	0.53	0.30	0.99

InsIndex = insulinogenic index; DI_o = oral disposition index; Δ = change in trait between year 1 and baseline. *SKAT analysis stratified by treatment group is in **Online Supplemental Table 2**.

least one minor allele of the 39 rare variants had a mean ΔInsIndex of 0.034 (95% CI, −0.027 to 0.096), while carriers of no rare variants had −0.079 (95% CI, −0.187 to −0.028) ($P = .03$). No statistically significant associations were seen between the individual rare variants and these traits (**Online Supplemental Table 3**).

Three SNPs (8 118252314, 8 118252435, rs16889462), two of which were novel, modified the effect of total zinc intake on diabetes risk, but had no clear additive trend with each additional minor allele (**Online Supplemental Table 8**).

Discussion

We identified 44 novel variants through targeted sequencing of the T2D candidate gene *SLC30A8* in the DPP. In aggregate, rare variants appear to influence diabetes risk and related traits, illustrating that both rare and common genetic variation may influence diabetes risk. Zinc intake did not appear to modify the genetic predisposition to diabetes at this locus, suggesting a limited role for dietary manipulations in modifying genetic risk.

We identified 39 rare variants, unique to certain ethnicities (**Online Supplemental Table 4**). Despite the "probably damaging" prediction by bioinformatic analysis, the individual missense variants and the missense variant GRS were not associated with diabetes incidence or quantitative traits. Further functional studies where point mutations are introduced into *SLC30A8* constructs for transfection into beta cells may elucidate whether these variants attenuate or enhance protein function. Similarly, phenotyping individuals with definite loss-of-function mutations should be informative with regard to the direction of *SLC30A8* variation on glycemic regulation in humans. We did not identify any loss of function variants in *SLC30A8* in this multiethnic cohort which underscores the drive for conservation and therefore relevance of this gene for metabolism.

Although none of the common SNPs was associated with diabetes incidence, we found associations between *SLC30A8* variants and insulin secretion traits. The minor alleles of rs2464591, rs2466296, rs2466297, and rs2466299 were associated with an improvement in β-cell function, illustrated by an increase in DI_o. Conversely, the rs2466293 minor allele was associated with a decrease in β-cell function. Given the nonsignificant treatment×genotype interaction, it appears that these variants influence glycemia similarly among all the intervention groups. These findings exemplify that *SLC30A8* variation comparably influences improvements or deteriorations of β-cell function over a year's follow-up, independent of insulin-sensitizing interventions that reduce diabetes risk.

We employed five methods to examine the contribution of rare genetic variation on diabetes risk and glucose- and insulin-related traits. The GRS was associated with ΔDI_o and the CMC method revealed an association between carriers of rare variants and ΔInsIndex. These results suggest that rare *SLC30A8* variation may have functional significance beyond the index SNP, rs13266634. The GRS and CMC methods are limited in that they presume that the rare allele is deleterious, which may not always be true despite our ascertainment having been largely conducted in participants who went on to develop diabetes. Therefore, this assumption may dilute the true impact of the rare GRS. This limitation is addressed with the SKAT method, which allows variants to have different directions and magnitude of effects (19); here we did not see an association with β-cell function. Although limited by power, none of the rare SNPs appear to have very large effects, but the aggregate burden of rare *SLC30A8* variation influences β-cell function. These findings provide the basis for future studies with the Exome chip implemented in larger populations where the rare variants can be adequately tested individually.

Limitations. We were able to enhance statistical power by constructing aggregate variant scores, but this method does not model the behavior of individual variants. As these methods continue to evolve, functional experiments

will be needed to further elucidate the mechanism by which rare variants influence phenotypes. Additionally, this study lacks a validation cohort and nominal associations found in this study warrant follow-up elsewhere. Furthermore, our sequencing efforts started prior to the introduction of next-generation sequencing techniques and only sequenced targeted regions of *SLC30A8*; thus, novel variants may have been overlooked.

Our study showed that an aggregate of rare variants in *SLC30A8* may increase diabetes risk and influence measures of β-cell function. This study supports the pursuit of rare variation to better understand the genetics of complex traits.

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* The full list of DPP Research Group investigators is provided in the Online Supplemental Data

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Author Contributions: LKB formulated the analysis plan, cleaned sequencing/genotyping data, interpreted the results, and wrote the manuscript under the guidance of JCF. KAJ formulated the analysis plan, carried out the analyses, and wrote and edited the manuscript. RJA assembled manuscript tables and reviewed the manuscript. AT, RRF, JBM, CG performed the sequencing and genotyping of the samples and reviewed the manuscript. LMD, DD, SEK, PWF, RLH, NMM, AS, EJM-D, WCK reviewed the analysis plan, contributed to discussion, and reviewed/edited the manuscript. JCF designed the experiment, formulated the analysis plan with LKB, and reviewed/edited the manuscript; he is the guarantor of this manuscript.

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