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SHORT COMMUNICATION

Association of the *SLC30A8* missense polymorphism R325W with proinsulin levels at baseline and after lifestyle, metformin or troglitazone intervention in the Diabetes Prevention Program

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Abstract
Aims/hypothesis Individuals with impaired glucose tolerance have increased proinsulin levels, despite normal glucose or C-peptide levels. In the Diabetes Prevention Program (DPP), increased proinsulin levels predicted type 2 diabetes and proinsulin levels were significantly reduced following treatment with metformin, lifestyle modification or troglitazone compared with placebo. Genetic and physiological studies suggest a role for the zinc transporter gene *SLC30A8* in diabetes risk, possibly through effects on insulin-processing in beta cells. We hypothesised that the risk allele at the type 2 diabetes-associated missense polymorphism rs13266634 (R325W) in *SLC30A8* would predict proinsulin levels in individuals at risk of type 2 diabetes and may modulate response to preventive interventions.
Methods We genotyped rs13266634 in 3,007 DPP participants and examined its association with fasting proinsulin and fasting insulin at baseline and at 1 year post-intervention.
Results We found that increasing dosage of the C risk allele at *SLC30A8* rs13266634 was significantly associated with higher proinsulin levels at baseline (p=0.002) after adjustment for baseline insulin. This supports the hypothesis that risk

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alleles at *SLC30A8* mark individuals with insulin-processing defects. At the 1 year analysis, proinsulin levels decreased significantly in all groups receiving active intervention and were no longer associated with *SLC30A8* genotype (p=0.86) after adjustment for insulin at baseline and 1 year. We found no genotype × treatment interactions at 1 year.
Conclusions/interpretation In prediabetic individuals, genotype at *SLC30A8* predicts baseline proinsulin levels independently of insulin levels, but does not predict proinsulin levels after amelioration of insulin sensitivity at 1 year.

Keywords Diabetes Prevention Program · Genetic association · Proinsulin · Single nucleotide polymorphisms · *SLC30A8* · Zinc transporter

Abbreviations

ANCOVA	Analysis of covariance
DPP	Diabetes Prevention Program
IGT	Impaired glucose tolerance
SNP	Single nucleotide polymorphism
ZnT-8	Zinc transporter 8

Introduction

The first published genome-wide association study for type 2 diabetes showed the single nucleotide polymorphism (SNP) rs13266634 in *SLC30A8* (OR 1.26, $p=5.0\times10^{-7}$) to be a diabetes-associated locus [1]. *SLC30A8* encodes zinc transporter 8 (ZnT-8), a 369 amino acid transmembrane zinc transporter protein produced at high levels only in the pancreas. Within the pancreatic beta cell, zinc is a necessary component for the formation of insulin granules as it provides the nucleus for crystallisation of insulin proteins within secretory vesicles. The *SLC30A8* non-synonymous SNP (C to T) results in a missense R325W substitution. The production pattern and putative function of ZnT-8 suggest that it may increase diabetes risk by impairing insulin secretion. Indeed a defect in first-phase insulin secretion was observed in homozygotes for the high-risk C allele in response to intravenous glucose tolerance test (19% decrease in first-phase insulin release, p=0.007), but not during an OGTT [2]. However, homology-based structural modelling of ZnT-8 does not indicate an obvious conformational defect in the docking or transporter segments of the protein [3]. In a recently reported beta cell-specific knockout of *Slc30a8* in mice, the defect in first-phase insulin secretion was repeated, and glucose intolerance and elevated proinsulin levels were demonstrated [4]. A study of overweight non-diabetic men from a Finnish cohort (30% of whom had impaired glucose tolerance [IGT]) found that individuals with the high-risk genotype at *SLC30A8* showed elevated proinsulin/insulin ratios while fasting as well as during OGTT [5].

These studies suggest that *SLC30A8* variants link to diabetes risk through alterations in proinsulin to insulin conversion. Previous studies have shown that elevated fasting proinsulin levels are associated with increased risk of developing type 2 diabetes independently of insulin levels [6, 7]. However, elevated proinsulin levels in carriers of the *SLC30A8* risk genotype may be an epiphenomenon of increased beta cell stress (from insulin resistance or beta cell death), rather than reflecting a specific deficit in polypeptide processing. Such non-specific mechanisms cannot be ruled out as mediators of the *SLC30A8*-associated increase in diabetes susceptibility. We reasoned that if *SLC30A8*-associated proinsulin elevations were largely mediated by a proinsulin-to-insulin conversion defect, interventions that decreased insulin resistance should lower proinsulin in high- and low-risk genotype groups to equal degrees. However, if increased beta cell stress (i.e. increased insulin resistance) was the major determinant of *SLC30A8*-associated proinsulin levels, interventions that ameliorate insulin resistance should show a selectively beneficial effect in carriers of the *SLC30A8* risk genotype as compared with the protective genotype.

In the Diabetes Prevention Program (DPP), we have previously shown that genotype at *SLC30A8* rs13266634 is not associated with diabetes incidence, with no significant interaction of genotype with intervention being observed with regard to this outcome [8]. To assess the putative association of *SLC30A8* with proinsulin levels and their response to treatments designed to lower insulin resistance, we examined the effect of *SLC30A8* genotype on proinsulin levels in the DPP at baseline and 1 year after preventive interventions.

Methods

The DPP (ClinicalTrials.gov number NCT00004992; for a list of investigators see electronic supplementary material [ESM]) enrolled 3,234 US American participants at high risk of developing diabetes (on the basis of overweight, increased fasting glucose and impaired glucose tolerance) and randomised them to placebo, metformin 850 mg twice daily or a lifestyle intervention aimed at ≥7% weight loss and ≥150 min of physical activity per week. A fourth arm of 585 participants who were initially randomised to troglitazone was terminated early because of concerns with hepatotoxicity. Of the above, 3,007 participants who had consented to genetic investigation and for whom valid *SLC30A8* genotypes and data at 1 year were available were studied here.

The main endpoint was development of diabetes (as indicated by fasting glucose or 2 h glucose after an OGTT) confirmed by a second measurement. In total, 551

participants developed diabetes; the DPP showed that participants treated with metformin or with a lifestyle intervention were respectively 31% or 58% less likely to develop diabetes after an average of 3.2 years of follow-up [9]. To assess the effects of genotype at *SLC30A8* rs13266634 on proinsulin levels in the DPP cohort, we analysed participants at baseline using an analysis of covariance (ANCOVA) model with proinsulin as the dependent variable, and fasting insulin, age, sex, self-reported ethnicity and genotype at *SLC30A8* rs13266634 (obtained from our prior study as previously described [8]) as independent variables.

To evaluate the effect of genotype at *SLC30A8* rs13266634 on treatment-mediated reductions in proinsulin after 1 year of DPP interventions, we performed a prospective cohort analysis using ANCOVA models with fasting proinsulin at baseline and year 1 as the dependent variables. Participants who developed diabetes at 1 year were excluded from year 1 analyses. Nominal two-sided *p* values adjusted for multiple comparisons are reported for post-hoc active treatment vs placebo statistical tests. All models included age, sex, self-reported ethnicity and genotype at *SLC30A8* rs13266634 as independent variables. Mixed models ANCOVA was used to assess the effect of DPP treatments and genotype at *SLC30A8* on proinsulin levels independently of insulin levels; the baseline proinsulin model was additionally adjusted for baseline fasting insulin; the year 1 proinsulin model was adjusted

Table 1 Baseline characteristics and association results by rs13266634 genotype

Baseline characteristics	Genotype			*p* value
	CC (*n*=1,726)	CT (*n*=1,087)	TT (*n*=194)	
Treatment group, *n* (%)				
Placebo	522 (59.3)	312 (35.4)	47 (5.3)	0.28[a]
Metformin	503 (56.6)	335 (37.7)	51 (5.7)	
Lifestyle	513 (56.6)	322 (35.5)	72 (7.9)	
Troglitazone	188 (57.0)	118 (35.8)	24 (7.3)	
Self-reported ethnicity, *n* (%)				
White	855 (50.0)	713 (41.7)	141 (8.3)	<0.001[a]
African-American	475 (80.2)	110 (18.6)	<15[d]	
Hispanic	298 (59.7)	173 (34.7)	28 (5.6)	
Asian/Pacific Islanders	48 (37.2)	68 (52.7)	<15[d]	
American Indians	50 (64.1)	23 (29.5)	<15[d]	
Demographics				
Age (years)	50.6±10.6	51.1±10.4	53.7±11.2	<0.01[b]
Male sex, *n* (%)	557 (32.3)	389 (35.8)	71 (36.6)	0.11[a]
Baseline associations				
BMI (kg/m^2)	34.0±6.7	33.8±6.5	33.9±6.7	0.83[b]
Fasting glucose (mmol/l)	5.9±0.4	5.9±0.4	5.9±0.4	0.62[b]
HbA_{1c} (%)	5.9±0.5	5.8±0.5	5.9±0.4	<0.001[b]
HbA_{1c} (mmol/mol)	41±5.5	40±5.5	41±4.3	
Fasting insulin (pmol/l)[e]	159.7 (16.6, 1152.8)	159.7 (20.8, 1277.9)	166.7 (42.4, 666.7)	0.68[b]
Proinsulin (pmol/l)[f]	15.81 (15.14, 16.51)	15.56 (14.82, 16.34)	15.29 (13.94, 16.77)	0.38[c]
Proinsulin adjusted[g] (pmol/l)[f]	12.36 (11.77, 12.97)	11.80 (11.17, 12.47)	11.30 (10.19, 12.53)	0.002[c]
Proinsulin adjusted[h] (pmol/l)[f]	15.92 (15.41, 16.44)	15.25 (14.70, 15.82)	14.43 (13.46, 15.46)	0.001[c]
Year 1 association				
Proinsulin adjusted[i] (pmol/l)[f]	12.25 (11.72, 12.81)	11.98 (11.39, 12.61)	12.06 (10.94, 13.30)	0.86[c]

Values are mean ± SD, unless indicated otherwise

All models adjusted for age, sex and ethnicity. The 1 year model was also adjusted for baseline fasting insulin

[a] χ^2; [b] *F* test from ANOVA; [c] ANCOVA

[d] To preserve confidentiality in accordance with DPP publication policy, absolute numbers are not provided in cells with <15 participants

[e] Fasting insulin values are median (minimum, maximum); [f] values are mean (95% CI)

Adjusted [g] for fasting insulin, [h] for fasting insulin and fasting glucose, [i] for year 1 fasting insulin

for year 1 fasting insulin. Additional adjustment of the year 1 model for baseline fasting proinsulin and insulin was performed to model change after controlling for the main effect of this SNP on proinsulin. Genotype × treatment interaction tests were performed for all year 1 models with the intention of analysing treatment groups together if there were no significant genotype × treatment interactions.

Results

At baseline, the C risk allele at *SLC30A8* rs13266634 was significantly associated with higher fasting proinsulin levels in the DPP cohort. This association was in an allele dose-dependent fashion after adjustment for baseline fasting insulin (p=0.002; Table 1) and remained statistically significant after further adjustment for fasting glucose (p=0.001; Table 1).

After 1 year of randomised DPP intervention, fasting proinsulin levels were significantly decreased (p<0.001) in all active intervention groups compared with placebo, even after adjustment for 1 year fasting insulin, age, sex and ethnicity (Fig. 1). While intervention groups had significantly lower proinsulin levels than placebo group at 1 year, the lifestyle intervention group had the largest decrease and was significantly lower than the metformin (p<0.001) or troglitazone (p=0.002) groups. The metformin and troglitazone intervention groups had similar proinsulin levels at 1 year (p=0.86). There was no genotype × treatment interaction (p=0.78), suggesting that the dosage of *SLC30A8* risk alleles did not modify the effectiveness of any treatment. As there were no significant genotype × treatment interactions, the four groups were also analysed together. At 1 year after DPP interventions, fasting proinsulin levels were no longer associated significantly with genotype at the *SLC30A8* locus when adjusted for 1 year fasting insulin, age, sex and ethnicity (p=0.86; Table 1).

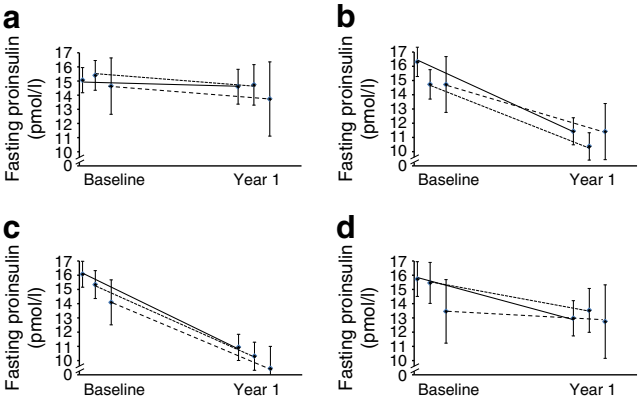

Fig. 1 Fasting proinsulin levels at baseline and 1 year after treatment in placebo (**a**), metformin (**b**), lifestyle (**c**), and troglitazone (**d**) groups. Proinsulin levels in each treatment panel were stratified by genotype at *SLC30A8* rs13266634. Lines coded by genotype connect proinsulin levels at baseline and 1 year for each genotype group (CC continuous lines, CT dotted lines, TT dashed lines) in each treatment arm. Fasting proinsulin decreased significantly in all treatment arms (p<0.001 by ANCOVA). There was no genotype × treatment interaction (p=0.78 by ANCOVA). Genotype at *SLC30A8* rs13266634 did not predict fasting proinsulin levels at 1 year (p=0.86 by ANCOVA). All models were adjusted for sex, age and self-reported ethnicity. Baseline values were adjusted for baseline fasting insulin, year 1 values were adjusted for 1 year fasting insulin. Values are mean ± 95% CI

Discussion

Our results demonstrate that genotype at *SLC30A8* was associated with fasting proinsulin levels at baseline in the prediabetic DPP cohort; proinsulin levels were positively correlated with dosage of the C diabetes risk allele. These findings are consistent with a previous study demonstrating elevated fasting proinsulin levels in Finnish individuals with IGT [5]. Because fasting proinsulin in our study was adjusted for fasting insulin, this trait represents an elevation of proinsulin that is out of proportion to the hyperinsulinaemia induced by insulin resistance. The persistence of this finding after adjustment for fasting glucose suggests that the effect of genotype on proinsulin is not mediated by ambient glycaemia and its impact on beta cells.

After 1 year of DPP treatments, all of which provided an insulin-sensitising effect, proinsulin levels fell in all treatment groups compared with placebo [10]. This decrease in proinsulin levels remained significant after adjustment for 1 year fasting insulin, suggesting that the decrease in proinsulin levels did not simply reflect a fall in insulin. This indicates that the notable amelioration in insulin sensitivity also signals improved beta cell function.

Genotype at *SLC30A8* rs13266634 was no longer associated with proinsulin levels in the DPP cohort after 1 year of treatment. This was true for the placebo as well as the active intervention groups. We also note that genotype at *SLC30A8* rs13266634 was no longer associated with proinsulin levels at 1 year even in a model unadjusted for baseline proinsulin levels (data not shown). This lack of association could be due to a loss of power to detect proinsulin differences by genotype when the cohort was divided into intervention groups. But these data are also consistent with the postulate that the various insulin-sensitising interventions leading to lower proinsulin levels could have been potent enough to decrease the main effect of *SLC30A8* rs13266634 genotype on proinsulin.

Since elevated proinsulin levels are an independent risk factor for the eventual development of type 2 diabetes, our data suggest that susceptibility to diabetes posed by high-risk alleles at the *SLC30A8* locus could be attenuated by

preventive treatment prior to the development of diabetes. In the case of the *SLC30A8* locus, interventions such as lifestyle could be targeted even before elevated proinsulin levels or IGT manifest in carriers of the risk genotypes.

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Contribution statement J.B.M. directed the genotyping with supervision from J.C.F. Recruitment and phenotyping were performed previously by the Diabetes Prevention Program Research Group. K.A.J. conducted statistical analyses with input from A.R.M. and J.C.F. A.R.M. wrote the manuscript with supervision from J.C.F. All authors were involved in the analysis and interpretation of results, contributed to the discussion, and reviewed and edited the manuscript. All the authors approved the final version of the manuscript.

Duality of interest J. C. Florez has received consulting honoraria from Daiichi-Sankyo and AstraZeneca. All other authors declare that there is no duality of interest associated with this manuscript.

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