

# Reduced Insulin Exocytosis in Human Pancreatic $\beta$ -Cells With Gene Variants Linked to Type 2 Diabetes

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The majority of genetic risk variants for type 2 diabetes (T2D) affect insulin secretion, but the mechanisms through which they influence pancreatic islet function remain largely unknown. We functionally characterized human islets to determine secretory, biophysical, and ultrastructural features in relation to genetic risk profiles in diabetic and nondiabetic donors. Islets from donors with T2D exhibited impaired insulin secretion, which was more pronounced in lean than obese diabetic donors. We assessed the impact of 14 disease susceptibility variants on measures of glucose sensing, exocytosis, and structure. Variants near *TCF7L2* and *ADRA2A* were associated with reduced glucose-induced insulin secretion, whereas susceptibility variants near *ADRA2A*, *KCNJ11*, *KCNQ1*, and *TCF7L2* were associated with reduced depolarization-evoked insulin exocytosis. *KCNQ1*, *ADRA2A*, *KCNJ11*, *HHEX/IDE*, and *SLC2A2* variants affected granule docking. We combined our results to create a novel genetic risk score for  $\beta$ -cell dysfunction that includes aberrant granule docking, decreased  $Ca^{2+}$  sensitivity of exocytosis, and reduced insulin release. Individuals with a high risk score displayed an impaired response to intravenous glucose and deteriorating insulin secretion over time. Our results underscore the importance of defects in  $\beta$ -cell exocytosis in T2D and demonstrate the potential of cellular phenotypic characterization in the elucidation of complex genetic disorders. *Diabetes* 61:1726–1733, 2012

**T**he rapid increase in type 2 diabetes (T2D) incidence results from a combination of lifestyle factors and genetics. Recent genome-wide association studies have identified close to 50 loci associated with T2D risk (1–4). Many of the risk variants are associated with reduced insulin secretion in vivo and are located in the vicinity of genes that are expressed in

pancreatic  $\beta$ -cells (1–4). The insulin secretory defect in T2D is multifactorial and may involve a reduction in  $\beta$ -cell mass, impaired  $\beta$ -cell glucose sensing, and defects in the  $\beta$ -cell secretory machinery downstream of glucose metabolism (5–7). These defects are not easily distinguished in vivo studies, and the specific disease mechanisms coupled to T2D risk loci are therefore incompletely understood.

A comprehensive understanding of the pathophysiology of T2D requires detailed phenotype characterization at the cellular level in addition to the systemic profiling traditionally investigated in genetic association studies. Such investigations may result in the identification of patient subgroups with distinct cellular defects that can be targeted by specific therapies. An obstacle to achieving this goal is our limited knowledge about human  $\beta$ -cells and how they become perturbed in T2D. Most mechanistic studies published to date have relied on animal models or cell lines. However, the recent demonstration of important differences between human and rodent  $\beta$ -cells in terms of electrophysiological and secretory properties highlights the potential pitfalls of extrapolating observations in model systems to man (8).

In this study, we have correlated the function and genotype of human islets obtained from diabetic and nondiabetic (ND) donors. We have analyzed a panel of 14 gene variants robustly associated with T2D susceptibility identified by recent genetic association studies. We have identified four genetic variants that confer reduced  $\beta$ -cell exocytosis and six variants that interfere with insulin granule distribution. Based on these observations, we calculate a genetic risk score for islet dysfunction leading to T2D that involves decreased docking of insulin-containing secretory granules, impaired insulin exocytosis, and reduced insulin secretion.

## RESEARCH DESIGN AND METHODS

**Human islets.** Experimental procedures were approved by the local ethical committees. Islets were obtained (with research consent) at Lund University Diabetes Centre and the Oxford Centre for Islet Transplantation from deceased donors. Donors with known T2D or  $HbA_{1c} > 6.0\%$  and no GAD antibodies were defined as having T2D. Islets were cultured for 1–9 days in CMRL1066 (ICN Biomedicals, Irvine, CA) supplemented with 10 mmol/L HEPES, 2 mmol/L L-glutamine, 50  $\mu$ g/mL gentamicin, 0.25  $\mu$ g/mL Fungizone (GIBCO, New York, NY), 20  $\mu$ g/mL ciprofloxacin (Bayer, Leverkusen, Germany), and 10 mmol/L nicotinamide at 37°C.

**Study populations.** ND individuals (604) from the Botnia study (9), characterized with intravenous glucose tolerance tests (IVGTTs), were genotyped. The prospective part included 2,770 ND family members (9). The corrected insulin response (CIR) was estimated from an oral glucose tolerance test (10).

**Islet isolation and in vitro insulin release.** Insulin secretion was measured as described previously (11). In brief, batches of 10–12 islets were pre-incubated in Krebs-Ringer bicarbonate buffer (KRBB) supplemented with 2.8 (Lund) or 1 mmol/L glucose (Oxford) followed by a 1-h incubation in KRBB with 16.7 (Lund) or 20 mmol/L glucose (Oxford).

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**Islet cell preparation and electrophysiology.** The electrophysiological measurements were conducted as described previously (8).  $\beta$ -cells were identified based on their size (8). For linopirdine experiments, cells were incubated with 50  $\mu\text{mol/L}$  of the compound for 1 h prior to experiments but linopirdine was not present during the recordings.

**Transmission electron microscopy.** Cultured islets were preincubated in KRBB with 2.8 mmol/L glucose for 30 min and then incubated in KRBB containing 16.7 mmol/L glucose for 1 h, fixed in glutaraldehyde, and added to freshly prepared Millonig buffer. Millonig buffer contains 2.26%  $\text{NaH}_2\text{PO}_4$  and 2.52% NaOH (pH 7.2). Islets were postfixated in osmium tetroxide (1%) and embedded for electron microscopy as previously described (12). The insulin granule diameter was  $285 \pm 11$  nm ( $n = 6,957$  granules from 22 individuals).

**ADP-ATP and insulin content measurements.** The ADP-ATP ratio was measured in human islets as previously reported (13). In brief, islets were incubated in Connaught Medical Research Laboratories medium, washed in PBS, and then mixed with a nucleotide-releasing reagent. ATP levels were measured using a luminometer. The ADP was then converted to ATP by adding an ADP-converting reagent followed by ATP measurements. The insulin-DNA ratio was measured as previously described (14) and expressed as picomoles per microgram. Islets were sonicated and transferred to acidic ethanol, followed by insulin quantification by ELISA (Merckodia). DNA was quantified using Picogreen (Invitrogen).

**RNA interference.** Islets were transfected with *KCNQ1* small interfering RNA (siRNA) according to the protocol described previously (11). Gene silencing was assessed by quantitative PCR using TaqMan (Applied Biosystems).

**Genotyping.** Seventeen single-nucleotide polymorphisms (SNPs) were genotyped from genomic DNA on an ABI 7900 (Applied Biosystems).

**Statistical procedures.** The experimental data showed non-Gaussian distributions. Gaussian distribution was obtained using logarithm transformation. All statistical analyses were therefore performed using  $\log_{10}$ -transformed data. Linear regression was used for the insulin secretion comparisons using the average secretion from each individual. The single-cell data, including electrophysiological recordings and granule distribution, were analyzed using a linear model in which all single-cell recordings were included as discrete observations but were grouped using donor ID as the subject cluster variable.  $\beta$  values for genotype-trait associations were obtained from the linear model. Capacitance recordings were obtained from at least four different cells per donor (median seven cells). Electron micrographs were analyzed from at least three different cells per donor (median seven cells). Complete phenotype data on insulin secretion, electrophysiology, and granule distribution were not available from all donors included in the study, which is why the number of donors used for the different trait analyses vary (see  $n$  for the different series). Exocytosis normalized to cell size was in the same range in donors from Oxford and Lund, but to account for any systematic differences, a binary covariate denoting the center was used in all analyses. The data presented denote nominal  $P$  values without correction for multiple comparisons. There were no interaction effects of the SNPs on the traits investigated. The risk score was calculated by adding the number of risk alleles. A linear model was used for the association analyses with the risk score and the traits. All statistical analyses were performed using SPSS Statistics (version 18.0).

## RESULTS

**Characterization of human islets from ND and diabetic donors.** Human pancreatic islets from 72 ND and 28 T2D donors were collected at two international centers (Supplementary Tables 1 and 2). We attempted to measure as many parameters as possible, but islet numbers set a limit to the number of experiments that could be performed on each preparation. Insulin secretion from size-matched islets was measured under static conditions. In ND donors, high glucose stimulated insulin secretion 5.1-fold ( $n = 42$  donors) over that seen at basal conditions (Fig. 1A). In islets from T2D donors, there was an  $\sim 50\%$  reduction of glucose-induced insulin secretion ( $P = 0.003$ ;  $n = 42$  ND and 17 T2D donors) (Fig. 1A).

$\beta$ -Cell mass may be reduced in T2D (7,15). Indeed, we observed a tendency toward a reduced insulin content in T2D islets compared with those from ND control subjects; islet insulin content averaged  $5.6 \pm 0.5$  ( $n = 41$ ) vs.  $4.0 \pm 0.6$  pmol insulin per  $\mu\text{g}$  DNA ( $n = 12$ ) in ND and T2D islets, respectively. Although insulin content is not equivalent to  $\beta$ -cell mass, the  $\sim 30\%$  decrease is close to the 35% reduction in islet  $\beta$ -cell mass recently reported (7) but did not reach

statistical significance ( $P = 0.11$ ). However, insulin release at high glucose was still reduced (by 50%) from T2D islets after correction for islet insulin content ( $P = 0.008$ ). Thus, both insulin secretion and content are reduced in T2D.

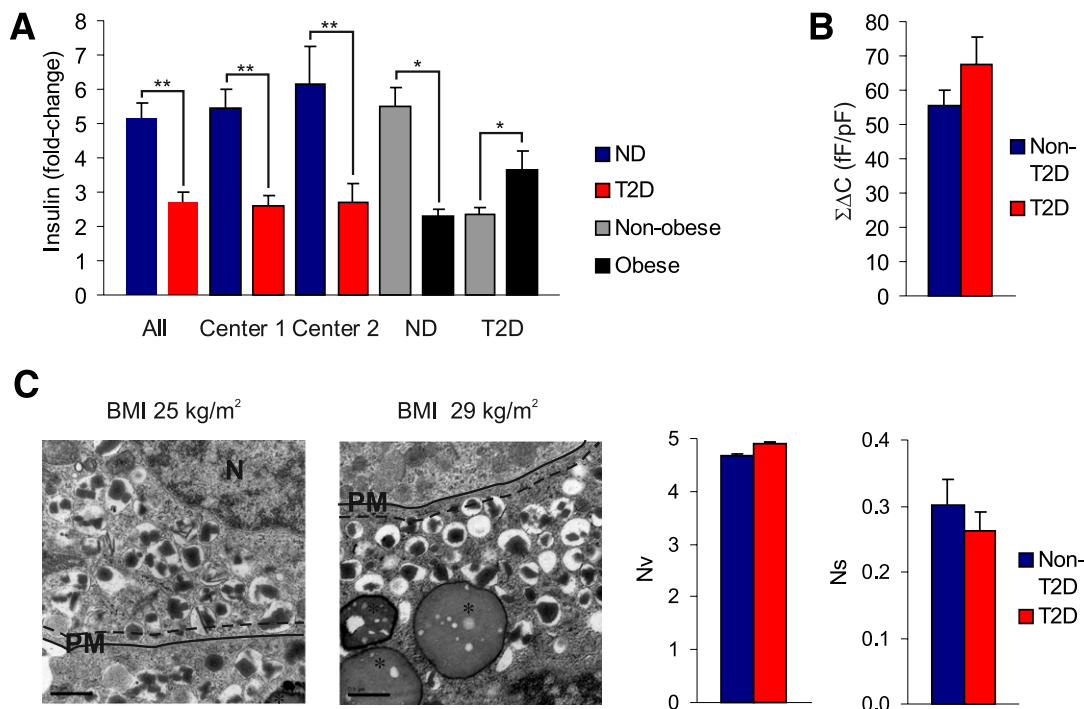
It has been suggested that insulin secretion in vivo may be upregulated in obese individuals to compensate for insulin resistance (7,15). We therefore divided the donors into those with a BMI above or below the upper quartile ( $31 \text{ kg/m}^2$ ). There was no significant difference in glucose-induced insulin secretion between nonobese and obese donors in the combined dataset of all donors ( $1.4 \pm 0.1$  ng/islet/h vs.  $1.9 \pm 0.6$  ng/islet/h for nonobese and obese donors, respectively; not statistically different). However, when stratified by diabetes status, the nonobese diabetic donors ( $n = 12$ ) displayed reduced insulin release compared with islets from obese diabetic subjects ( $n = 5$ ;  $P = 0.034$ ) (Fig. 1A).

**Characterization of single  $\beta$ -cell exocytosis.** Insulin is released in a characteristic biphasic manner: a rapid and transient first phase is followed by a slowly developed but sustained second phase. The consensus model for glucose-induced insulin secretion suggests that glucose triggers insulin secretion via increased cytosolic ATP, closure of ATP-sensitive potassium channels ( $\text{K}_{\text{ATP}}$  channels), initiation of electrical activity, and  $\text{Ca}^{2+}$ -dependent exocytosis from the  $\beta$ -cells (Supplementary Fig. 1) (16). In addition, several mechanisms fine-tune the release competence and intracellular distribution of the insulin granules. These two processes are referred to as the triggering and amplifying pathways. A current hypothesis postulates that first-phase insulin secretion reflects the release of granules docked beneath the plasma membrane, whereas second phase requires physical recruitment of granules from the cell interior and/or chemical modification (priming) of granules already in place beneath the membrane (16), although this hypothesis is not uncontested (17). There is evidence that T2D interferes with both the proximal (triggering) and distal (amplifying) mechanisms (18).

We examined exocytosis by measurements of cell capacitance that monitor the increase in cell area that results when secretory granules fuse with the plasma membrane. The standard whole-cell configuration was used, and the cytoplasmic ATP and cAMP levels were thus clamped to the concentrations of the pipette-filling solutions, allowing the exocytotic capacity to be analyzed without the confounding effect of glucose metabolism and variation in receptor signaling mediated by cAMP (e.g., glucagon-like peptide 1). Despite the clear difference in insulin secretion between islets from T2D and non-T2D donors, there was no overall change in  $\beta$ -cell exocytosis between the two groups (Fig. 1B).

We also analyzed electron micrographs of human islets. There was a strong correlation between BMI and the number of lipid droplets in  $\beta$ -cells ( $P = 0.008$ ;  $n = 14$  ND and 10 T2D donors). In agreement with a recent study (19), there was a correlation between the amount of lipofuscin bodies in  $\beta$ -cells and donor age ( $P = 0.012$ ). However, no differences were observed in the total granule number or number of granules docked below the plasma membrane between ND and T2D islets (Fig. 1C). The islet ADP-ATP ratio at 5.6 mmol/L glucose was not different between ND and T2D islets and averaged  $0.08 \pm 0.01$  ( $n = 42$ ) vs.  $0.07 \pm 0.01$  ( $n = 14$ ), respectively.

**Analysis of islet physiology in genetic subgroups.** T2D may result from a combination of genetic factors that differentially affect glucose sensing, exocytosis, and  $\beta$ -cell



**FIG. 1.** Characterization of islets from diabetic and ND individuals. **A:** Fold-stimulation of insulin secretion by glucose in batch-incubated islets from non-T2D ( $n = 42$ ) and T2D ( $n = 17$ ) donors, nonobese ( $n = 37$ ) and obese ( $n = 5$ ) ND donors, as well as nonobese ( $n = 12$ ) and obese T2D donors ( $n = 5$ ). Fold-stimulation of insulin secretion in non-T2D and T2D donors separated by center (1, Lund; 2, Oxford) is also displayed. **B:** Total exocytosis evoked by a train of 10 depolarizations from  $-70$  to  $0$  mV ( $\Sigma\Delta C$ ) ( $n = 189$  and  $60$  cells from  $28$  non-T2D and  $8$  T2D donors, respectively). **C:** Electron micrographs of human islets from non-T2D donors with low ( $25$  kg/m<sup>2</sup>) and high ( $29$  kg/m<sup>2</sup>) BMI. Lipid droplets (\*), nucleus (N), and plasma membrane (PM, solid line) are indicated; dashed lines show  $150$ -nm distance from PM. Scale bars,  $0.5$   $\mu$ m. The histograms show the total granule number as volume density (Nv; granules/ $\mu$ m<sup>3</sup>) and the number of docked granules as surface density (Ns; granules/ $\mu$ m<sup>2</sup>) in  $\beta$ -cells from non-T2D and T2D donors ( $n = 75$  and  $42$  cells from  $12$  and  $6$  donors, respectively). A granule was defined as docked when the center of the granule was located within  $150$  nm from the plasma membrane. Data are presented as means  $\pm$  SEM. \* $P < 0.05$  and \*\* $P < 0.01$ .

mass. Disease heterogeneity might thus explain our inability to detect any specific defect accounting for the lowered glucose-induced insulin secretion when analyzing the entire cohort of diabetic donors. Therefore, we examined whether stratification of the donors based on genetic susceptibility could facilitate the identification of defects in single-cell phenotypes.

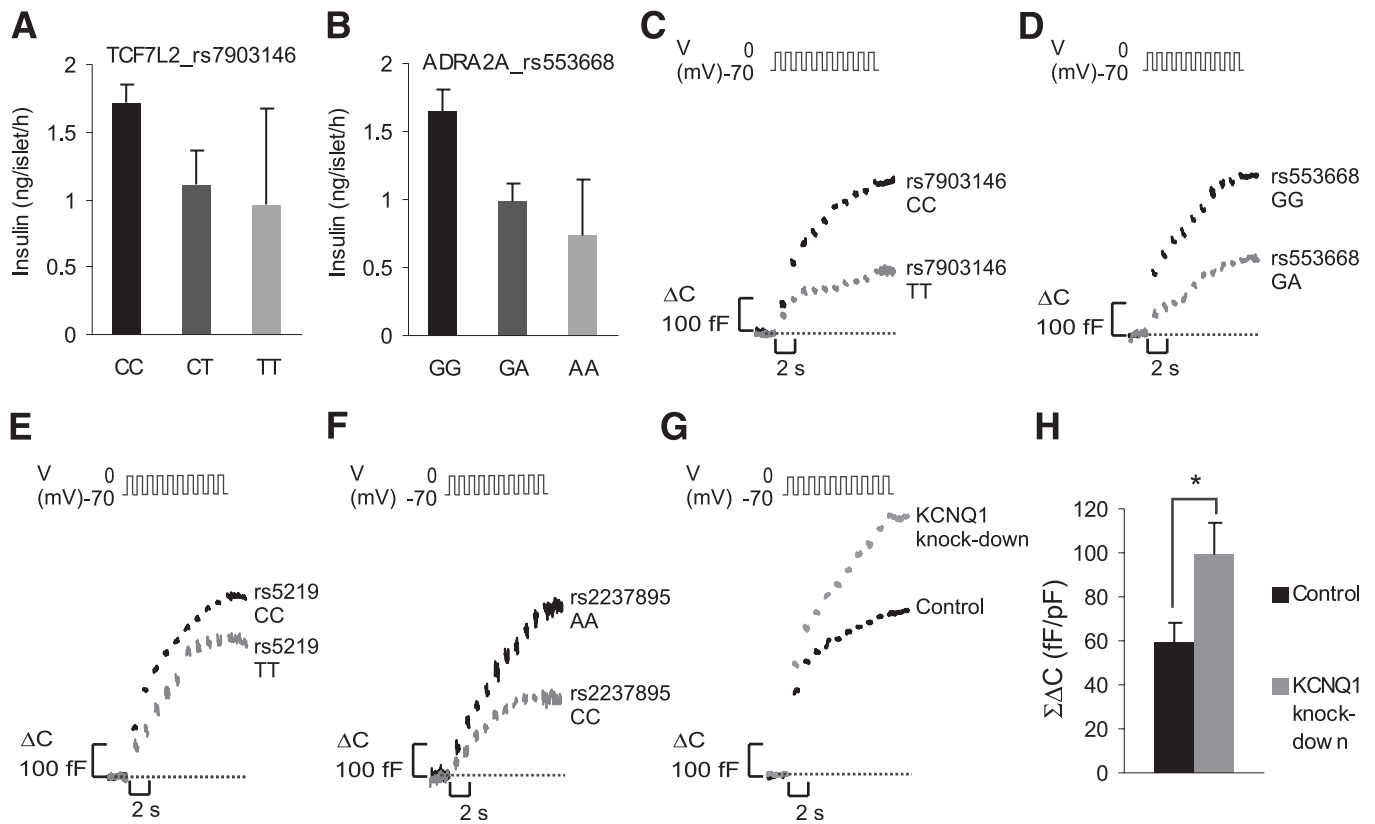
Fourteen SNPs (Supplementary Table 3) were selected based on their association with reduced insulin secretion in vivo in previous genetic studies (1–4,11). There are multiple ways by which these variants could potentially affect insulin secretion, and it is currently unclear whether some T2D susceptibility variants reduce insulin secretion through direct effects on insulin exocytosis in human  $\beta$ -cells.

There was no interaction of the genotype-phenotype associations with T2D status among the donors as analyzed by a linear interaction model. Based on that observation and for reasons of statistical power, both ND and T2D islets were included in the genotype analyses. However, diabetes status was used as a binary covariate in all analyses. BMI and age were also used as covariates. Associations were analyzed by a linear model where the  $\beta$  values indicate the effect of genotype on the studied parameters. **Genetic variants associated with insulin secretion.** First we investigated the effect of T2D-associated alleles on glucose-stimulated insulin secretion in isolated islets. Human islets from donors with the *TCF7L2* rs7903146 risk genotype, the genetic variant with the largest effect on T2D susceptibility known to date (20,21), displayed significantly reduced insulin secretion ( $P = 0.002$ ;  $\beta = -0.199$  [95% CI  $-0.324$  to  $-0.075$ ];  $n = 47$  donors) (Fig. 2A and Table 1).

There was also a significant reduction in insulin secretion in islets from risk carriers for *ADRA2A* rs553668 ( $P = 0.017$ ;  $\beta = -0.165$  [ $-0.300$  to  $-0.030$ ];  $n = 50$ ) (Fig. 2B and Table 1), in accordance with previous data (11).

**Identification of genetic variants influencing  $\beta$ -cell exocytosis and insulin granule docking.** Exocytosis and insulin granule distribution were studied in  $\beta$ -cells from different genotype carriers. Total exocytosis was reduced in *TCF7L2* rs7903146 risk allele carriers ( $P = 0.007$ ;  $n = 249$  cells from  $36$  donors) (Fig. 2C, Table 1, and Supplementary Table 4), whereas intracellular granule distribution was unaffected (Table 1, Supplementary Table 5, and Supplementary Fig. 2), echoing recent studies in rodent cells (22). The *TCF7L2* risk allele did not affect the depolarization-evoked integrated  $Ca^{2+}$  current ( $P = 0.5$ ;  $\beta = -0.031$  [95% CI  $-0.114$  to  $-0.052$ ];  $n = 188$  cells from  $28$  donors) (Supplementary Table 4). We therefore conclude that the *TCF7L2* risk variant interferes with exocytosis at a late stage. In keeping with this idea, the  $Ca^{2+}$  sensitivity of exocytosis (estimated from the ratio between exocytosis and the integrated  $Ca^{2+}$  current during the first depolarization) was reduced in nonobese (BMI  $< 31$  kg/m<sup>2</sup>) risk allele carriers ( $P = 0.002$ ;  $\beta = -0.321$  [ $-0.526$  to  $-0.116$ ];  $n = 90$  cells from  $13$  donors) (Supplementary Table 4).

The *ADRA2A* rs553668 risk allele tended to reduce exocytosis ( $P = 0.059$ ;  $n = 249$  cells from  $36$  donors) (Fig. 2D and Table 1), an effect more pronounced in  $\beta$ -cells from nonobese donors where it attained statistical significance ( $P = 2 \times 10^{-6}$ ;  $n = 170$  cells from  $24$  donors;  $\beta = -0.433$  compared with  $-0.160$  for the entire cohort) (Supplementary Table 4). Both rapid exocytosis (estimated as the



**FIG. 2.** Genotype effects on insulin secretion and  $\beta$ -cell exocytosis. **A:** Glucose-stimulated insulin secretion in islets from different genotype carriers of rs7903146 (T is risk allele). Data from 28 CC, 17 CT, and 2 TT carriers. **B:** Glucose-stimulated insulin secretion in islets from donors with different genotype for rs553668 (A is risk allele). Data from 36 GG, 12 GA, and 2 AA carriers. **C–F:** Depolarization-evoked increase in cell capacitance ( $\Delta C$ ) in  $\beta$ -cells representative for individuals carrying risk or nonrisk alleles for rs7903146 (*TCF7L2*), rs553668 (*ADRA2A*), rs5219 (*KCNJ11*), and rs2237895 (*KCNQ1*). **G** and **H:** Exocytotic response in human  $\beta$ -cells treated with control siRNA or siRNA targeting *KCNQ1*. Histogram shows average total exocytosis ( $\Sigma\Delta C$ ) from 22 and 21 cells per group, respectively. \* $P < 0.05$ .

response to the first two depolarizations in the train) and slow exocytosis (the response to pulses 3–10), proposed to correlate with first- and second-phase insulin secretion (16), were affected ( $P = 0.004$  and  $P = 8 \times 10^{-8}$ ). There was no

change in the integrated  $Ca^{2+}$  currents, whereas the  $Ca^{2+}$  sensitivity of exocytosis was reduced in nonobese risk allele carriers ( $P = 3 \times 10^{-5}$ ) (Supplementary Table 4). Furthermore, the risk allele was associated with impaired docking

**TABLE 1**  
Genotype effects on  $\beta$ -cell phenotypes in the complete cohort

SNP	Nearest gene	Insulin secretion ( $n = 47$ –50 donors)		Total exocytosis* ( $n = 249$ cells from 36 donors)		Ns† ( $n = 97$ cells from 18 donors)	
		<i>P</i> value	Effect‡	<i>P</i> value	Effect	<i>P</i> value	Effect
<b>rs7903146</b>	<b><i>TCF7L2</i></b>	<b>0.002</b>	<b>−0.530</b>	<b>0.007</b>	<b>−6.40</b>	<b>0.40</b>	<b>−0.004</b>
<b>rs553668</b>	<b><i>ADRA2A</i></b>	<b>0.017</b>	<b>−0.575</b>	<b>0.059</b>	<b>−15.4</b>	<b>0.03</b>	<b>−0.14</b>
<b>rs5219</b>	<b><i>KCNJ11</i></b>	<b>0.776</b>	<b>0.109</b>	<b>0.021</b>	<b>−7.00</b>	<b>0.05</b>	<b>−0.12</b>
<b>rs2237895</b>	<b><i>KCNQ1</i></b>	<b>0.484</b>	<b>−0.171</b>	<b>0.016</b>	<b>−10.1</b>	<b>0.04</b>	<b>−0.10</b>
rs10946398	<i>CDKAL1</i>	0.153	−0.159	0.414	−1.73	0.187	0.081
rs2191349	<i>DGKB</i>	0.229	0.401	0.301	−14.3	0.381	0.101
rs560887	<i>G6PC2</i>	0.519	0.226	0.676	−4.97	0.06	−0.108
rs4607517	<i>GCK</i>	0.213	0.333	0.667	8.33	0.74	0.002
rs10423928	<i>GIPR</i>	0.972	−0.053	0.849	−4.56	0.8	0.004
rs1111875	<i>HHEX/IDE</i>	0.164	0.121	0.086	−10.9	0.016	−0.054
rs231362	<i>KCNQ1</i>	0.312	0.130	0.319	6.03	0.011	0.073
rs10830963	<i>MTNR1B</i>	0.851	0.001	0.655	0.129	0.074	0.095
rs11920090	<i>SLC2A2</i>	0.829	0.040	0.57	13.4	0.037	0.058
rs13266634	<i>SLC30A8</i>	0.662	0.084	0.819	−1.71	0.72	0.018

SNPs in boldface were used to calculate the genetic risk score. \*Exocytosis measured as the total increase in single  $\beta$ -cell capacitance in response to 10 depolarizations. †Number of docked insulin granules. ‡Effect of each additional risk allele on insulin secretion (in ng/islet/h), exocytosis (fF/pF), or granule distribution (Ns, granules/ $\mu m^2$ ) estimated from the linear model.

of insulin granules studied by electron microscopy ( $P = 0.025$ ;  $n = 97$  cells from 18 donors) (Table 1 and Supplementary Fig. 2). These data add novel insights into the effect of *ADRA2A* rs553668 on human  $\beta$ -cell function by demonstrating that the risk variant interferes with late-stage exocytosis via impaired granule docking. The defect was particularly pronounced in nonobese individuals, which is in line with patient data showing a stronger effect of rs553668 on T2D risk in lean subjects (11).

Carriers of the *KCNJ11* rs5219 T allele display reduced  $\beta$ -cell exocytosis ( $P = 0.021$ ;  $n = 249$  cells from 36 donors), particularly during the first two pulses of the train (Fig. 2E and Table 1). In addition, the  $\text{Ca}^{2+}$  sensitivity of exocytosis was reduced ( $P = 0.06$  for entire cohort and  $P = 0.004$  for nonobese donors;  $n = 24$ ) (Supplementary Table 4). *KCNJ11* encodes Kir6.2, which forms part of the  $\text{K}_{\text{ATP}}$  channel that regulates  $\beta$ -cell membrane potential. Activating mutations in *KCNJ11* are a cause of neonatal diabetes (23), and the nonsynonymous rs5219 risk allele of the channel (E23K) combined with the S1369A risk allele of the neighboring *ABCC8* (encoding the sulfonylurea receptor 1) exhibits decreased ATP inhibition (24). Since membrane potential is clamped in the capacitance measurements of exocytosis, our findings cannot be explained by immediate effects on electrical activity. Rather, we favor the idea that the risk allele results in long-term changes in electrical activity that may perturb granule distribution. The findings that  $\beta$ -cells from risk genotype donors had a decreased number of docked granules (35% reduction per risk allele;  $P = 0.05$ ;  $n = 97$  cells from 18 donors) (Table 1, Supplementary Table 5, and Supplementary Fig. 2) and that the exocytotic defect was restricted to the first two pulses support this hypothesis. There is some evidence that  $\text{K}_{\text{ATP}}$  channels may locate to the secretory granules, a finding that may be related to the proposal that sulfonylureas modulate insulin release by a direct effect on exocytosis (25).

The risk allele of rs2237895 (26,27), located in intron 15 of *KCNQ1*, was also associated with impaired exocytosis ( $P = 0.016$ ) (Fig. 2F and Table 1), an effect that was particularly pronounced among nonobese risk allele carriers ( $P = 0.001$ ) (Supplementary Table 4). The reduction was stronger for rapid exocytosis ( $P = 0.011$  for pulses 1 and 2 and  $P = 0.15$  for pulses 3–10 in the entire cohort and  $P = 0.003$  and 0.008, respectively, in nonobese donors). Moreover,  $\beta$ -cells from risk allele carriers exhibited a reduced number (25% per risk allele) of docked granules ( $P = 0.037$ ;  $n = 97$  cells from 18 donors) (Table 1 and Supplementary Fig. 2). Another SNP in *KCNQ1* (rs231362), which is independently associated with T2D risk (4), did not affect exocytosis and increased (30%) rather than reduced the number of docked granules (Table 1).

*KCNQ1* encodes a voltage-gated  $\text{K}^+$  channel, mutations of which cause both long- and short-QT syndromes (28). Previous investigations into its role in insulin secretion are contradictory (29,30). Preincubation with the *KCNQ1* antagonist linopirdine stimulated exocytosis in human  $\beta$ -cells ( $P = 0.03$  for rapid exocytosis;  $n = 21$ ). siRNA silencing of *KCNQ1* ( $64 \pm 3\%$  [ $n = 3$ ] reduction of mRNA, 74% protein reduction as measured by immunocytochemistry, and 54% reduction as measured by Western blot) (Supplementary Fig. 3) likewise enhanced exocytosis from  $59 \pm 9$  fF/pC to  $99 \pm 15$  fF/pC ( $P = 0.011$ ) (Fig. 2G and H). Collectively, these data suggest that *KCNQ1* is expressed in human  $\beta$ -cells and that activity of the channel regulates exocytosis by an effect unrelated to any impact this channel may have on action potential firing.

In addition, the risk allele for rs1111875 (*HHEX/IDE*) significantly decreased the number of docked granules ( $P = 0.016$ ), an effect that correlated with a tendency for reduced exocytosis ( $P = 0.086$ ). Moreover, risk genotype for rs11920090 (*SLC2A2*) tended to increase the number of docked granules ( $P = 0.037$ ) (Table 1). Rs13266634 (*SLC30A8*) is the second most strongly associated T2D variant identified to date (1), but previous population studies have shown inconsistent effects of the variant on insulin secretion (31,32). Studies reporting global knockout of this gene have shown abnormalities in zinc accumulation, insulin granule morphology, and insulin secretion from isolated  $\beta$ -cells, but effects on in vivo glucose homeostasis have been less consistent (33,34). In contrast,  $\beta$ -cell-specific *Slc30a8* knockout mice have shown defects in insulin secretion both in vivo and in vitro (35). However, we found no association between the risk variant and either insulin secretion or exocytosis in human islets (Table 1).

mRNA levels of the four genes associated with defects in  $\beta$ -cell function were measured in human islets (Supplementary Table 6). Interestingly, *KCNJ11* expression was reduced by 30% in islets from donors with T2D ( $n = 15$ ) compared with those from normoglycemic donors ( $n = 56$ ), whereas *ADRA2A* and *KCNQ1* transcripts did not differ between islets from ND and T2D donors. For *TCF7L2*, previous reports on its expression in islets from donors with T2D are inconsistent (21,36,37); both silencing and overexpression of this gene have been associated with impaired glucose tolerance (38,39). Of note, we did not detect any change in the levels of *TCF7L2* expression, including a newly discovered isoform (40), in T2D donors. **Genetic risk score for  $\beta$ -cell dysfunction.** Finally, we investigated whether we could combine our findings to construct a genetic risk score for defective  $\beta$ -cell function. For each individual, we added the number of risk alleles for the four SNPs associated with secretion/exocytosis phenotypes (highlighted in bold in Table 1). Thus, each donor was assigned a score from 0 to 8 (an individual being homozygous for all risk alleles would have a score of 8). There was a significant association between the risk score and reduced glucose-stimulated insulin secretion in islets ( $P = 0.008$ ;  $n = 47$  donors) (Table 2) whereas there was no effect on insulin content ( $P = 0.8$ ). The risk score was linearly associated with reduced  $\beta$ -cell exocytosis ( $P = 5 \times 10^{-6}$ ) (Fig. 3A), which was more pronounced in nonobese individuals ( $P = 4 \times 10^{-8}$ ). Both rapid and slow exocytosis were decreased (Table 2), an effect not associated with reduced  $\text{Ca}^{2+}$  influx. Donors with the highest risk score showed reduced  $\text{Ca}^{2+}$  sensitivity of exocytosis ( $P = 0.003$ ), which was paralleled by a strong correlation between the risk score and a reduced number of docked insulin granules ( $P = 0.0004$ ) (Fig. 3B; for quantification see Table 2). For reasons of statistical power, both T2D and ND donors were included in the genotype analyses, and diabetes status was used as a covariate. When only ND donors were analyzed, all associations exhibited similar trends with respect to the  $\beta$  values, but the  $P$  values in most cases did not reach statistical significance (Supplementary Table 7). However, the risk score was associated with reduced glucose-stimulated insulin release ( $P = 0.013$ ;  $\beta = -0.068$ ;  $n = 33$  donors), decreased  $\beta$ -cell exocytosis ( $P = 0.001$ ;  $\beta = -0.060$ ;  $n = 199$  cells from 29 donors), and reduced number of docked granules ( $P = 2 \times 10^{-5}$ ;  $\beta = -0.182$ ;  $n = 60$  cells from 12 donors) also when considering ND donors alone.

To investigate if this risk score could predict insulin secretion capacity in an independent patient cohort, we

TABLE 2  
Effect of genetic score on cellular phenotypes

Parameter	<i>P</i> value	Effect*
Glucose-induced insulin secretion (ng/islet/h)	0.008	-0.235
Total exocytosis (fF/pF)	$5 \times 10^{-6}$	-6.09
Rapid exocytosis (fF/pF)	$2 \times 10^{-4}$	-6.82
Slow exocytosis (fF/pF)	$4 \times 10^{-4}$	-4.70
Integrated $\text{Ca}^{2+}$ current (pC)	0.70	-0.008
Exocytosis/charge <sup>†</sup> (fF/pC)	0.003	-1.34
Ns <sup>‡</sup> (granules/ $\mu\text{m}^2$ )	$4 \times 10^{-4}$	-0.080

\*A linear model was used to analyze the association between the genetic risk score (0–8) and physiological measures in islets from the donors. The effect of each additional risk allele on the phenotypes is given in units as indicated. <sup>†</sup>The capacitance increase in response to the first depolarization normalized to the charge, indicating the  $\text{Ca}^{2+}$  sensitivity of exocytosis. <sup>‡</sup>Number of docked granules.

studied the four T2D variants in 604 ND individuals for whom IVGTT data were available. Interestingly, the genetic risk score was associated with reduced fasting insulin ( $P = 0.034$ ) and impaired insulin secretion in response to an IVGTT (Fig. 3C and Supplementary Table 8). Moreover, in 2,770 subjects from the Botnia Prospective cohort, individuals with a high risk score exhibited reduced CIR over time, with suppressed CIR at the follow-up visit ( $P = 0.035$ ;  $\beta = -0.030$ ).

## DISCUSSION

This study is the first to investigate the association between T2D genetic risk variants and detailed  $\beta$ -cell phenotypes. The data demonstrate the potential of investigating genetic subgroups of patients in combination

with in vitro cellular phenotypes to better characterize the mechanisms underlying reduced systemic insulin levels. It is of interest that the negative impact of T2D loci on  $\beta$ -cell function was particularly evident in islets from nonobese individuals. This suggests that the functional effects of the T2D-associated SNPs identified to date may be more pronounced in lean than in obese individuals. It may seem counterintuitive that obese individuals with T2D exhibit greater insulin secretion than their lean counterparts. It is likely that this reflects compensation, albeit insufficient to prevent diabetes, in the obese donors. In addition, lean donors that have developed T2D are likely to be those with the lowest insulin secretory capacity or else they would not have become diabetic.

A number of mechanisms could contribute to the reduced insulin secretion in vivo that has been associated with several T2D susceptibility variants. Dissection of the underlying cellular pathology requires 1) access to relevant human tissues from nonrisk and risk genotype carriers, which facilitates the correct translation of association signals compared with studying genetically modified animals, and 2) characterization of the effect of genotype on detailed cellular phenotypes. There are fundamental electrophysiological and secretory differences between human and rodent  $\beta$ -cells, making the study of human islets essential to investigate the influence of T2D susceptibility variants on  $\beta$ -cell function. The biophysical and ultrastructural examination of human  $\beta$ -cells in the current study identified four T2D variants that were associated with reduced exocytosis and enabled characterization of the mechanisms for the exocytotic impairment. The results shed new light on the pathophysiology linked with these risk variants, near *TCF7L2*, *ADRA2A*, *KCNJ11*, and *KCNQ1*, and demonstrate that defective  $\beta$ -cell exocytosis can be an important pathogenic mechanism in genetic subgroups of T2D. The data

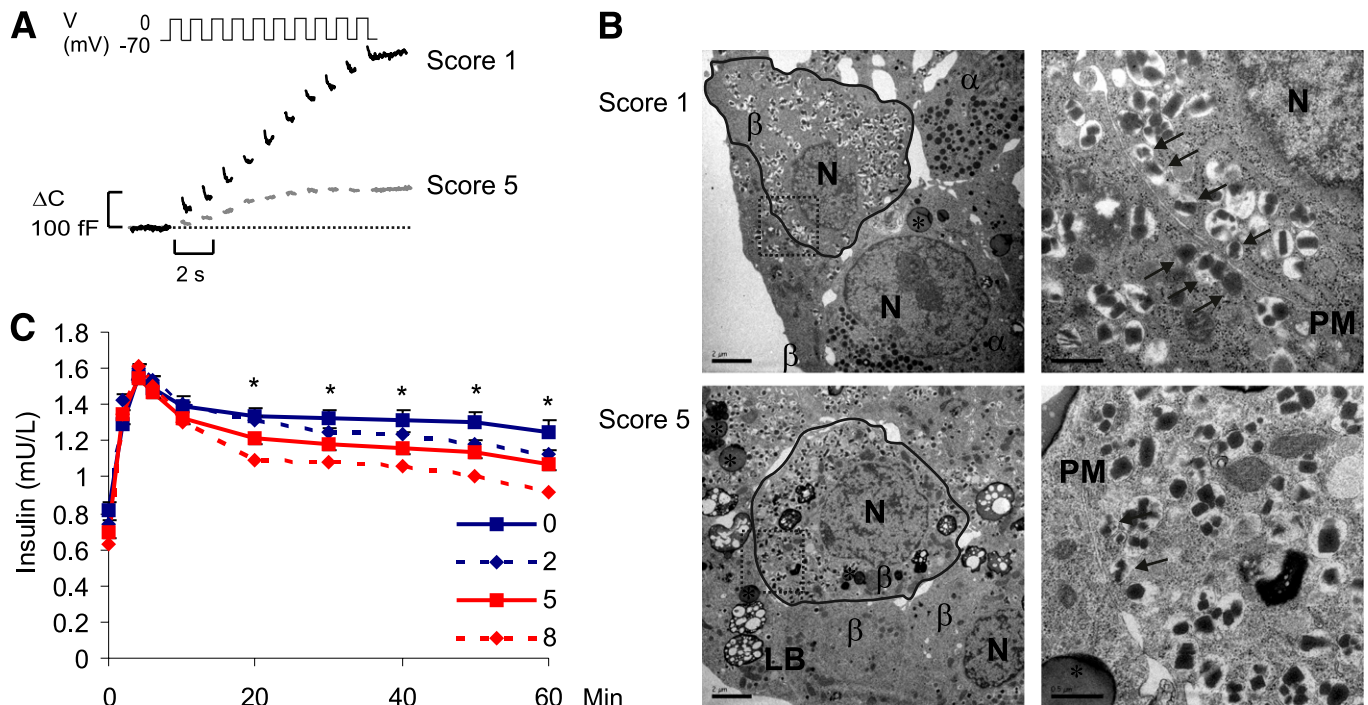


FIG. 3. Effects of the genetic risk score. **A**: Depolarization-evoked exocytosis in  $\beta$ -cells from donors with different scores. **B**: Electron micrographs of human islet sections from an individual with low (*top*) and high (*bottom*) score. Docked insulin granules (arrows), lipid droplets (\*), nuclei (N), lipofuscin bodies (LB), and plasma membrane (PM) are indicated. Left scale bars, 2  $\mu\text{m}$ ; right, 0.5  $\mu\text{m}$ . **C**: Effects of the risk score on insulin secretion during IVGTT in 604 individuals. Data are means  $\pm$  SEM. \* $P < 0.05$ .

suggest that there may be considerable heterogeneity in the cellular pathways that lead to reduced insulin secretion, which may explain why the reduction of exocytosis is evident only in genetic subgroups and not in the entire T2D cohort. Stratification based on genetic variants may therefore be useful to better resolve the disease mechanisms. Similar approaches may therefore be valuable to study the T2D susceptibility variants that were not associated with defective  $\beta$ -cell exocytosis in the current study (Table 1) and may instead impair systemic insulin release through effects on  $\beta$ -cell mass and/or glucose sensing or indirectly via incretins and innervation.

We acknowledge that although this study is the largest to date (totally including islets from 100 donors), the number of donors and consequently the statistical power is limited compared with that of genetic population studies. However, the combination of genetics and cellular physiology identified four risk alleles that associate with impaired  $\beta$ -cell exocytosis and enabled us to form a novel genetic risk score for single  $\beta$ -cell dysfunction that involves impaired granule docking and defective  $\text{Ca}^{2+}$  sensitivity of exocytosis. These findings give new insights on the pathophysiology of T2D that may open up possibilities for identifying subgroups of patients who would benefit from treatment specifically aimed at improving  $\beta$ -cell exocytosis. In this context, it may be relevant that glucagon-like peptide 1, via elevation of intracellular cAMP (41), amplifies  $\text{Ca}^{2+}$ -induced exocytosis in human  $\beta$ -cells.

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A.H.R., M.B., and L.E. designed the project, performed the research, analyzed the data, and wrote the manuscript. T.M., M.S., E.Z., A.S.A., A.E., A.J., R.R., Y.T., J.N.W., and A.B. performed the research. S.A.A. and M.G.P. performed the research, analyzed the data, and contributed to the manuscript. M.E.T. performed the research and wrote the manuscript. P.A. and C.L. designed the project and analyzed the data. P.R.V.J. provided human islets. V.L. analyzed the data. M.I.M., L.G., A.L.G., E.R., and P.R. designed the project and wrote the manuscript. A.S. designed the project, performed the research, and analyzed the data. A.H.R. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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