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<td>Author(s)</td>
<td>Fløyel, Tina; Brorsson, Caroline; Nielsen, Lotte B.; Miani, Michela; Bang-Berthelsen, Claus Heiner; Friedrichsen, Martin; Overgaard, Anne Julie; Berchtold, Lukas A.; Wiberg, Anna; Poulsen, Pernille; Hansen, Lars; Rosinger, Silke; Boehm, Bernhard O.; Ram, Ramesh; Nguyen, Quang; Mehta, Munish; Morahan, Grant; Concannon, Patrick; Bergholdt, Regine; Nielsen, Jens H.; Reinheckel, Thomas; Herrath, Matthias von; Vaag, Allan; Eizirik, Decio Laks; Mortensen, Henrik B.; Størling, Joachim; Pociot, Flemming</td>
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CTSH regulates β-cell function and disease progression in newly diagnosed type 1 diabetes patients

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Over 40 susceptibility loci have been identified for type 1 diabetes (T1D). Little is known about how these variants modify risk and progression. Here, we combined in vitro and in vivo experiments with clinical studies to determine how genetic variation of the candidate gene cathepsin H (CTSH) affects disease mechanisms and progression in T1D. The T allele of rs3825932 was associated with lower CTSH expression in human lymphoblastoid cell lines and pancreatic tissue. Proinflammatory cytokines decreased the expression of CTSH in human islets and primary rat β-cells, and overexpression of CTSH protected insulin-secreting cells against cytokine-induced apoptosis. Mechanistic studies indicated that CTSH exerts its antiapoptotic effects through decreased JNK and p38 signaling and reduced expression of the proapoptotic factors Bim, DPs, and c-Myc. CTSH overexpression also up-regulated Ins2 expression and increased insulin secretion. Additionally, islets from Cts−/− mice contained less insulin than islets from WT mice. Importantly, the TT genotype was associated with higher daily insulin dose and faster disease progression in newly diagnosed T1D patients, indicating agreement between the experimental and clinical data. In line with these observations, healthy human subjects carrying the T allele have lower β-cell function, which was evaluated by glucose tolerance testing. The data provide strong evidence that CTSH is an important regulator of β-cell function during progression of T1D and reinforce the concept that candidate genes for T1D may affect disease progression by modulating survival and function of pancreatic β-cells, the target cells of the autoimmune assault.

The T1D susceptibility locus on chromosome 15q25.1 contains four known protein-coding genes: ADAMTS7, cathepsin H (CTSH), MORF4L1, and RASGRF (3, 7). The most significantly associated SNP, rs3825932, is located in intron 1 of the CTSH gene (7) and has been reported to correlate with the CTSH transcript level (http://eqtl.uchicago.edu/cgi-bin/gbrowse/eqtl/). CTSH is a ubiquitously expressed lysosomal cysteine protease. The family of cathepsins is involved in overall protein turnover and specific cellular processes, such as apoptosis, antigen presentation, and prohormone processing (8, 9). Experiments with nonobese diabetic mice deficient in cathepsin B, L, or S showed that these proteases are important for the onset of autoimmune diabetes (10, 11). Additionally, several cathepsins have been implicated in proinsulin processing (12) or insulin secretion, suggesting that genetic susceptibility to T1D may affect disease progression by modulating survival and function of the β-cells.

Significance

In type 1 diabetes (T1D), the insulin-producing pancreatic β-cells are destroyed by the immune system. Both genetic and environmental factors contribute to T1D risk. Candidate genes for T1D identified by genome-wide association studies have been proposed to act at both the immune system and the β-cell levels. This study shows that the risk variant rs3825932 in the candidate gene cathepsin H (CTSH) predicts β-cell function in both model systems and human T1D. Collectively, our data indicate that higher CTSH expression in β-cells may protect against immune-mediated damage and preserve β-cell function, thereby representing a possible therapeutic target. Our study reinforces the concept that candidate genes for T1D may affect disease progression by modulating survival and function of the β-cells.


The authors declare no conflict of interest.

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Against this background, we investigated how genetic variations affect CTSH expression, disease mechanisms, and disease progression in children with newly diagnosed T1D. By combining in vitro and in vivo experimental studies and functional observations in healthy and T1D individuals, we show that the T1D candidate gene CTSH is an important regulator of β-cell function during progression of T1D.

Results

rs3825932 Genotypes Correlate with CTSH mRNA and Protein Expression. Previous expression quantitative trait locus (eQTL) studies have reported effects of rs3825932 on the CTSH transcript level (19, 20). These datasets show a clear signal surrounding the linkage disequilibrium (LD) block at the transcriptional start site of CTSH, where rs3825932 is among the SNPs with the most significant eQTL signals in CTSH (Fig. S1). There was no eQTL effect on the expression of the three additional genes at the 15q25.1 locus.

Real-time quantitative PCR (qPCR) analysis of the HapMap B-lymphoblastoid cell lines (BLCLs) confirmed that the T allele of rs3825932 correlated with decreased CTSH mRNA in an allele dose-dependent manner (P = 1.3 × 10⁻², ANOVA) (Fig. 1A). Of all analyzed SNPs in the LD block, rs3825932 was most significantly associated with CTSH expression (Table S1) and captured the eQTL effect of the top SNPs, which was shown by forward stepwise regression. When tag SNPs for the entire region were included, rs3825932 explained most of the eQTL effects observed.

Additionally, rs3825932 also affected the protein expression of CTSH in the HapMap BLCLs. The TT genotype was associated with a lower CTSH protein level compared with the CC and CT genotypes (P = 0.014, ANOVA) (Fig. 1B and C). Similar to the HapMap BLCLs, the T allele of rs3825932 was correlated with a decreased CTSH mRNA level in BLCLs from patients with T1D (P = 0.006). Analysis of pancreatic tissue from five donors with T1D supported that the effect is present in primary disease-relevant tissue, because the CTSH mRNA level was one-third in the three TT genotype carriers compared with the two CC/CT carriers (Fig. S2).

Cytokines Suppress CTSH Expression in Human Islets and Rat β-cells. Proinflammatory cytokines, particularly IL-1β, IFN-γ, and TNF-α, contribute to β-cell dysfunction and apoptosis in T1D (1, 21). We, therefore, investigated if cytokines affect the expression of CTSH in pancreatic islets. Exposure of human islets to IL-1β, IFN-γ, and TNF-α decreased the expression of CTSH compared with untreated islets (Fig. 2A). The cytokine-mediated suppression of CTSH expression was confirmed specifically in β-cells, which was shown by a decrease in Csh mRNA in purified primary rat β-cells after exposure to IL-1β and IFN-γ (Fig. 2B).

To confirm that CTSH is expressed at the protein level in human β-cells, we performed immunohistochemical staining of pancreatic sections from nondonor diabetics. CTSH was expressed in both exocrine tissue and pancreatic islets (Figs. S3 and S4), including β-cells, where it co-stained with insulin (Fig. S3).

Overexpression of CTSH Decreases Cytokine-Induced Apoptosis. To investigate whether CTSH is involved in cytokine-induced β-cells apoptosis, insulin-secreting INS-1 cells with stable overexpression of CTSH were generated, and two CTSH-overexpressing clones [plasmid encoding CTSH #1 (pCTSH #1) and pCTSH #2] were selected (Fig. 3A). Overexpression of CTSH decreased cytokine-induced apoptosis by ~50% compared with cells transfected with an empty vector (Fig. 3B). Basal apoptosis rate was not significantly affected. These findings were supported by data showing that overexpression of CTSH decreased cytokine-induced caspase-3/7 activity (Fig. 3C).

CTSH Regulates Cytokine Signaling. NF-κB, signal transducer and activator of transcription 1, and the MAPKs JNK, p38, and ERK are critical mediators of cytokine signal transduction in β-cells (21, 22). We, therefore, examined if CTSH affects cytokine signaling through these components. Cytokine-induced degradation of IκB, phosphorylation of signal transducer and activator of transcription 1, and phosphorylation of ERK1/2 were unaffected by CTSH overexpression (Fig. S5). In contrast, phosphorylation of JNK1/2 and p38 was diminished in cells overexpressing CTSH compared with control cells (Fig. 4A and B). In line with these data, phosphorylation of c-Jun and JunD, transcription factors downstream of JNK, was decreased in CTSH-overexpressing cells after exposure to cytokines (Fig. 4C and D). A downstream target of NF-κB and MAPKs in β-cells is inducible nitric oxide synthase (iNOS) (21, 22). Overexpression of CTSH caused an early transient reduction in cytokine-induced iNOS mRNA expression, which translated into reduced iNOS protein (Fig. 4E and F).

Finally, we examined if overexpression of CTSH affects the expression of the proapoptotic factors Bim, Bak, Bid, death protein 5 (DP5), p53 up-regulated modulator of apoptosis (Puma), and c-Myc. Although overexpression of CTSH abolished cytokine-induced expression of Bcl2l11 (Bim), c-Myc, and DP5 (Hrk) mRNA (Fig. 4G–I), it failed to significantly affect Bak, Bid, and Puma (Bcl3) mRNA expression (Fig. S5).

CTSH Regulates Insulin Transcription. We also examined if CTSH affects insulin secretion. Overexpression of CTSH increased the
medium-accumulated insulin by ~50–70% compared with control cells (Fig. 5A); however, CTSH did not affect glucose-stimulated insulin secretion (Fig. 5B). The CTSH-overexpressing cells had a higher Ins2 mRNA expression compared with the control cells (Fig. 5B), whereas Ins1 was not significantly affected (Fig. 5C).

To further validate our findings, mirror experiments were performed in primary islets from Ctsh−/− and WT mice. Medium-accumulated insulin was lower in isolated islets from Ctsh−/− mice compared with WT littermates (Fig. 5D). These data are in line with a trend for a higher fasting blood glucose level in Ctsh−/− mice (P = 0.2) (Fig. 5E). Additionally, the insulin intensity was significantly lower in islets from Ctsh−/− mice compared with islets from WT mice (Fig. 6 and Table S2), which was evaluated by immunohistochemical staining of pancreatic sections. The difference was not caused by a deficit of Ins2 in Ctsh−/− mice, which was shown by an identical ratio of Ins1 and Ins2 mRNA in pancreas from Ctsh−/− and WT mice. In contrast, there was no difference in the glucagon intensity between Ctsh−/− and WT mice (Fig. 6).

**rs3825932 Affects Disease Progression in Children with T1D.** To gain insight into possible in vivo effects caused by the rs3825932 variant, we examined if rs3825932 affects residual β-cell function and glycemic control in children with newly diagnosed T1D. These children display a varying degree of decline in residual β-cell function, which could partly be determined by genetic risk factors. Of interest, carriers of the TT genotype required a higher daily insulin dose 9 and 12 mo after disease onset compared with the CC and CT carriers (9 mo: estimate: 0.18 U/kg, P = 0.009; 12 mo: estimate: 0.22 U/kg, P = 0.009) (Fig. 7A). There was no difference in glycemic control (HbA1c) between the groups. The insulin dose-adjusted HbA1c (IDAA1c) levels were, however, higher 9 and 12 mo after disease onset in carriers of the TT genotype compared with the CC and CT carriers (9 mo: estimate: 0.08, P = 0.042; 12 mo: estimate: 1.1, P = 0.026) (Fig. 7B). Of note, there was a tendency toward fewer TT carriers entering remission (as assessed by an IDAA1c ≤ 9) (23), which is a state of temporary improvement in disease progression caused by improved β-cell function. Indeed, by 12 mo after disease onset, none of the TT carriers were in remission compared with 15% and 21% of the CC and CT carriers, respectively (Fig. 7C) (P = 0.068, recessive model).

**rs3825932 Affects β-Cell Function in Healthy Subjects.** We next investigated if rs3825932 affects β-cell function in a group of young, clinically well-characterized healthy twins. Insulin secretion was assessed by oral glucose tolerance test (OGTT) (Table 1) and i.v. glucose tolerance test (IVGTT) (Table S3), whereas insulin sensitivity was evaluated by a euglycemic–hyperinsulinemic clamp (Table S3). There was a significant effect of rs3825932 on the insulinogenic index, a measure of β-cell function. The effect size was 0.79 (P = 0.03), corresponding to a 21% decrease in β-cell function for each T allele (Table 1). However, rs3825932 did not affect insulin sensitivity (Table S3), suggesting that the observed effect is caused by differences in β-cell function.

**Discussion**

We have shown that CTSH is a T1D candidate gene that regulates β-cell function and disease progression in newly diagnosed patients. We confirmed that the previously reported eQTL signals for SNPs located in the T1D susceptibility locus on chromosome 15q25.1 affect the expression of CTSH. Specifically, we observed that the T allele and the TT genotype of rs3825932 significantly correlate with a lower CTSH expression at the mRNA and protein levels, respectively; rs3825932 has no effect on the expression of the other candidate genes in the region, supporting CTSH as the causal gene of this locus. Although rs3825932 is superior in explaining the eQTL signal in CTSH expression, it cannot be excluded that other SNPs or structural variants in tight LD with this variant also carry causal effects.

Exposure of isolated human islets and purified primary β-cells to proinflammatory cytokines decreased CTSH mRNA expression. This observation is in concordance with recently published RNA sequencing data used to identify transcripts under cytokine regulation in human islets (4). Importantly, we confirmed that CTSH is expressed at the protein level in human β-cells in pancreatic sections, which is consistent with a previous study that showed that CTSH is localized to the secretory granules of α- and β-cells (24).

The overexpression experiments revealed CTSH as a negative regulator of cytokine-induced apoptosis in insulin-secreting cells. Interestingly, deletion of CTSH in a mouse model of pancreatic islet cell cancer led to a reduction in tumor incidence and tumor growth because of increased apoptosis (17). Taken together, these results support an antiapoptotic effect of CTSH. Subsequent mechanistic experiments indicated that CTSH exerts its antiapoptotic effects through inhibition of specific cytokine-induced signals in β-cells. Thus, overexpression of CTSH in INS-1 cells diminished signaling through the JNK and p38 pathways, key modulators of cytokine-induced β-cell apoptosis (21). We also observed a clear inhibitory effect of CTSH overexpression on cytokine-induced transcription of the proapoptotic factors Bim, DP5, and c-Myc. The expressions of Bim, c-Myc, and DP5 are induced by cytokines in β-cells, and Bim and DP5 are regulated, at least in part, through JNK and p38 pathways, key modulators of cytokine-induced β-cell apoptosis (21). We also observed a clear inhibitory effect of CTSH overexpression on cytokine-induced transcription of the proapoptotic factors Bim, DP5, and c-Myc. The expressions of Bim, c-Myc, and DP5 are induced by cytokines in β-cells, and Bim and DP5 are regulated, at least in part, through JNK and p38 pathways, key modulators of cytokine-induced β-cell apoptosis (21). We also observed a clear inhibitory effect of CTSH overexpression on cytokine-induced transcription of the proapoptotic factors Bim, DP5, and c-Myc. The expressions of Bim, c-Myc, and DP5 are induced by cytokines in β-cells, and Bim and DP5 are regulated, at least in part, through JNK and p38 pathways, key modulators of cytokine-induced β-cell apoptosis (21).

Our data also showed effects of CTSH on insulin gene transcription and chronic insulin release. Overexpression of CTSH causes an increase in chronic insulin release from INS-1 cells that is paralleled by increased transcription of Ins2. Accordingly, islets from Ctslh−/− mice contain less insulin than islets from WT littermates. Ctslh−/− mice also tend to have a higher fasting blood glucose.

The clinical data from patients with newly diagnosed T1D supported the in vitro results. Carriers of the rs3825932 TT genotype require a significantly higher insulin dose to maintain glycemic control and have higher IDAA1c levels, thus indicating a negative effect of the TT genotype on the residual β-cell function in T1D patients. Interestingly, the CTSH protein expression data support the clinical observation that the TT
genotype differs from the CC and CT genotypes. The lack of association between CTSH genotypes and C-peptide or proinsulin levels might relate to the fact that these patients already suffer from severe β-cell loss, rendering it difficult to detect subtle differences in the β-cell mass and function. Nevertheless, the results indicate that TT carriers have a faster disease progression, leading to a more prominent β-cell dysfunction, which is consistent with our observation that fewer TT carriers enter remission.

In line with these observations, healthy human subjects carrying the T allele had lower β-cell function. We found no effect of rs3825932 on the first-phase insulin secretion, which was assessed through the IVGTT, but in the OGTT, rs3825932 significantly affected the insulinogenic index, showing that the β-cell function was decreased by 21% for each T allele. The data suggest that rs3825932 primarily affects insulin secretion in response to oral as opposed to i.v. glucose ingestion. It is conceivable that the differences between the two tests are explained by rs3825932 primarily affecting the second-phase insulin secretion response. Importantly, a recent study identified a type 2 diabetes candidate gene that only affects second-phase insulin secretion (29).

Several T1D candidate genes have been implicated in β-cell function and survival (28, 30–32), but to our knowledge, this study is the first demonstration that a genome-wide association study-identified genetic variation affects β-cell function in both model systems and human T1D. Thus, this study strengthens CTSH as a causal risk gene in T1D and reinforces the concept that candidate genes for T1D may affect disease progression by modulating survival and function of the pancreatic β-cells (33). We suggest that SNPs, which cause a low expression of CTSH, sensitize β-cells to immune-mediated β-cell dysfunction and death, leading to a more rapid and severe loss of functional β-cell mass. This functional knowledge of CTSH may be used for development of new diagnostic and preventive strategies to preserve β-cell function in T1D.
Materials and Methods

Cell Lines. BLCLs from 60 HapMap CEPH founders, obtained from the Coriell Institute (www.ccr.coriell.org), were cultured in RPMI1640 with Glutamax, 10% (vol/vol) heat-inactivated FBS, 1% (vol/vol) penicillin, and 100 μg/mL streptomycin (Invitrogen). qPCR, immunoblotting, and eQTL analysis are described in SI Materials and Methods. BLCLs from 20 T1D and 25 nondiabetic subjects were available from the Type 1 Diabetes Genetics Consortium (www.t1dgc.org). The generation and culturing of these cell lines have been described previously (34). Microarray, genotyping, and eQTL analysis are in SI Materials and Methods. INS-1 cells were cultured as described (35) and stably transfected with a plasmid pCTSH (OriGene) or an empty control vector (pcDNA 3.1) using Dharmafect 1 (Thermo Scientific). Two posttransfection, 200 μg/mL G418 (Sigma) was added to the culture medium for selection of stably transfected cells. After 1 wk, the concentration of G418 was reduced to 100 μg/mL, which was used continuously during culturing. INS-1 cells were treated with recombinant mouse IL-1β (BD Pharmingen) and recombinant rat IFN-γ (R&D Systems). qPCR, immunoblotting, apoptosis, and insulin measurements are described in SI Materials and Methods.

Human Pancreas. Human islets from four nondiabetic donors were provided through the Juvenile Diabetes Research Foundation Islet Distribution Program. The islets were cultured in RPMI1640 supplemented with 2% (vol/vol) human serum (Lonza) and precultured for ~5 d to allow islet depletion of passenger cells, including immune cells. Islets were treated with IL-1β (1 ng/mL), IFN-γ (20 ng/mL), and TNF-α (8 ng/mL) and used for microarray analysis (SI Materials and Methods). Six-micrometer fresh frozen pancreatic samples from five donors with T1D (disease durations ranging from 1 to 8 y) were obtained through the Network for Pancreatic Organ Donors with Diabetes or donated by Thomas Kay (St. Vincent’s Institute, Fitzroy, Victoria, Australia). The samples were used for qPCR and genotyping (SI Materials and Methods). Adult human paraffin-embedded, formaldehyde-fixed pancreatic tissues were obtained from four nondiabetic donors through the multicenter European Union-supported program on β-cell transplantation in diabetes and used for immunostaining (SI Materials and Methods).

Animals. Isolation and culturing of primary rat β-cells have been described previously (36) and followed the guidelines of the Belgian Regulation for Animal Care. The preparations contained 93 ± 1% β-cells (n = 6) and were treated with recombinant human IL-1β (R&D Systems) and recombinant rat IFN-γ (R&D Systems), qPCR is described in SI Materials and Methods. Ctsdh−/− mice (strain Ctsdh<sup>tm1Thre</sup>) were generated as described (37) on a 129P2/OlaHsd genetic background and backcrossed for 10 generations to the C57BL/6N strain. For these experiments, Ctsdh<sup>−/−</sup> mice were bred, offspring was genotyped (37), and Ctsdh<sup>−/−</sup> and Ctsdh<sup>+/−</sup> littermates were selected for study. The absence of Ctsdh in pancreases from Ctsdh<sup>−/−</sup> mice was verified using qPCR (SI Materials and Methods). The generation and phenotype analyses of Ctsdh<sup>−/−</sup> mice were conducted in accordance with the German law of animal protection. The protocols were approved by the ethics committee of the governmental regional board, Freiburg or the Danish Animal Experiment Inspectorate as appropriate. Pancreatic islets were isolated from Ctsdh<sup>+/−</sup> and Ctsdh<sup>−/−</sup> mice (equal mix of males and females; 5–16 wk of age) by the collagenase digestion method (Collagenase P; Roche), handpicked, and cultured in RPMI1640 with Glutamax, 10% (vol/vol) heat-inactivated FBS, 100 μU/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). The islets were precultured for 3 d. Insulin measurements and immunostaining are in SI Materials and Methods.

Study Populations. The study population of T1D children was collected through The Hvidoeire Study Group on Childhood Diabetes as described (38). The cohort included 126 girls and 131 boys with new-onset T1D. Age at diagnosis was 9.1 ± 3.7 y (mean ± SEM). Glycemic control was measured at 1, 3, 6, 9, and 12 mo after diagnosis (38). IDAA1c defined as actual HbA<sub>1c</sub> (+ x insulin dose in units per kilogram per 24 h) was calculated and used as a surrogate marker of residual β-cell function. A calculated IDAA1c ≤ 9 corresponds to an estimated maximal C-peptide level above 300 pmol/L and was used to define clinical remission (23). The twin study population originally included 55 young (22–31 y) and 43 old (57–66 y) monozygotic and same sex dizygotic twin pairs without known diabetes (39); however, in this study, we focused on the young twins, because they are most likely more compatible with the T1D children; 108 subjects had normal glucose tolerance, and 2 subjects had impaired glucose tolerance. The clinical examination included a 75-g OGGT, a 30-min IVGTT (0.3 g/kg body weight), and a 2-h euglycemic-hyperinsulinemic clamp (40 μU/m<sup>2</sup>·min<sup>−1</sup>) (39).

Ethical Approval. All human studies were approved by the regional ethical committee and conducted according to the principles of the Declaration of Helsinki. Animal studies were approved by the local ethical committees as described in detail above.

Statistical Analyses. Statistical analysis was performed using a two-tailed t test unless otherwise stated. P < 0.05 was considered statistically significant. Genotype-specific levels of CTSH were analyzed using two-way ANOVA. The difference in intensity of the insulin staining between islets from WT and Ctsdh<sup>−/−</sup> mice was evaluated using χ<sup>2</sup> test with 5 degrees of freedom (groups 6 and 7 were merged because of few measurements). The distributions are shown in Table S2. All statistical tests on the two study populations were performed in SAS (version 9.1; SAS Institute). In the Hvidoeire Study Group, stimulated C peptide (logarithmic), proinsulin (logarithmic), HbA<sub>1c</sub>, daily insulin dose per kilogram, and IDAA1c (logarithmic) were analyzed as dependent variables in separate multiple regression repeated measurement models with unstructured variance, with sex, age, HLA risk groups, and CTSH genotype as explanatory variables. The assumption of constant effect of genotype was checked by first allowing for interaction between genotype and disease duration. In the twin study population, the proc mixed procedure was used to adjust for twin pair and zygosity status as well as other contributing variables, including age, sex, VO<sub>2max</sub>, and total fat percentage. All response variables were log-transformed to avoid skewness of the residuals, which also resulted in per-allele effects expressing percentage-wise and not absolute changes of the response variable.


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Table 1. Oral glucose tolerance test measures in healthy subjects

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<th>n (men/women)</th>
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<td>51</td>
<td>22/29</td>
<td>17</td>
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<tr>
<td>Age (y)</td>
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<td>27.7 (1.9)</td>
<td>28.8 (1.7)</td>
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<tr>
<td>InsBASAL (pmol/L)*</td>
<td>36.3 (22.5)</td>
<td>32.8 (15.3)</td>
<td>33.1 (18.2)</td>
<td>1.03</td>
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<td>InsBASAL</td>
<td>198 (282)</td>
<td>138 (112)</td>
<td>79 (29)</td>
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<td>AUC0–30 min</td>
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<td>5,684 (3,249)</td>
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*Insulinogenic index was calculated as (Ins0 – Ins30)/(Glu0 – Glu30).

†The total insulin area under the curve (AUC) was calculated for the initial 30-min period.

‡Homeostasis model assessment (HOMA) of β-cell function was calculated as Ins0 × 0.144 × 20/(Glu0 – 3.5).

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