Increase in Pancreatic Proinsulin and Preservation of β-Cell Mass in Autoantibody-Positive Donors Prior to Type 1 Diabetes Onset

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Type 1 diabetes is characterized by the loss of insulin production caused by β-cell dysfunction and/or destruction. The hypothesis that β-cell loss occurs early during the prediabetic phase has recently been challenged. Here we show, for the first time in situ, that in pancreas sections from autoantibody-positive (Ab+) donors, insulin area and β-cell mass are maintained before disease onset and that production of proinsulin increases. This suggests that β-cell destruction occurs more precipitously than previously assumed. Indeed, the pancreatic proinsulin-to-insulin area ratio was also increased in these donors with prediabetes. Using high-resolution confocal microscopy, we found a high accumulation of vesicles containing proinsulin in β-cells from Ab+ donors, suggesting a defect in proinsulin conversion or an accumulation of immature vesicles caused by an increase in insulin demand and/or a dysfunction in vesicular trafficking. In addition, islets from Ab+ donors were larger and contained a higher number of β-cells per islet. Our data indicate that β-cell mass (and function) is maintained until shortly before diagnosis and declines rapidly at the time of clinical onset of disease. This suggests that secondary prevention before onset, when β-cell mass is still intact, could be a successful therapeutic strategy.

PATHOPHYSIOLOGY

Type 1 diabetes is defined as an autoimmune disease in which clinical symptoms arise as a result of β-cell loss. Genetic and environmental factors might render β-cells susceptible to attack by the immune system or could contribute to β-cell dysfunction (1,2). More than three decades ago, Eisenbarth and colleagues (3) described a linear loss of first-phase insulin release after intravenous glucose administration in individuals with islet-cell antibodies who were monitored for 10 years before diagnosis. However, elevations in fasting blood glucose and peak glucose during oral glucose tolerance tests were only seen in the year before onset. This sustained loss of β-cell function in individuals with prediabetes strongly correlated with the time to overt diabetes and led to Eisenbarth's (4) landmark article in which the stages of type 1 diabetes were presented and the steady decrease in insulin secretion was linked to a linear reduction in β-cell mass that continued after diagnosis.

Although this model remained a reference for many years, new studies have suggested that β-cell mass is not lost in a linear fashion during the prediabetic phase, and a debate about the discrepancy between β-cell mass and function ensued (2). Subsequent studies have also detected a loss of glucose tolerance in the months preceding diagnosis (5,6). β-Cell dysfunction might occur early in the disease process, at the point at which the individual becomes autoantibody positive (Ab+), but an actual decline in β-cell mass might occur later. In the Diabetes Virus Detection (DiViD) study, a transient β-cell dysfunction was detected in live cells obtained at diagnosis, which improved in a nondiabetic culture milieu (7). Increasing dysfunction would prompt an increase in insulin demand (8,9), which could eventually cause a more cataclysmic decline in β-cell mass around the clinical onset of diabetes. However, the cause of the decline in function and the precise time course of events have remained largely undefined.

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Studies from the Network for Pancreatic Organ Donors with Diabetes (nPOD) have recently shown that β-cell mass is not diminished in Ab+ donors and that single β-cells and islets containing insulin can be found in donors with long-standing type 1 diabetes (10). The time course from seroconversion to onset of clinical diabetes has been further characterized in longitudinal studies. After autoantibody seroconversion, 14.5% of single Ab+ and 67.9% of multiple Ab+ patients progressed to type 1 diabetes in a 10-year follow-up study in three geographically different cohorts (11). Another study also revealed that 11% of multiple Ab+ children would progress to clinical disease each year (12). However, the exact triggers and progression to clinical onset are not fully understood.

Proinsulin is an important autoantigen in type 1 diabetes in humans and mice (13) because it shapes the autoreactive CD8 T-cell repertoire (14,15). Importantly, recent studies have shown that several epitopes within its precursor (preproinsulin) and proinsulin itself are recognized by islet-infiltrating CD4 and/or CD8 T cells isolated from patients with type 1 diabetes (16–20), suggesting a potential role for this antigen in disease pathogenesis. Preproinsulin is processed into proinsulin and signal peptide (21). Only a marginal fraction of proinsulin is secreted to the circulation, but it accounts for 30–50% of the protein production in β-cells and increases in response to higher insulin demand. Because of this high metabolic demand, β-cells are prone to endoplasmic reticulum (ER) stress and proinsulin misfolding, which could lead to β-cell failure (22). ER stress may also be induced by viral infection (23), which was recently detected in the islets of Langerhans at diagnosis (24). Interestingly, Cianciaruso et al. (25) demonstrated that cytokine-induced ER stress enhances the exosomal release of proinsulin. Several reports present evidence of high circulating proinsulin and proinsulin intermediates, with or without accompanying hyperglycemia, in patients at risk for developing the disease and after diagnosis (26,27). Proinsulin and proinsulin–to–C-peptide (PI-to-C) ratios in combination with autoantibody concentration have been suggested as potential biomarkers for type 1 diabetes, capable of identifying with high sensitivity individuals at risk for developing disease 1 to 40 months before clinical onset (28,29). However, the link between proinsulin levels in serum and their content in the pancreas in β-cells themselves has not been investigated in human specimens in situ.

The objective of this study was to add refinement to the classic model of linear β-cell loss and to fill an important gap in our understanding of the prediabetic phase in human type 1 diabetes. We studied, for the first time in the human pancreas, the distribution of proinsulin in single- and double-Ab+ individuals preceding the onset of disease as well as in patients with recent-onset type 1 diabetes and correlated it with loss of insulin content, β-cell mass, and PI area–to–insulin (PI-to-INS) area ratio. These studies underline the need for development of biomarkers as well as for preventive therapies focusing on normalizing β-cell dysfunction during the prediabetic stage.

RESEARCH DESIGN AND METHODS

Subjects

Human pancreas sections were collected from cadaveric organ donors through nPOD. Six micrometer formalin-fixed paraffin-embedded sections from the head, body, and tail of the pancreas were obtained from control donors without diabetes who were single Ab+ (n = 8), double Ab+ (n = 5), and Ab− (n = 9), and from one donor at onset of type 1 diabetes. In addition, 4-μm formalin-fixed paraffin-embedded sections from living donors with type 1 diabetes (n = 6) were obtained through the DiViD study by tail resection (30). Overall, 22 sections from the head, 22 from the body, and 29 from the tail of the pancreas were analyzed (n = 73 sections). Table 1 summarizes the demographic information for each group. Detailed donor information can be found in Supplementary Table 1. All experimental procedures were approved by the La Jolla Institute for Allergy and Immunology Institutional Review Board, approved protocol number DI3-054-0216. For the DiViD study, participants provided written informed consent, and more details can be found in Krogvold et al. (30).

Table 1—Donor demographic information including age, percentage of males and females, ethnicity, BMI, disease duration, and C-peptide levels

	Control (n = 9)	Ab+ (n = 13)	Type 1 diabetes (n = 7)	Total (N = 29)
Age, mean ± SD (years)	33.6 ± 12.5	36.1 ± 13.5	28.2 ± 4.9	32.6 ± 4.1
Sex, n (%)				
Female	4 (44.5)	8 (61.5)	3 (42.8)	15 (51.7)
Male	5 (55.5)	5 (38.5)	4 (57.2)	14 (48.3)
Ethnicity, n (%)				
African American	0 (0)	2 (15.4)	0 (0)	2 (6.9)
Caucasian	7 (77.7)	8 (61.5)	7 (100)	22 (75.9)
Hispanic	2 (22.3)	3 (23.1)	0 (0)	5 (17.2)
BMI, mean ± SD (kg/m^2)	28.2 ± 6	26.2 ± 5	25 ± 3.2	26.5 ± 1.6
Disease duration, mean ± SD (weeks)			4.4 ± 2.7	
C-peptide, mean ± SD (ng/mL)	6.7 ± 7.2	6.1 ± 6.2	—	

Immunofluorescence

Pancreas sections were stained for insulin, proinsulin, and glucagon after a standard triple indirect immunofluorescence staining. After deparaffinization and rehydration in descending ethanol concentrations, sections were exposed to heat-based antigen retrieval (citrate buffer). Staining was performed using a polyclonal guinea pig anti-insulin antibody (1:500; Dako, Carpinteria, CA), monoclonal mouse anti-proinsulin (DSHB clone GS-9A8, 1:50), and monoclonal mouse anti-glucagon (clone K79bB10, 1:300; Abcam) conjugated in-house to Alexa Fluor 647. Secondary antibodies included $F(ab')_2$ fragment of goat anti-guinea pig IgG conjugated to Alexa Fluor 488 (1:800; Jackson ImmunoResearch) and goat anti-mouse IgG (H+L) conjugated to Alexa Fluor 555 (1:1,000; Life Technologies, Grand Island, NY) incubated at room temperature for 30 min. Sections were counterstained with Hoechst (1:400; Life Technologies) for 10 min and then mounted with ProLong Gold Antifade Mountant (Life Technologies).

Image Acquisition and Analysis

Sections were scanned with an Axio Scan Z.1 slide scanner (Carl Zeiss Microscopy, Thornwood, NY) using a fluorescent Orca Flash 4.0 v2 (HXP 120 V lamp) camera with a ×20/0.8 NA objective for immunofluorescence. Images were acquired with ZEN2 software slidescan module. Acquisition settings were kept constant between specimens to allow for quantitative comparison between samples. ZEN2 software blue edition was used to process the images before analysis. Lower and upper thresholds were defined for each channel. For insulin and proinsulin, similar thresholds were set for comparison. Then, whole-tissue section images were exported and reduced at 30% or 45%, depending on their size, into .tiff files for automatic software analysis. Custom macros were developed to measure tissue area (macro 1) and islet size and count (macro 2) and calculate the percentage of insulin, proinsulin, and glucagon areas (macro 3; only cytoplasmic staining above background levels was measured). To classify and calculate the number of α- and β-cells, the image of the whole tissue section was exported into 20 × 20 .tiff files to improve resolution and then processed by a different custom macro (macro 4) (Supplementary Fig. 1). All of the macros were developed for Fiji, an image-processing software program developed for ImageJ (National Institutes of Health). R software was used to systematically analyze the data (R is available as free software under the terms of the Free Software Foundation's GNU General Public License in source code form). In addition, three cases per group were analyzed by high-resolution confocal microscopy using an LSM 880 confocal microscope with Airyscan technology (Carl Zeiss, Jena, Germany) and a ×63 objective.

Statistical Analysis

Differences between group pairs were analyzed with a Student *t* test or Mann-Whitney test. Group differences were analyzed using one-way ANOVA, followed by a Holm-Šídák multiple comparisons test or Kruskal-Wallis test, followed by a Dunn multiple comparisons test. To assess the plausibility of using the PI-to-INS area ratio as a discriminator for disease status, a logistic regression was fitted to the data using R version 3.3.0 (9).

The scores for this model were calculated as the distance from each point (plotted using their percentage of proinsulin and insulin area as coordinates) to the line proinsulin = insulin (i.e., $y = x$). Briefly:

$$score = \frac{Insulin - Proinsulin}{\sqrt{2}}$$

The significance of the overall model was calculated by comparing it with a model with just the intercept (i.e., a null model). Besides the logistic regression, a receiver operating characteristic curve analysis using the R package ROCR version 1.0-7 (10) was performed to investigate the predictive power of the model.

Statistical analysis was performed using GraphPad Prism version 6 (GraphPad Software, La Jolla, CA). Data in graphs and tables are presented as mean ± SD unless otherwise indicated. Findings were assumed statistically significant at $P \leq 0.05$.

RESULTS

Proinsulin Area Is Significantly Increased in Ab+ Donor Islets Compared With Islets From Control Donors Without Diabetes, Whereas Insulin Area Remains Similar

We systematically measured insulin, proinsulin, and glucagon staining from the head, body, and tail regions of pancreatic tissue sections. Interestingly, there was a small increase in total insulin area in Ab+ donors that did not reach statistical significance (Fig. 1*A–C*, left panel). Conversely, a significant increase in the proinsulin area was observed in the head (54%, $P = 0.0233$), body (57%, $P = 0.0143$), and tail (60%, $P = 0.0087$) regions in Ab+ individuals compared with control sections (Fig. 1*A–C*, central panel). Lastly, there were no major differences in glucagon area between both groups for any of the regions (Fig. 1*A–C*, right panel). To better understand whether this was caused by a shift in the subcellular localization of proinsulin and/or by an increase in proinsulin content, a super high–resolution confocal microscope with Airyscan technology was used. Proinsulin in control donors was mainly localized close to the nucleus, with a staining pattern consistent with the Golgi apparatus, and was minimally present in other compartments. In multiple Ab+ donors, proinsulin was more widely localized to the juxtanuclear region (Golgi) and vesicular compartment, confirming a change in subcellular localization (Fig. 2 and Supplementary Fig. 2).

β-Cell Mass Is Not Reduced in Single or Double Ab+ Donors

The combination of insulin area from the head, body, and tail sections of the pancreas, normalized by the size of the respective tissues, was multiplied by the total weight of the pancreas. Mean β-cell mass was almost identical in control

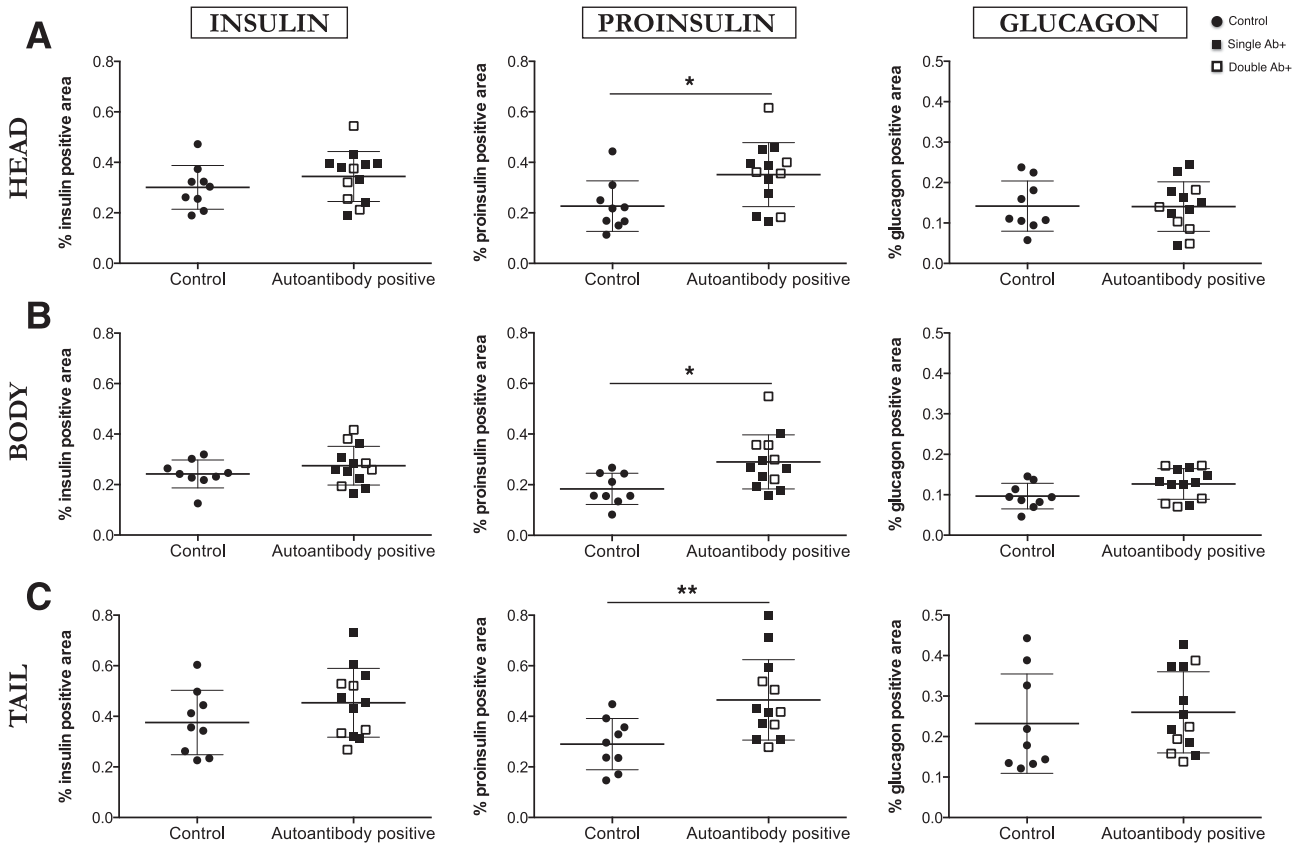

Figure 1—Proinsulin area but not insulin area is significantly increased in the pancreas of Ab+ donors compared with control donors without diabetes. Insulin (left panel), proinsulin (middle panel), and glucagon (right panel) areas expressed as percentage of positive area were measured in whole-tissue sections from the head (*A*), body (*B*), and tail (*C*) region of the pancreas obtained from control donors without diabetes ($n = 9$) and from single Ab+ ($n = 8$) and double Ab+ ($n = 5$) cadaveric organ donors without diabetes. *$P \leq 0.05$; **$P \leq 0.01$.

and Ab+ donors (245.0 ± 60.5 mg control donors vs. 267.7 ± 80.2 mg Ab+) (Fig. 3*A*). However, when proinsulin area was used as reference instead of insulin, a significant increase in β-cell mass in the Ab+ donor group was seen (187.4 ± 49.7 mg control donors vs. 266.9 ± 99.7 mg Ab+) (Fig. 3*B*). Lastly, α-cell mass, calculated as the adjusted percentage of glucagon area multiplied by the total weight of the pancreas, was similar for Ab+ and control groups (121.5 ± 42.6 mg control donors vs. 141.2 ± 65.7 mg Ab+) (Fig. 3*C*). β-Cell mass did not correlate with age ($r = -0.018$; $P = 0.9341$), BMI ($r = 0.377$; $P = 0.0833$), or time in the intensive care unit ($r = -0.056$; $P = 0.8250$) (data not shown).

The PI-to-INS Area Ratio Is Increased in Ab+ Donors and Constitutes a Potential Indicator of β-Cell Dysfunction

To study the direct relation between insulin and proinsulin area in the pancreas, the PI-to-INS area ratio was calculated for each section, region, and donor. Interestingly, the ratio was increased for Ab+ donors compared with control donors for the head (38%; $P = 0.0031$), body (40%; $P = 0.0005$), and tail (32%; $P = 0.0004$) regions of the pancreas (Fig. 4*A*). To evaluate the use of the area ratio as a potential indicator of β-cell dysfunction and to directly compare Ab+ donors with control donors, an arbitrary reference value of 1:1 (proinsulin area–to–insulin area) was chosen and graphically represented as a line to separate control donors from "at-risk" donors. The distance from the donor's area values to the line was used as a score to estimate the risk of disease and to classify and distinguish control donors from Ab+ donors (Fig. 4*B*). Then, a logistic regression was performed, and the area under the curve (AUC) was calculated (Fig. 4*C*). High AUC values, significant coefficients, and a good model fit were obtained for the head (AUC 0.85, coefficient $P = 0.0348$, model $P = 0.00225$), body (AUC 0.87, coefficient $P = 0.0213$, model $P = 0.00073$), and tail (AUC 0.9, coefficient $P = 0.0196$, model $P = 0.00045$), indicating that the pancreatic PI-to-INS area ratio could identify individuals at risk for developing disease.

To see whether the insulin area, the proinsulin area, or the PI-to-INS area ratio could correlate with the risk of developing type 1 diabetes, a risk index was calculated for all of the donors based on their age, HLA, and autoantibody status (Supplementary Fig. 3). To calculate the risk index, a score of 0 (low risk), 1 (medium risk), or 2 (high risk) was assigned as follows based on the risk of developing type 1 diabetes:

- Age: >40 (0), 30–40 (1), 0–30 (2).
- HLA: no risk alleles (0), DR4 or DR3 only (1), DR4 DQ8 or DR3 DQ2 or DQ8 (2).
- Autoantibodies: 0 (0), 1 (1), 2 (2).

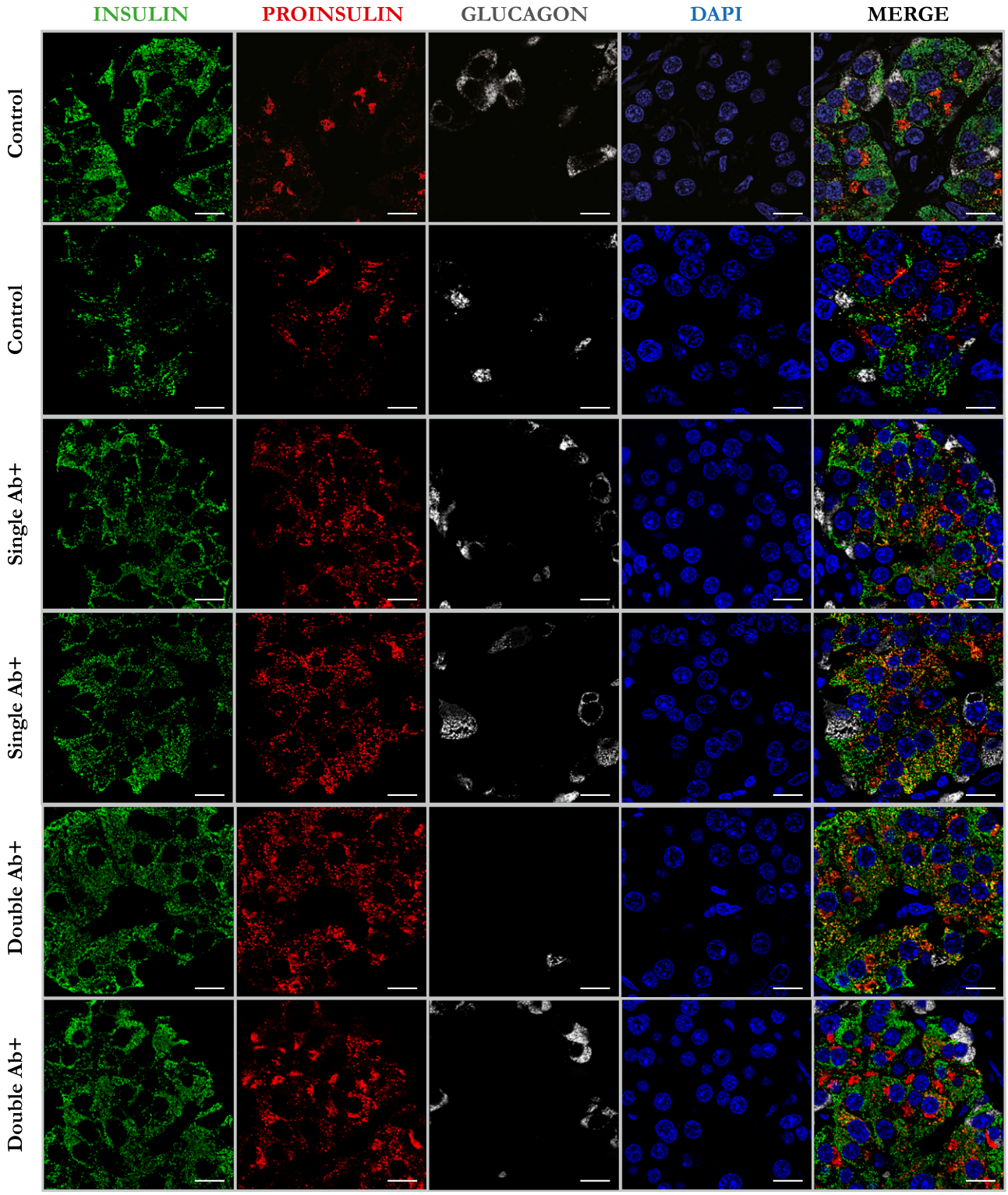

Figure 2—Proinsulin accumulates in the cytoplasmic compartment in β-cells from Ab+ donors. Pancreatic sections from control (upper rows), single Ab+ (middle rows), and double Ab+ (lower rows) cadaveric organ donors without diabetes were stained for insulin (green), proinsulin (red), glucagon (white), and DAPI (blue) after a standard immunofluorescence staining protocol. The merged image can be seen on the right panel. Images were taken using a Zeiss LSM 880 confocal microscope with Airyscan and a ×63 objective. Scale bar: 10 μm.

The risk index was then calculated as the sum of the values obtained in each category for each donor (range 0 [minimum] to 6 [maximum]). A strong positive correlation was found between the risk index and the PI-to-INS area ratio (Supplementary Fig. 3, right panel), but there was a weak correlation with proinsulin area

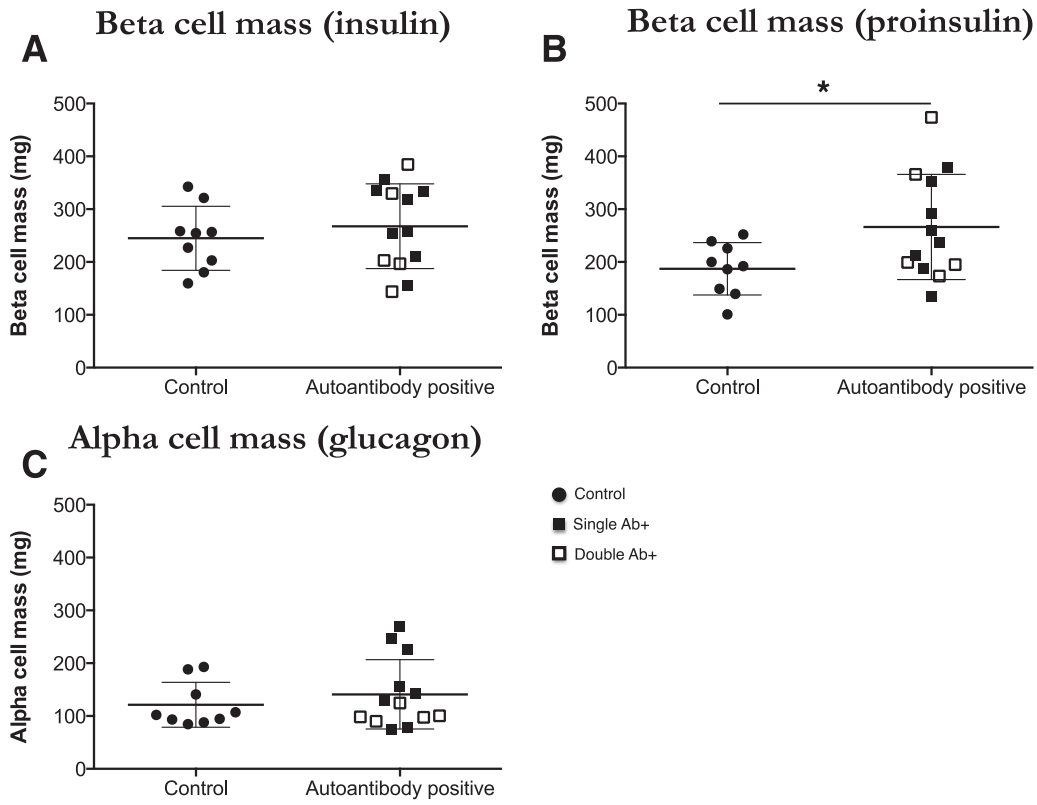

Figure 3—β-Cell mass is not reduced in single or double Ab+ donors. β-Cell mass was calculated as total pancreas weight multiplied by insulin area (*A*) and as total pancreas weight multiplied by proinsulin area (*B*), and α-cell mass was calculated as total pancreas weight multiplied by glucagon area (*C*) in control donors without diabetes (n = 9) and in single Ab+ (n = 8) and double Ab+ (n = 5) donors without diabetes. *$P \leq 0.05$.

(central panel) and no correlation with insulin area (left panel).

Patients With Recent-Onset Type 1 Diabetes Have More Glucagon, Less Insulin, and Less Proinsulin Area but Increased PI-to-INS Area Ratio

To investigate the timing of the increase in proinsulin area and the inversion of the PI-to-INS area ratio when compared with control donors, pancreas tissue from a set of recently diagnosed patients with type 1 diabetes (0–9 weeks postdiagnosis) was studied (Fig. 5). Only the tail region was analyzed, because all samples but one were obtained by tail resection, as previously described (30). (One section from a donor with type 1 diabetes at onset obtained from nPOD was also included for comparison.) Patients with type 1 diabetes were compared with control donors, and single Ab+ and double Ab+ donors separately (Fig. 5*A*). The insulin and proinsulin areas were lower than in the rest of the groups owing to a reduction in the islets that contained insulin, whereas glucagon area was increased (Fig. 5*C*, *D*, and *F*). Interestingly, the PI-to-INS area ratio was increased in patients with type 1 diabetes and almost identical to that of at-risk double Ab+ donors (0.77 ± 0.13 control donors vs. 0.99 ± 0.15 single Ab+ vs. 1.06 ± 0.10 double Ab+ vs. 1.07 ± 0.18 type 1 diabetes) (Fig. 5*E*, left panel).

Systematic Analysis of Islet Size Reveals Heterogeneity Within the Pancreas and Subtle Differences Between Ab+ Donors and Control Donors

Small morphological alterations as well as in differences in islet number, size, and distribution might occur many years before diagnosis in individuals at risk for developing disease. First, the number of islets was counted, and the total area of the tissue section was measured (see RESEARCH DESIGN AND METHODS for details and Supplementary Fig. 1). Total islet number and tissue size were variable across the pancreas but were similar in control donors and Ab+ donors (data not shown). There were no major differences in mean islet density between donors without diabetes (head 2.5 ± 0.5 vs. body 1.8 ± 0.2 vs. tail 2.8 ± 0.7 islets/mm^2) and Ab+ donors (head 2.6 ± 0.7 vs. body 2.1 ± 0.7 vs. tail 3.1 ± 0.9 islets/mm^2) (Fig. 6*A*). Next, the islet size distribution was analyzed. Significant differences were found between control donors without diabetes and double Ab+ donors for the head, body, and tail (Fig. 6*B* and Supplementary Fig. 4). Lastly, sections from patients with type 1 diabetes were analyzed. Larger islets were found in these sections (Fig. 5*A* and Supplementary Fig. 4, tail region only), but the islet density was lower (Fig. 5*B*), as expected. Interestingly, the tail of the pancreas presented a distinct islet distribution compared with the head and

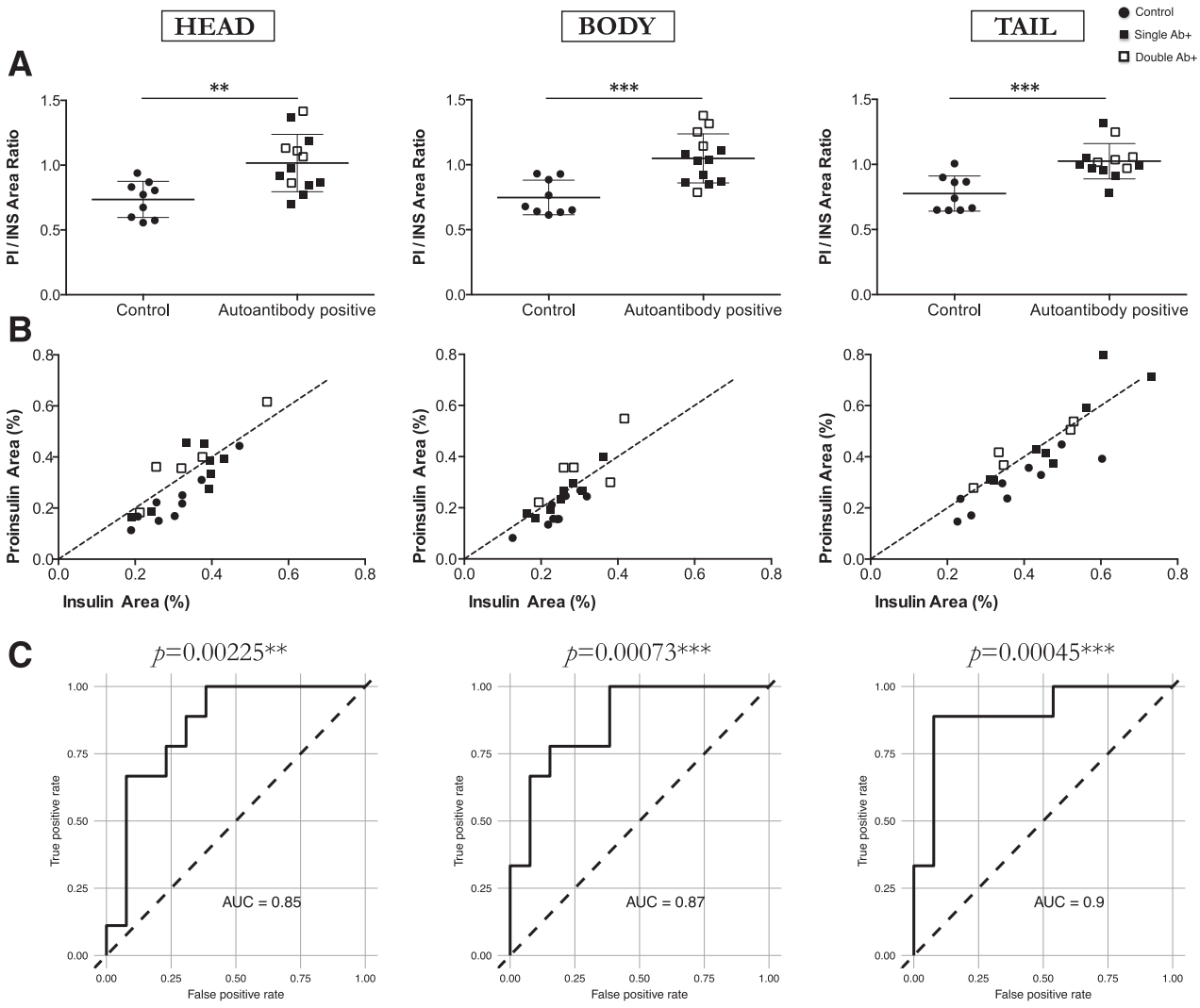

Figure 4—The PI-to-INS area ratio is increased in Ab+ donors and constitutes a potential indicator of β-cell dysfunction. *A*: The PI-to-INS area ratio was calculated for head (left panel), body (middle panel), and tail (right panel) regions of the pancreas from control donors (n = 9) and single Ab+ (n = 8) and double Ab+ (n = 5) cadaveric organ donors without diabetes. *B*: Proinsulin area vs. insulin area *xy* plot: a theoretical reference value of 1:1 (proinsulin-to-insulin) was chosen and graphically represented as a line capable of separating control from "at-risk" donors. The area under this line represents a ratio smaller than 1 and vice versa. The distance from the donor's area values to the line was used as a score to estimate the risk of developing disease and to classify and distinguish control donors from Ab+ donors (single and double combined). *C*: Receiver operating characteristic curve for the head (left panel), body (middle panel), and tail (right panel) regions of the pancreas. The AUC was calculated for the classifier described in panel *B*. The *P* values show the significance of the logistic regression model, including the predictor, when compared with a model with just the intercept. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

body regions for all groups, with a predominance of large islets and fewer small islets (Fig. 6*C* and Supplementary Fig. 4), confirming important regional differences within the pancreas.

Changes in the Number of α- and β-Cells Occur During the Prediabetic Phase and After Onset of Disease

The number of insulin- and glucagon-expressing cells per islet was counted, and the ratio between both cell populations was calculated (Supplementary Fig. 5*A*). Although no significant differences were found, double Ab+ donors presented higher β-cell–to–α-cell ratios in the head, body, and tail regions (head median = 3.8 control vs. 5.3 single Ab+ vs. 6.4 double Ab+/body median = 4.0 control vs. 3.8 single Ab+ vs. 7.1 double Ab+/tail median = 3.6 control vs. 3.8 single Ab+ vs. 4.9 double Ab+). Very heterogeneous cell distribution patterns were found among the Ab+ donors, with some individuals having a higher abundance of α-cells per islet (Supplementary Fig. 5*B*, central panel) and others with a clear predominance of β-cells (Supplementary Fig. 5*B*, right panel) compared with control sections (Supplementary Fig. 5*B*, left panel).

Patients with type 1 diabetes had a significantly lower β-cell–to–α-cell ratio (median = 1.1 type 1 diabetes, tail region only) (Fig. 5*E*, right panel). Lastly, the percentage of islets containing only β-cells was calculated, which

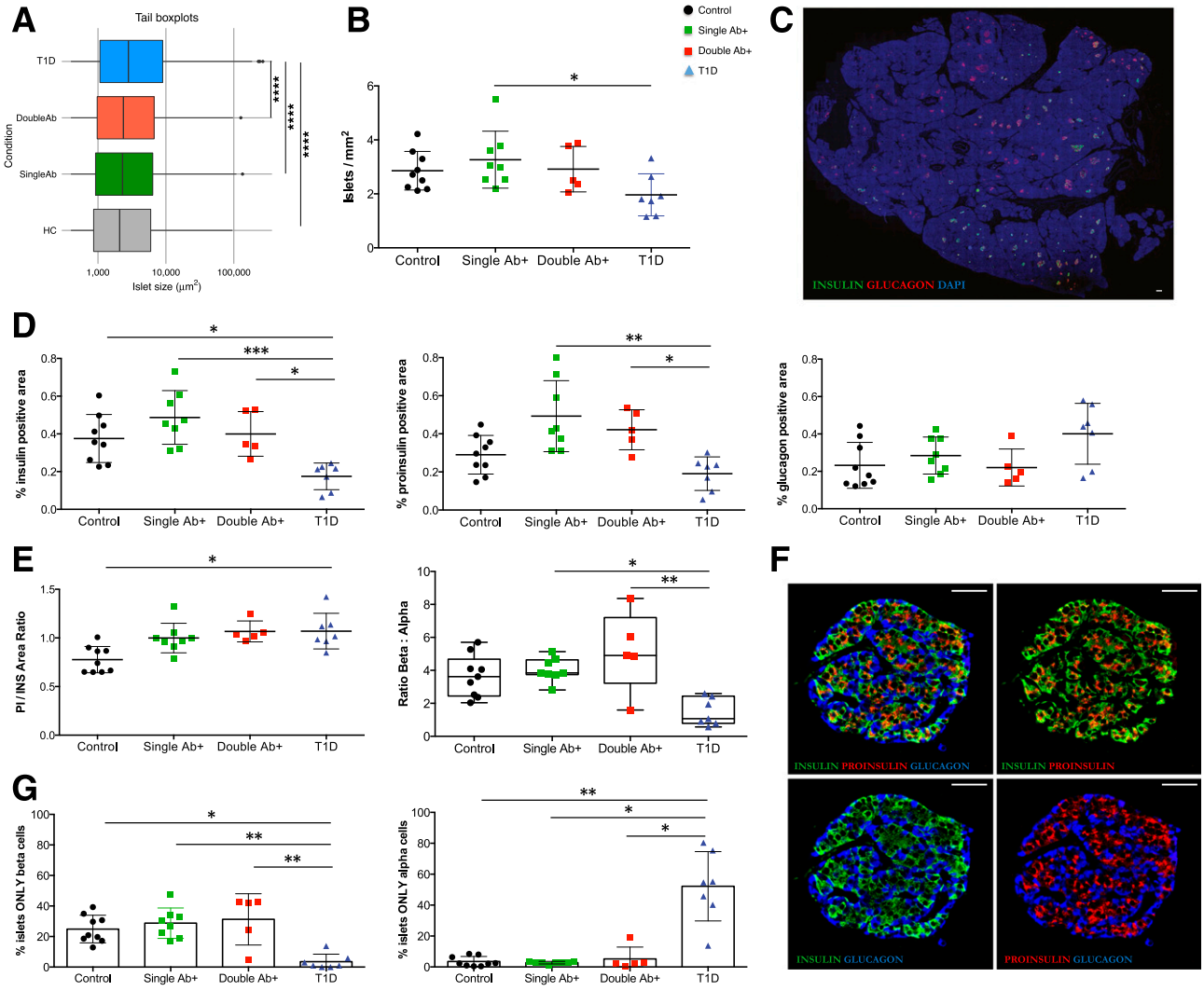

Figure 5—Higher glucagon and lower insulin and proinsulin areas but increased PI-to-INS area ratio in patients with recent-onset type 1 diabetes. *A*: Box plots represent islet size distribution for control donors without diabetes (HC, *n* = 5,109), single Ab+ (SingleAb, *n* = 4,692) and double Ab+ (DoubleAb, *n* = 2,859) donors, and donors with type 1 diabetes (T1D, *n* = 1,748) in the tail region of the pancreas. *B*: Islet density was calculated as the total number of islets per section divided by the total area of the tissue for the tail region of the pancreas. *C*: Representative image from whole-tissue section of donor #6362, with type 1 diabetes, at onset. Insulin is shown in green, glucagon in red, and DAPI in blue. Note the presence of insulin-deficient and insulin-containing islets scattered across the pancreas parenchyma. Scale bar: 500 μm. *D*: Insulin (left panel), proinsulin (middle panel), and glucagon (right panel) areas expressed as a percentage of the positive area were measured in whole-tissue sections from the tail region of the pancreas. *E*: The PI-to-INS area ratio (left panel) and the β-cell–to–α-cell ratio (right panel) were calculated for the pancreas tail region. *F*: Representative image of an islet from a recent-onset donor (DiViD study). Insulin is shown in green, proinsulin in red, and glucagon in blue. Scale bar: 50 μm. *G*: The percentage of islets containing only β-cells (left panel) and only α-cells (right panel) is shown. All panels: control donors without diabetes (*n* = 9), single Ab+ (*n* = 8) and double Ab+ (*n* = 5) donors without diabetes, and donors with type 1 diabetes (*n* = 7). *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$.

again was similar for the control (24.9% ± 9.0), single (28.8% ± 9.9), and double Ab+ group (31.3% ± 16.8) and lower for the group with type 1 diabetes (3.5% ± 4.9) (Fig. 5*G*, left panel). Islets with only α-cells were not common (control 3.5% ± 3.2 vs. single Ab+ 2.8% ± 0.8 vs. double Ab+ 5.2% ± 7.7). Only one double Ab+ donor (#6267, 18.9%) and the donors with type 1 diabetes (52.2% ± 22.4) presented evident increases in the percentage of islets containing only α-cells compared with the rest of the donor groups (Fig. 5*G*, right panel).

DISCUSSION

The recent access to human pancreata for research purposes and the subsequent histological studies have filled important gaps in our understanding of pancreatic pathology (10,31–33). However, many fundamental questions remain to be answered. In this study, we aimed to fully characterize β- and α-cells, investigating insulin, proinsulin, and glucagon content and distribution across the head, body, and tail regions of the human pancreas in healthy individuals as well as in single Ab+ and multiple

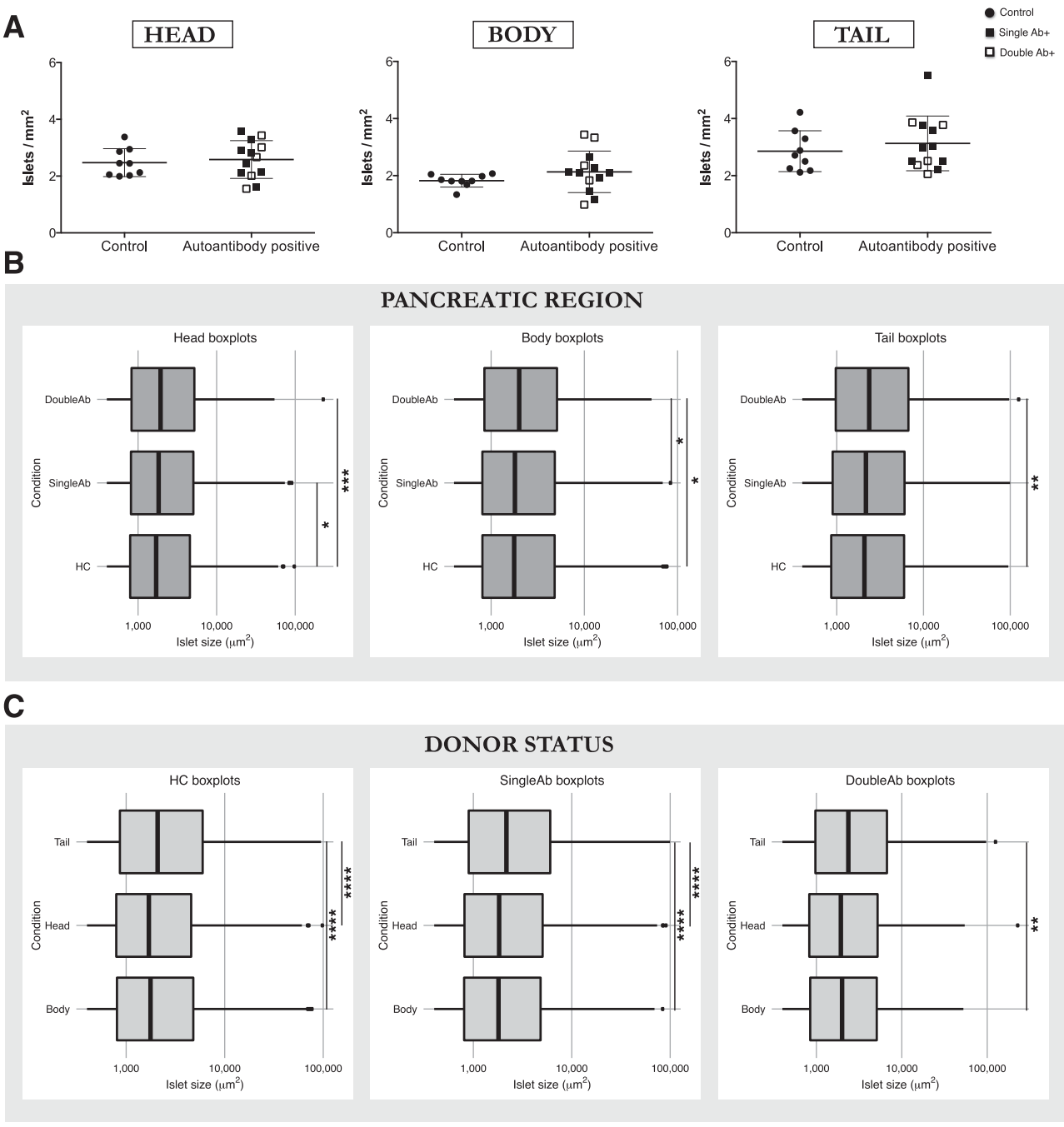

Figure 6—Systematic analysis of islet distribution reveals heterogeneity within the pancreas and subtle differences between Ab+ donors and control donors. *A*: Islet density was calculated as the total number of islets per section divided by the total area of the tissue for the head, body, and tail regions of the pancreas of control donors (n = 9), single Ab+ (SingleAb) donors (n = 8), and double Ab+ (DoubleAb) donors (n = 5). *B*: Box plots represent islet size distribution for healthy control (HC) donors, single Ab+ donors, and double Ab+ donors in the head (left panel), body (middle panel), and tail (right panel) region of the pancreas. *C*: Box plots represent islet size distribution for head, body, and tail regions in HC donors (left panel), single Ab+ (middle panel) donors, and double Ab+ (right panel) donors. Number of islets: head (HC, n = 4,390; SingleAb, n = 4,021; DoubleAb, n = 2,067), body (HC, n = 3,446; SingleAb, n = 4,052; DoubleAb, n = 2,043), and tail (HC, n = 5,109; SingleAb, n = 4,692; DoubleAb, n = 2859). *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$.

Ab+ donors. In addition, we had access to a very unique subset of pancreatic tissues from living patients with recent-onset type 1 diabetes who participated in the DiViD study in which a small piece of the tail of the pancreas was surgically removed immediately in the months after diagnosis (30). Our study is the first to show an increase in pancreatic proinsulin area in individuals with prediabetes and confirms the value of the PI-to-INS area ratio as an indicator of early β-cell dysfunction. In addition, we confirm previous findings that β-cell mass is not reduced in Ab+ individuals. We

therefore add critical refinement to the original model of β-cell loss described by Eisenbarth (4) three decades ago. We did not find any significant differences in the overall insulin-positive area between healthy control donors and Ab+ donors to support a reduction in insulin content long before the onset of disease. Moreover, β-cell mass was essentially identical in both groups, demonstrating that β-cells are preserved in Ab+ individuals until shortly before diagnosis and in agreement with previous publications that reported no differences in β-cell mass between donors without diabetes and Ab+ donors (34–36).

Increases in the proinsulin area were found in some of the single Ab+ pancreata, which could explain why the first-phase insulin response is already abnormal in some of these individuals (4). Proinsulin primarily accumulates in the Golgi apparatus in resting β-cells and is further processed to insulin and C-peptide in immature secretory granules (37). Our data show significant increases in the proinsulin area in Ab+ individuals in the head, body, and tail regions of the pancreas. Using a high-resolution confocal microscope to study a subset of samples, we observed that the increase in the proinsulin area was partially caused by a shift in proinsulin subcellular localization from the Golgi area in control individuals (juxtanuclear) to secretory vesicles in multiple Ab+ individuals (cytosolic). Although an increase in proinsulin area suggests an increase in the amount of proinsulin protein, additional experiments are needed to accurately measure the protein content. Nevertheless, this increase in area could be caused by an increase in proinsulin production (to cover insulin demand) or by a defect in the processing and maturation of the existing pool of proinsulin to insulin, which is consequently released to the circulation. This is in agreement with studies that have detected proinsulinemia in patients with prediabetes and in patients with type 1 diabetes (38). In addition, an increase in proinsulin synthesis could lead to ER stress, protein misfolding, and loss of glucose-stimulated insulin secretion (39). Other extrinsic factors, for example, recurrent autoimmune attacks or other forms of metabolic stress (40,41), may affect β-cell function and proinsulin processing early in the disease process of prediabetes. In normal β-cells, up to 20% of proinsulin can be misfolded. However, only under pathological conditions and ER dysfunction, and once a certain level of misfolded proinsulin has been accumulated, do β-cell toxicity and death occur (22).

In a case-control study analysis by Sims et al. (42), an elevation of the PI-to-C ratio preceded disease onset in high-risk subjects and could be detected at least 12 months before diagnosis. This is in agreement with the data presented here, in which the pancreatic PI-to-INS area ratio was increased in Ab+ individuals. Among the single Ab+ group, two donors (#6170 and #6184) consistently presented an elevated PI-to-INS area ratio. This suggests that β-cells in these two individuals could have had a functional defect. Conversely, all but one double Ab+ donor (#6080) presented an increased ratio in at least two of the three regions of the pancreas, supporting the notion of an early functional defect in β-cells during the prediabetic phase and before diagnosis that does not necessarily imply β-cell loss. Donor #6080 was positive for GAD and insulin Abs but was 69 years old and therefore unlikely to have developed the disease. In conclusion, our in situ results correlate well with those found in serum and confirm the potential of monitoring PI-to-C or PI-to-INS area ratios as indicators of β-cell dysfunction.

The samples obtained from living individuals with recent-onset type 1 diabetes through the DiViD study (30) showed an expected significant decrease in the insulin and proinsulin area resulting from a reduced number of insulin-containing islets; however, PI-to-INS area ratios were elevated in four of six patients, suggesting that insulin therapy at onset might not fully alleviate the dysfunctional β-cells. Our findings indicate that potential therapies should target β-cells early before onset, when they still have the ability to be functionally rescued and when the immune system has not been fully activated.

We further characterized islet distribution and composition to study whether small differences in islet pathology could be observed early in the prediabetic phase. The tail of the pancreas contained larger islets and higher islet density in all donor groups, which could point to important developmental and architectural differences in vascularization and innervation of the islets in this region (43,44). Interestingly, subtle differences were found in double Ab+ at-risk individuals, in whom larger islets were found in the body and tail region of the pancreas compared with control individuals. Moreover, this tendency was accentuated in individuals with recently diagnosed type 1 diabetes, where even larger islets could be found in the tail region, many of them containing only α-cells. This is in agreement with previous studies in diabetic NOD mice in which small islets were preferentially lost and a subsequent expansion of large islets was seen (45). An increase in the size of the islet and number of β-cells in multiple Ab+ donors could be a compensatory mechanism caused by the chronic increase in insulin demand.

Our observations in the pancreas of individuals at risk for developing type 1 diabetes point to important and very early (at the first sign of autoimmunity) pathological changes in β-cells without an evident loss of β-cell mass. This confirms the potential benefit of estimating the PI-to-C or PI-to-INS area ratios in Ab+ individuals to identify those patients at high risk at a stage where β-cells might still respond to preventive therapies and to enroll patients in clinical trials at a point in the disease when they would benefit the most. Whether a consequence of an increase in insulin demand, a primary cellular defect, or a change in the orchestrated interplay between the immune system and the islet, the higher accumulation of proinsulin in β-cells without a reduction in insulin content might ultimately lead to β-cell exhaustion and death, with the subsequent release of β-cell antigens that initiate the autoimmune process. Whether type 1 diabetes is a primary autoimmune disease or autoimmunity is secondary to metabolic or functional defects that render β-cells susceptible to autoimmune destruction remains unknown.

Future studies on proinsulin and insulin dynamics as well as a better characterization of β-cells themselves will provide the necessary answers to fully understand the pathological changes that precede the clinical onset of type 1 diabetes.

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References

1. Pugliese A. The multiple origins of type 1 diabetes. Diabet Med 2013;30:135–146
2. van Belle TL, Coppieters KT, von Herrath MG. Type 1 diabetes: etiology, immunology, and therapeutic strategies. Physiol Rev 2011;91:79–118
3. Srikanta S, Ganda OP, Gleason RE, Jackson RA, Soeldner JS, Eisenbarth GS. Pre-type I diabetes. Linear loss of beta cell response to intravenous glucose. Diabetes 1984;33:717–720
4. Eisenbarth GS. Type I diabetes mellitus. A chronic autoimmune disease. N Engl J Med 1986;314:1360–1368
5. Sosenko JM, Skyler JS, Herold KC, Palmer JP; Type 1 Diabetes TrialNet and Diabetes Prevention Trial–Type 1 Study Groups. The metabolic progression to type 1 diabetes as indicated by serial oral glucose tolerance testing in the Diabetes Prevention Trial–Type 1. Diabetes 2012;61:1331–1337
6. Helminen O, Aspholm S, Pokka T, et al. HbA1c predicts time to diagnosis of type 1 diabetes in children at risk. Diabetes 2015;64:1719–1727
7. Krogvold L, Skog O, Sundström G, et al. Function of isolated pancreatic islets from patients at onset of type 1 diabetes: insulin secretion can be restored after some days in a nondiabetogenic environment in vitro: results from the DiViD Study. Diabetes 2015;64:2506–2512
8. Marré ML, James EA, Piganelli JD. β cell ER stress and the implications for immunogenicity in type 1 diabetes. Front Cell Dev Biol 2015;3:67
9. Eizirik DL, Cnop M. ER stress in pancreatic beta cells: the thin red line between adaptation and failure. Sci Signal 2010;3:pe7
10. Campbell-Thompson M, Fu A, Kaddis JS, et al. Insulitis and β-cell mass in the natural history of type 1 diabetes. Diabetes 2016;65:719–731
11. Ziegler AG, Rewers M, Simell O, et al. Seroconversion to multiple islet autoantibodies and risk of progression to diabetes in children. JAMA 2013;309:2473–2479
12. Bonifacio E. Predicting type 1 diabetes using biomarkers. Diabetes Care 2015;38:989–996
13. Narendran P, Mannering SI, Harrison LC. Proinsulin-a pathogenic autoantigen in type 1 diabetes. Autoimmun Rev 2003;2:204–210
14. Thayer TC, Pearson JA, De Leenheer E, et al. Peripheral proinsulin expression controls low-avidity proinsulin-reactive CD8 T cells in type 1 diabetes. Diabetes 2016;65:3429–3439
15. Pearson JA, Thayer TC, McLaren JE, et al. Proinsulin expression shapes the TCR repertoire but fails to control the development of low-avidity insulin-reactive CD8+ T cells. Diabetes 2016;65:1679–1689
16. Toma A, Laïka T, Haddouk S, et al. Recognition of human proinsulin leader sequence by class I-restricted T-cells in HLA-A*0201 transgenic mice and in human type 1 diabetes. Diabetes 2009;58:394–402
17. Toma A, Haddouk S, Briand JP, et al. Recognition of a subregion of human proinsulin by class I-restricted T cells in type 1 diabetic patients. Proc Natl Acad Sci U S A 2005;102:10581–10586
18. Mallone R, Martinuzzi E, Blancou P, et al. CD8+ T-cell responses identify beta-cell autoimmunity in human type 1 diabetes. Diabetes 2007;56:613–621
19. Michels AW, Landry LG, McDaniel KA, et al. Islet-derived CD4 T-cells targeting proinsulin in human autoimmune diabetes. Diabetes 2017;66:722–734
20. Babon JA, DeNicola ME, Blodgett DM, et al. Analysis of self-antigen specificity of islet-infiltrating T cells from human donors with type 1 diabetes. Nat Med 2016;22:1482–1487
21. Liu M, Wright J, Guo H, Xiong Y, Arvan P. Chapter Two – proinsulin entry and transit through the endoplasmic reticulum in pancreatic beta cells. Vitam Horm 2014;95:35–62
22. Sun J, Cui J, He Q, Chen Z, Arvan P, Liu M. Proinsulin misfolding and endoplasmic reticulum stress during the development and progression of diabetes. Mol Aspects Med 2015;42:105–118
23. Op de Beeck A, Eizirik DL. Viral infections in type 1 diabetes mellitus–why the β cells? Nat Rev Endocrinol 2016;12:263–273
24. Krogvold L, Edwin B, Buanes T, et al. Detection of a low-grade enteroviral infection in the islets of Langerhans of living patients newly diagnosed with type 1 diabetes. Diabetes 2015;64:1682–1687
25. Cianciaruso C, Phelps EA, Pasquier M, et al. Primary human and rat beta -cells release the intracellular autoantigens GAD65, IA-2, and proinsulin in exosomes together with cytokine-induced enhancers of immunity. Diabetes 2017;66:460–473
26. Røder ME, Knip M, Hartling SG, Karjalainen J, Akerblom HK, Binder C; The Childhood Diabetes in Finland Study Group. Disproportionately elevated proinsulin levels precede the onset of insulin-dependent diabetes mellitus in siblings with low first phase insulin responses. J Clin Endocrinol Metab 1994;79:1570–1575
27. Hartling SG, Knip M, Roder ME, Dinesen B, Akerblom HK, Binder C; Study Group on Childhood Diabetes in Finland. Longitudinal study of fasting proinsulin in 148 siblings of patients with insulin-dependent diabetes mellitus. Eur J Endocrinol 1997;137:490–494
28. Truyen I, De Pauw P, Jørgensen PN, et al.; Belgian Diabetes Registry. Proinsulin levels and the proinsulin:c-peptide ratio complement autoantibody measurement for predicting type 1 diabetes. Diabetologia 2005;48:2322–2329
29. Watkins RA, Evans-Molina C, Terrell JK, et al. Proinsulin and heat shock protein 90 as biomarkers of beta-cell stress in the early period after onset of type 1 diabetes. Transl Res 2016;168:96–106.e101

30. Krogvold L, Edwin B, Buanes T, et al. Pancreatic biopsy by minimal tail resection in live adult patients at the onset of type 1 diabetes: experiences from the DiViD study. Diabetologia 2014;57:841–843
31. Kaddis JS, Pugliese A, Atkinson MA. A run on the biobank: what have we learned about type 1 diabetes from the nPOD tissue repository? Curr Opin Endocrinol Diabetes Obes 2015;22:290–295
32. Pugliese A, Vendrame F, Reijonen H, Atkinson MA, Campbell-Thompson M, Burke GW. New insight on human type 1 diabetes biology: nPOD and nPOD-transplantation. Curr Diab Rep 2014;14:530
33. Campbell-Thompson M. Organ donor specimens: what can they tell us about type 1 diabetes? Pediatr Diabetes 2015;16:320–330
34. Wagner R, McNally JM, Bonifacio E, et al. Lack of immunohistological changes in the islets of nondiabetic, autoimmune, polyendocrine patients with beta-selective GAD-specific islet cell antibodies. Diabetes 1994;43:851–856
35. In't Veld P, Lievens D, De Grijse J, et al. Screening for insulitis in adult autoantibody-positive organ donors. Diabetes 2007;56:2400–2404
36. Diedisheim M, Mallone R, Boitard C, Larger E. β-cell mass in nondiabetic autoantibody-positive subjects: an analysis based on the Network for Pancreatic Organ Donors Database. J Clin Endocrinol Metab 2016;101:1390–1397
37. Haataja L, Snapp E, Wright J, et al. Proinsulin intermolecular interactions during secretory trafficking in pancreatic β cells. J Biol Chem 2013;288:1896–1906
38. Hostens K, Ling Z, Van Schravendijk C, Pipeleers D. Prolonged exposure of human beta-cells to high glucose increases their release of proinsulin during acute stimulation with glucose or arginine. J Clin Endocrinol Metab 1999;84:1386–1390
39. Wang S, Kaufman RJ. The impact of the unfolded protein response on human disease. J Cell Biol 2012;197:857–867
40. Oresic M, Simell S, Sysi-Aho M, et al. Dysregulation of lipid and amino acid metabolism precedes islet autoimmunity in children who later progress to type 1 diabetes. J Exp Med 2008;205:2975–2984
41. Sysi-Aho M, Ermolov A, Gopalacharyulu PV, et al. Metabolic regulation in progression to autoimmune diabetes. PLoS Comput Biol 2011;7:e1002257
42. Sims EK, Chaudhry Z, Watkins R, et al. Elevations in the fasting serum proinsulin–to–C-peptide ratio precede the onset of type 1 diabetes. Diabetes Care 2016;39:1519–1526
43. Amella C, Cappello F, Kahl P, Fritsch H, Lozanoff S, Sergi C. Spatial and temporal dynamics of innervation during the development of fetal human pancreas. Neuroscience 2008;154:1477–1487
44. Lammert E, Cleaver O, Melton D. Induction of pancreatic differentiation by signals from blood vessels. Science 2001;294:564–567
45. Alanentalo T, Hörnblad A, Mayans S, et al. Quantification and three-dimensional imaging of the insulitis-induced destruction of β-cells in murine type 1 diabetes. Diabetes 2010;59:1756–1764