β-cell Mass in Nondiabetic Autoantibody-Positive Subjects: An Analysis Based on the Network for Pancreatic Organ Donors Database

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Context: Little information is available about β-cell mass in antibody-positive (Ab+) nondiabetic subjects.

Objective: We have investigated whether the publicly available virtual slides of the Network for Pancreatic Organ Donors with Diabetes (nPOD) project can be used to assess β-cell mass and distribution in nondiabetic antibody-negative (Ab−) and antibody-positive (Ab+) subjects and in patients with recent-onset type 1 diabetes (T1D).

Subjects and Methods: We developed a semi-automated quantification method and applied it to 415 insulin-stained slides from 69 Ab− subjects, 101 slides from 18 Ab+ subjects, and 46 slides from eight recent-onset (<3 y) T1D subjects. Among these subjects, 48, 17, and seven had an available pancreatic mass, respectively, and were used for the quantification of β-cell mass.

Results: In Ab− subjects, the β-cell and endocrine mass were 0.66 ± 0.42 and 1.0 ± 0.65 g, respectively. Nonexocrine tissue represented 29% of pancreatic area, a proportion that increased with age. Proportional β-cell area relative to total pancreatic area was higher in the tail compared with head (0.83 vs 0.71%; P < .001). In Ab+ subjects, β-cell mass and β-cell area were similar to those of Ab− individuals, whereas these parameters were dramatically decreased in recent-onset T1D patients.

Conclusion: The virtual slides of the nPOD project can be used for quantification projects. In Ab+ nondiabetic subjects, the β-cell mass was not decreased. However, as this cohort is largely composed of donors from the general population, with a single autoantibody, future studies with a larger number of donors with multiple autoantibodies and predisposing human leucocyte antigen genes are required to better define the dynamics of β-cell destruction in the preclinical phases of T1D. (J Clin Endocrinol Metab 101: 1390–1397, 2016)

Analysis of pancreatic pathology in diabetes has been limited by the rare availability of pancreatic specimens. Thus, most data have been obtained from autopic material, with few rare exceptions (1–3). The Network for Pancreatic Organ Donors with Diabetes (nPOD) has been launched to provide access to high-quality pancreatic specimens for research purposes (4). Although initially created to promote research on type 1 diabetes (T1D), nPOD also collects pancreata from type 2 diabetes (T2D) and nondiabetic subjects. To date, the nPOD repository contains more than 300 pancreatic specimens, and high-magnification digital images (virtual slides) stained with several antibodies (Abs) are available on the nPOD website (www.jdrfnpod.org).

Abbreviations: Abs, antibodies; BMI, body mass index; HbA1c, glycated hemoglobin; nPOD, Network for Pancreatic Organ Donors with Diabetes; T1D, type 1 diabetes; T2D, type 2 diabetes.
Highlighting the difficulties in assessing the human diabetic pancreas, β-cell mass in T2D likewise varies, overlapping with that of normal individuals, although current data suggest a 20–60% reduction (5). Similarly, the question of the head-to-tail β-cell distribution remains debated given that the original study of E.L. Opie (6), with the tail reported as harboring more (7–10) or similar (2, 11, 12) β-cell numbers compared with the head.

Another important issue is raised by the pancreatic pathology of T1D. It is now well established that T1D is the late consequence of a β-cell-targeted autoimmune disease (13). However, the pancreas pathology is seldom available, except in rare cases of death at onset, and β-cell mass has been rarely quantified in recent-onset T1D patients (14–18). Moreover, there have been only three reports of pancreas pathology in normoglycemic Ab+ subjects (19–21), but the β-cell mass was not quantified in these studies. More recent reports using specimens from recent-onset cases available in the nPOD database have rather focused on inflammation (22, 23).

The purpose of this study was to investigate whether the nPOD virtual slides can be used to perform high-quality morphometric analyses. To this end, we developed a semi-automated quantification method and applied it to assess the β-cell mass in all pancreatic sections from nontobiotic (Ab+ and Ab−) and recent-onset T1D individuals available through the nPOD website. Although β-cell mass was reduced in T1D specimens, our results do not highlight significant differences in Ab+ vs Ab− nontobiotic subjects.

Design and Methods

Study subjects

We downloaded from the nPOD database all insulin-stained slides available as of July 2013 from nondiabetic Ab− brain-dead organ donors, ie, donors with normal glycated hemoglobin (HbA1c) (<6.5%) and no IA-2 or GAD Abs. We also downloaded insulin-stained slides of Ab+ nontobiotic subjects and of recent-onset (<3 y) T1D patients, available as of July 2014. The weight of the pancreas and clinical data were retrieved when available. The clinical data of 30 of 69 control Ab− subjects, 14 of 18 Ab+ nontobiotic subjects and of four of nine recent-onset T1D patients have been published elsewhere (22, 23) and are presented in Supplemental Table 1 with data from all the subjects included in the present report. The nPOD project has been approved by the University of Florida Health Center Institutional Review Board. The present study was approved by the Comité de Protection des Personnes Ile-de-France III and by the nPOD Tissue Prioritization Committee.

Pancreas sampling and processing

nPOD uses a standardized protocol for preparing pancreas specimens (4). Each pancreas is divided into three regions: the head, from the near-duodenal region up to the proximal isthmic region, located in front of the superior mesenteric vessels. Separation of the remaining pancreatic tissue into two equal parts defines the body and tail portions. Double immunohistochemistry staining was performed with Kit67 and insulin Abs (24) and revealed with peroxidase-diaminobenzidine and alkaline phosphatase-Fast Red polymer systems, respectively.

Virtual slides were analyzed at 100× magnification with a resolution of 1 μm/pixel. Depending on the number of slides available, we analyzed a median of 2.8 slides per region (range, 1–7). When several slides were available for the same region, mean results are reported.

Morphometric analysis

Morphometric analysis was carried out with a semiautomated quantification method, as described in Supplemental Methods and Supplemental Figure 1. This process allows analysis of the whole section. Lymph nodes (found in five slides from control Ab− subjects, 1%), and regions with edge artifacts or nonspecific staining were manually excluded. For each slide, we quantified the total tissue area and separated it into exocrine and nonexocrine tissue (adipose and mesenchymal tissues, large ducts, and vessels) vs the insulin-stained area. The percentage of the total tissue area was converted into mass by multiplying it by the weight of the pancreas when available. When the weight of the pancreas was not available, only the ratio of the endocrine area to the total area was calculated. We used Photoshop CS4 (Adobe Systems) to perform thresholding on whole sections, and measurements were performed using ImageJ (http://rsweb.nih.gov/ij/). Given that only the cytoplasm was stained for β-cells using the insulin Ab, we developed another Photoshop script to quantify the surface of the entire islet.

Statistical analysis

Data are expressed as mean ± SD when n is greater than 30 or median ± interquartile range when n is less than 30. Analyses were carried out using SigmaStat 3.5 (Systat Software,) and GraphPad Prism 6.02. Differences between groups were evaluated using nonparametric tests, namely Mann-Whitney U test for nonpaired analyses, Wilcoxon test for analysis of paired data, Kruskal-Wallis test for ANOVA, and Spearman rank test for correlations. Comparison of β-cell mass between Ab+ nontobiotic and Ab− subjects was made using a multiple logistic regression analysis that took into account the age of the subjects.

Results

Quantification of exocrine/endocrine and β-cell mass in control, Ab− subjects reveals higher β-cell mass as a function of age

Clinical characteristics of the studied subjects are presented in Table 1. As of July 2013, 496 slides from 95 nontobiotic Ab− individuals were available. We eliminated 49 slides corresponding to serial sections, 12 due to significant autolysis, two for significant nonspecific staining, and 18 from fetal pancreata (Table 2). Thus, 415 virtual slides from 69 individuals were analyzed: 232 from the head, 71 from the body, and 112 from the tail of the
pancreas. The islet area has been already quantified in 15 of 69 of these Ab− subjects (23). Also, for 48 individuals, slides from both head and tail of the pancreas were available.

The pancreas weight, which was available in 48 of 69 Ab− subjects, correlated significantly and independently with age ($r^2 = 0.76; P < .01$) and body mass index (BMI) ($r^2 = 0.56; P < .01$). Moreover, as expected (25), it increased during the first years of age and decreased after 40 years, while continuously increasing with BMI, but not significantly so when the analysis was restricted to subjects greater than 18 years old (Spearman $r = 0.396; P < .001$) and body mass index (BMI) ($r = -0.35; P < .005$) and age ($r = -0.40; P < .001$). When the nonexocrine fraction was studied by age group, the proportion of nonexocrine tissue was 24 ± 5% and stable from birth to 50 years, and increased to 47 ± 8% after 60 years (Figure 2C). The nonexocrine fraction was 32% higher in the head compared with the tail of the pancreas (33 ± 13 and 25 ± 4% respectively; $P < 10^{-4}$) (Figure 2D).

The nonexocrine tissue, because of its proportional importance and its uneven distribution throughout the pancreas, influenced the assessment of $\beta$-cell area. Thus, the $\beta$-cell ratio was higher in the tail than in the head when expressed as the proportion of $\beta$-cell area to tissue area ($P < .01; n = 48$). In contrast, when the $\beta$-cell ratio was expressed relative to exocrine tissue area, no difference was observed ($P = .28; n = 48$) (Figure 3).

Finally we analyzed the distribution of the islet size in the head and tail of the pancreas (Supplemental Figure 3). This distribution was skewed toward greater number of large islets in the tail: skewness, 5.6 ± 1.8 in the head compared with 4.4 ± 3.4 in the tail ($P = .02$).

**Quantification of the $\beta$-cell mass in Ab+ nondiabetic subjects**

Having established the validity of our morphometric analysis, we next analyze the same parameters in Ab+
subjects compared with Ab− subjects and recent-onset T1D patients.

As of July 2014, 192 slides from 20 Ab+ nondiabetic subjects and 122 slides from eight recent-onset T1D patients were available. We eliminated 28 slides corresponding to serial sections, 14 due to significant autolysis, four for significant nonspecific staining, and eight from fetal pancreas (Table 2), thus analyzing 18 Ab+ nondiabetic subjects and eight recent-onset T1D pa-

tients. We then randomly selected three slides from the head and three slides from the tail of the pancreas. The relative β-cell area was 0.04% [median and interquartile range, 0.01–0.06] and 0.67% [median and interquartile range, 0.40–0.75] in recent-onset T1D (n = 8) and Ab+ nondiabetic subjects (n = 18), respectively. The β-cell and is-

let mass were also calculated for the Ab+ and recent-onset T1D sub-
jects for whom the pancreas weight was available (n = 15 and 7, re-
respectively). As shown in Figure 4, the β-cell mass was close to zero in patients with recent-

onset T1D (P < .001 vs control subjects and P < .01 vs Ab+ subjects), whereas that of Ab+ subjects was un-

 distinguishable from Ab− subjects (P = .2), even after adjustment for age in a multiple logistic regression anal-

ysis. Finally, the β-cell mass of donors with single (n = 12) or with multiple autoantibodies (n = 3) did not differ (0.42 vs 0.58 g, respectively; P = .095).

Figure 1. Quantification of β-cell in healthy controls. A, β-cell mass in infants (< 2 y; n = 10), children (2–10 y; n = 10) and subjects > 10 y (n = 28) and (B) in lean (n = 14) and obese adults (n = 4). Bars represent median values with interquartile range. Statistical significance was calculated by ANOVA.

Figure 2. Quantification of nonexocrine tissue. A, Original image. B, Nonexocrine tissue highlighted in orange after digital processing. C, Percent nonexocrine area as a function of age (n = 69). Bars represent medians with interquartile range and data were analyzed with the Mann-Whitney U test. D, Percent nonexocrine area in the head and tail of the pancreas (n = 48). Bars represent means with SD and data were analyzed by Wilcoxon signed rank test.
Discussion

The main finding of our study was that the \( \beta \)-cell mass was not decreased in a large cohort of \( \text{Ab}^- \) nondiabetic subjects, whereas these parameters were, as expected, largely decreased in recent-onset T1D patients. To our knowledge, only \( \beta \)-cell area has been quantified in \( \text{Ab}^- \) nondiabetic subjects (21, 23). This difference is relevant because the \( \beta \)-cell area parameter does not take into account the fact that the weight of the pancreas is decreased in \( \text{Ab}^- \) nondiabetic subjects (27). A semiautomated method for morphometric quantification of the \( \beta \)-cell mass was applied and first validated on pancreatic section slides from control \( \text{Ab}^- \) subjects of the nPOD online repository. Although different, this method shares similarities with that developed by Wang et al (9) for immunofluorescent staining. This method allowed us to address pending questions regarding the \( \beta \)-cell distribution throughout the pancreas. In control \( \text{Ab}^- \) subjects, the \( \beta \)-cell mass we quantified and its relationship to BMI were similar to those published in the literature (12, 26), thus validating our quantification method. Moreover, we found that the head-to-tail distribution of \( \beta \)-cells can be differently appreciated depending on the way it is expressed: when the \( \beta \)-cell mass was calculated as a percentage of whole pancreatic tissue, the proportion of \( \beta \)-cells was higher in the head compared with tail, whereas no difference was observed when the \( \beta \)-cell proportion was calculated as percentage of exocrine tissue (ie, excluding fat, vessels, etc.). The islet size distribution also differed between head and tail, with larger islets in the tail as described by Wang et al (9). During validation of our quantification method, one expected result was that the proportion of nonexocrine tissue increased with age, mostly due to an increase in adipose tissue (25). Conversely, one unexpected finding was that the distribution of pancreatic nonexocrine tissue was uneven between the head and tail of the pancreas. This latter observation has already been reported long ago (28).

The nPOD project was launched to stimulate research on the pancreas pathology of T1D (4), providing access to high-quality specimens at no cost to investigators. When our study was started, 11,700 digitalized slides from 334 individuals were available. Insulin-stained slides were available for all pancreata. All the pancreas processing and immunohistochemical staining had been performed using standardized protocols and automated processing of the slides. Indeed, only 54 of 810 insulin-stained slides were not analyzed due to poor quality. However, significant fluctuations in staining intensities and in signal/noise ratios (29) do not allow for a fully automated analysis. As a result, we employed a more accurate semiautomated method that allowed us to assess the entire section of each slide.

The main advantage of this semiautomatic quantification technique is that the entire section can be analyzed at
high magnification. Larger areas are thus quantified when compared with the point-counting method using lattice, which usually quantifies approximately one quarter of the section (26). Furthermore, unlike some other semiautomated quantification methods, small islets and even single cells are quantified, thus limiting selection bias. Indeed, islet sections of less than five cells represented approximately 10% of the total β-cell mass. Published reports seldom specify whether these small islets are included in the assessment, and these small structures are sometimes not counted at all (8, 30). Following manual exclusion of nonpancreatic tissue, mainly peripancreatic lymph nodes and artifacts, we quantified endocrine, exocrine, and non-exocrine areas on whole sections. Surprisingly, the non-exocrine area accounts for approximately one third of the pancreas area, a factor that is not taken into account in several quantifications of β-cell mass (9, 11, 12, 30). For example, the choice of representative fields (31) may bias quantification, as these are probably chosen at distance from nonexocrine regions and therefore overestimating the real β-cell area. Furthermore, we have observed that nonexocrine tissue increased with age, reflecting the well-known fatty degeneration of the pancreas (25, 32). It has long been known that fatty degeneration is increased in both T1D and T2D patients (33). It is thus important to carefully take into account the nonexocrine tissue when assessing the endocrine mass in diabetic patients. Indeed, the distribution of nonexocrine tissue was uneven between the head and tail of the pancreas, which may have influenced previous analyses comparing β-cell area between these two regions (2, 7–12, 26).

One weakness of our study is that only three slides per pancreatic region were available on average, and that the pancreata were not sampled using an unbiased systematic method. However, an average of six slides were assessed for a total number of 2018 islets per patient, which is probably similar or higher than reported for other published analyses (2, 12, 26, 31). Moreover, the whole section was analyzed. Thus, the relatively few number of slides per subject was counterbalanced by the analysis of a large number of individuals.

β-cell mass was normal in Ab+ nondiabetic subjects, whereas it was markedly decreased in recent-onset T1D patients. β-cell mass has not been extensively quantified in recent-onset T1D patients, given that several reports have at best quantified the proportional β-cell area (18, 34, 35). Reports that insulin-positive islets are decreased by 50% at diagnosis (36) have not systematically quantified the β-cell mass, but only the fraction of islets with residual β-cells. This is important given that it has been reported that the mass of the pancreas is decreased in T1D patients, event at onset (37). Such a decrease in pancreatic mass can only be explained by a decrease in the exocrine tissue, which was first described at T1D onset by Foulis (34) and has seldom been studied since. β-cell mass has instead never been quantified in Ab+ nondiabetic subjects. Although some reports have employed biopsies (19), others have only quantified the insulin-positive area in patients with polyendocrine diseases and GAD Abs but no T1D (21). Finally, using the same nPOD database herein employed, von Herrath and colleagues have reported the islet area in some of the Ab+ subject herein studied (23), but they did not quantify the β-cell mass, despite knowledge that pancreatic mass is decreased in these individuals (27).

In this report (23), similar to our findings, the β-cell area of Ab+ was not decreased compared with Ab− subjects. Islet area (ie, the sum of insulin- and glucagon-stained areas), was only decreased by approximately 50% in recent-onset T1D individuals and only by approximately 15% in individuals with T1D for more than 5 years in the

same report (23), highlighting the fact that β-cell mass cannot be deduced from islet area. With regard to β-cell area, our data are consistent with the literature (18).

When interpreting our findings in Ab+ nondiabetic subjects, it should be kept in mind that the majority (14 of 18) of these individuals were positive for a single Ab, most commonly GAD Abs given the prevalent adult age of this study group (Supplemental Table 1). It is well established that GAD Abs alone are weak predictors of subsequent T1D development, with one third of these results being reported as only transiently positive (38). Indeed, no insulitis was observed in the subjects of this cohort that were previously analyzed (22). Our results seem to confirm the clinical observations that isolated GAD Abs may not be the sign of ongoing autoimmunity and question the pathogenic relevance of the autoimmune process in these patients (39). An alternative explanation may be that, in patients with ongoing autoimmunity, disease does not progress as long as immune regulatory mechanisms keep these responses at bay, or as long as immune-mediated β-cell destruction is compensated by β-cell renewal (13). This latter point further highlights the gap in knowledge about the kinetics of β-cell loss during the preclinical phase of T1D, which could reach a critical slope only at a late stage, as we already observed in the NOD mouse model (40).

In conclusion, the present study shows that the publicly available insulin-stained virtual slides of the nPOD project are suitable for quantitative analyses. We applied the quantification method to the largest known collection of Ab+ nondiabetic subjects and showed that the β-cell mass was not decreased compared with Ab− control subjects. Answering the question of the kinetics of β-cell loss during the latent autoimmune phase that may or may not lead to T1D will need collection of more donors that have autoantibodies and insulitis.

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