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PATHOGENESIS OF TYPE 1 DIABETES (A PUGLIESE AND SJ RICHARDSON, SECTION EDITORS)

Pancreas Pathology During the Natural History of Type 1 Diabetes

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Abstract

Purpose of review We provide an overview of pancreas pathology in type 1 diabetes (T1D) in the context of its clinical stages.

Recent findings Recent studies of pancreata from organ donors with T1D and non-diabetic donors expressing T1D-associated autoantibodies reveal pathological changes/disease mechanisms beyond the well-known loss of β cells and lymphocytic infiltrates of the islets (insulitis), including β-cell stress, dysfunction, and viral infections. Pancreas pathology evolves through disease stages, is asynchronous, and demonstrates a chronic disease that remains active years after diagnosis. Critically, β-cell loss is not complete at onset, although young age is associated with increased severity.

Summary The recognition of multiple pathogenic alterations and the chronic nature of disease mechanisms during and after the development of T1D inform improved clinical trial design and reveal additional targets for therapeutic manipulation, in the context of an expanded time window for intervention.

Keywords Type 1 diabetes · Insulitis · β cell · Pancreas · Islet autoimmunity

Abbreviations

AAb+	Autoantibody positive
EADB	Exeter Archival Diabetes Biobank
ER	Endoplasmic reticulum
DiViD	Diabetes Virus Detection Study
GAD	Glutamic acid decarboxylase
HA	Hyaluronan
HLAI	Human leukocyte antigen class I
IA-2	Islet antigen-2
ICI	Insulin-containing islet
IDI	Insulin-deficient islet
MODY	Maturity onset diabetes of the young
NOD	Non-obese diabetic mouse
nPOD	Network for Pancreatic Organ Donors with Diabetes
T2D	Type 2 diabetes

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Introduction

Type 1 diabetes (T1D) is a chronic autoimmune disease leading to severe loss of pancreatic β cells. The disease often manifests in children and adolescents, but many patients are diagnosed as adults [1, 2]. The prominent pancreas pathological features of T1D are loss of β cells and islet inflammation. The discovery of autoantibodies led to the recognition that autoimmunity may be triggered even in early life, and autoantibody conversion precedes clinical symptoms from months to years. All of the above and early pathology studies led to the belief that β-cell destruction is occurring over time, largely prior to the clinical onset, and that about 90% of the β cells are lost by the time symptoms manifest. Autoreactive T cells are considered the primary mediators of β-cell loss [3]. Since the mid-1980s, the design of clinical trials for preventing or reversing diabetes has been based on these views.

Here, we provide an updated view of pancreas pathology in T1D. We revisit earlier and recent studies to describe how our knowledge has evolved. Systematic efforts to provide greater access to the T1D pancreas to the scientific community, improved molecular methods, and collaboration have advanced our understanding of T1D pathogenesis and pathology, including the discovery of additional disease mechanisms, cellular players, and pathological features, all of which may be amenable to therapeutic manipulation. We also discuss current gaps in knowledge, which are especially critical during the prodromic phases of the disease, for which the characterization of pancreas pathology remains limited.

Sources of Human Pancreas for T1D Research

Access to the pancreas from patients with T1D has been historically limited, but it has been possible to obtain pancreata from patients through autopsy, biopsy, and organ donation. Currently, three pancreatic biobanks are actively supporting T1D research: the Exeter Archival Diabetes Biobank (EADB) in the UK, the Diabetes Virus Detection study (DiViD) in Norway, and the Network for Pancreatic Organ Donors with Diabetes (nPOD) in the USA. These are described below:

EADB Studies of autopsy pancreas were first, reflecting the higher probability of patients passing away following complications of ketoacidosis, which are now rare with improved therapies [4–6]. Established by Foulis in the 1980s, the EADB holds formalin-fixed, paraffin-embedded pancreas blocks from nearly 200 patients, of which about half are from young patients (< 20 years old) with recent-onset T1D. Thus, the EADB is the world's largest collection of autopsy pancreas samples recovered near a diagnosis of T1D.

DiViD Percutaneous biopsies were performed in Japan in the 1990s [7, 8]; although safe overall, the approach yielded little material, which limits investigations and their significance given that only a small area of pancreas can be examined. In 2014, the DiViD study reported obtaining specimens via laparoscopic pancreatic tail resection from six living adult patients with newly diagnosed T1D (24–35 years old) [9]. A significant amount of tissue was obtained, and samples were shared with many investigators around the world for collaborative studies. However, surgical complications led to the closure of the study and no additional biopsies were performed [10].

nPOD Established in 2007, nPOD has and continues to obtain pancreas and other tissues from organ donors with T1D and these are provided to the scientific community [11]. The T1D donors recovered cover a wide range of age and T1D duration. nPOD collects tissues from organ donors without diabetes and screens them to identify those with autoantibodies who might have been developing T1D. Thus, nPOD is attempting to obtain tissues that could inform about the preclinical stage of T1D. Samples available include tissues that are fixed, frozen, or fresh and are derived from the pancreas, spleen, pancreatic and non-pancreatic lymph nodes, blood (whole blood, serum, and plasma), duodenum, and thymus. Presently, nPOD is the largest biobank dedicated to T1D research; it has collected 185 non-diabetic donors, 36 autoantibody-positive (AAb+) donors, 168 donors with T1D, and donors with other forms of diabetes (T2D, MODY, GDM, cystic fibrosis).

Overall, these efforts have recovered pancreas from patients with T1D during the last 80 years, with varying age of onset and disease duration (Fig. 1a); these biobanks are complementary to each other and tremendously valuable for the T1D research community.

Key Features of Pancreas Pathology in T1D

Studies by LeCompte [13], Gepts [4], and others provided initial insight onto pancreas pathology in T1D. When Gepts described the T1D pancreas pathology in 1965 [4], T1D was referred to as "juvenile diabetes" and classified as acute (near diagnosis) and chronic (long duration); the role of autoimmunity was unknown, and islet cell antibodies were not discovered until 1974 [14]. Gepts evaluated the pancreas from 40 patients, 22 of whom were considered to have acute juvenile diabetes as they passed soon after diagnosis (disease duration range 3–180 days, < 90 days for 21/22 patients); this was a cohort of young children (mean age 10.89 years; ten children below age 10, nine teens aged 13–17, and only three adults aged 21, 22, and 30). Gepts made the following observations in these young patients with recent onset diabetes: 1) a drastic reduction in the number of β cells, estimated at less than 10% of well age-matched individuals without diabetes; 2) residual β cells with cytological signs of marked activity, presence of large islets, and signs of new islet formation; and 3) peri- and intra-insular inflammatory (termed "inflaminatory") infiltrates in 68% of the patients. Gepts also evaluated pancreata from 18 patients with chronic diabetes (disease duration 2–37 years, mean 17 years) who were on average 12.5 years old at diagnosis. In this cohort, the inflammatory process was not observed but β cells were completely absent, with few exceptions. Thus, islet inflammation and β-cell loss have been considered the main pathological features of T1D. The other major pathological feature reported in the T1D pancreas is the expression of elevated levels of HLA class I molecules, both in the cytoplasm and on the surface of islet cells, first reported by Bottazzo and Foulis in the mid-1980s [15–17]. These are considered the most typical features of T1D pancreas pathology and are reviewed below.

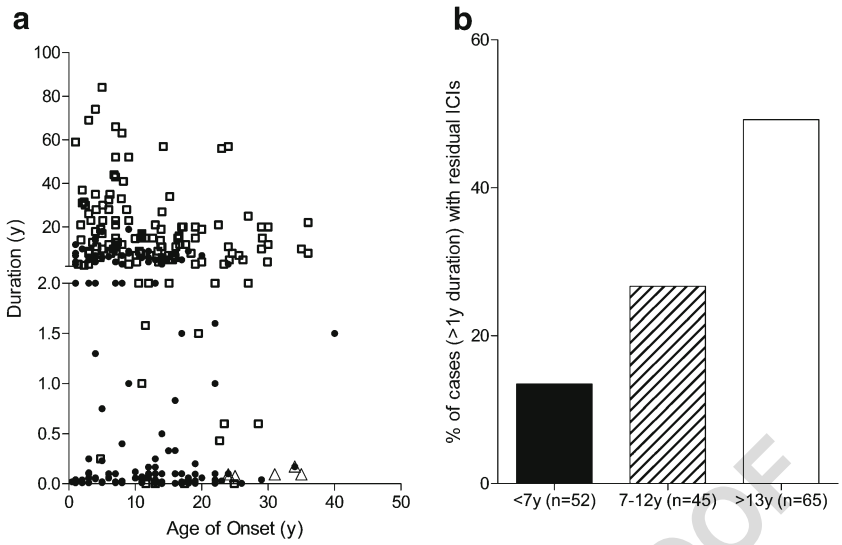

Fig. 1 Key features of the patients in the EADB, DiViD, and nPOD biobanks. **a** Dot plot illustrating the differences in age at onset and disease duration for the different three main pancreas biobanks, EADB (*black circles*), DiViD (*white triangles*), and nPOD (*white squares*). The EADB cohort is enriched for young-onset, short-duration T1D cases, whereas the nPOD cohort contains many donors with older onset and longer disease duration. **b** Age of onset strongly determines the proportion of cases with residual ICIs > 1 year post-diagnosis. Bar graph shows the % of cases with > 1 year duration of disease with residual ICIs divided based on the age at diagnosis: < 7 years (13.5%, *black bars*), 7–12 years (26.7%, *hatched bars*), and > 13 years (49.2%, *white bars*). Sources: http://foulis.vub.ac.be/; https://www.jdrfnpod.org/for-investigators/online-pathology-information/; Krogvold et al. 2014 [12•]

Insulitis This pathognomonic lesion consists of immune cell infiltrates within and around the pancreatic islets [18], and it supports the concept that T1D is a T-cell-mediated autoimmune disease. In the mid-1980s, expanding on the studies by Gepts, Foulis et al. [6] reported insulitis in 78% of young patients with recent onset disease (< 1 year). A 2011 meta-analysis by In't Veld [18] collected information from studies published since 1902 (213 cases with insulitis) and reported that insulitis occurs in 73% of young (< 14 years) patients with T1D who have a short duration of disease (< 1 month), in 60% of young patients with disease duration between 1 month and 1 year, and only in 4% of young patients with a duration of disease longer than 1 year. This scenario drastically changes in older patients. Only 29% of cases with onset between 15 and 40 years of age and disease duration < 1 month showed insulitis. Foulis reported that 23% of insulin-containing islets (ICIs) and only 1% of insulin-deficient islets (IDIs) had insulitis in young patients with < 1 year disease duration. Willcox and colleagues [19] examined the pancreas of 29 young patients (mean age 11.7 years) with disease duration between 1 day and 18 months; 23.8% of the islets contained insulin, of which 34.8% had insulitis, including 5% of IDIs. In the meta-analysis by In't Veld involving young patients with recent-onset disease (< 1 month), 34% of the islets stained for insulin on average, but only 33.6% of these islets had insulitis; in older patients with recent-onset disease, an average of 63% of the islets contained β cells, with 18% of the insulin-containing islets also showing insulitis [18]. In the DiViD study, the proportion of islets with insulitis ranged between 5 and 58% and only a single patient had insulitis in more than 50% of the islets [9, 12•]; in these adult patients, an average of 11% of the islets examined showed insulitis. In a study of nPOD organ donors with variable duration of T1D, 17 had insulitis with a broad range of disease duration (0–12 years) and age of onset (4–28 years); importantly, the frequency of insulitis had limited inverse correlation with diabetes duration and no correlation with age, whether at diagnosis or passing. Thus, the proportion of islets showing insulitis in the T1D pancreas is, overall, moderate to low; however, it varies significantly with age and disease duration. It is evident from the above that insulitis can be observed in many patients many years after diagnosis [20•].

According to the 2013 consensus [21], insulitis is defined by a predominantly lymphocytic infiltration of the islets consisting of at least 15 $CD45^+$ cells/islet (Fig. 2a) in a minimum of three islets, and the pancreas should also contain presence of IDIs or pseudoatrophic islets. Inflammatory infiltrates are more commonly detected in the islet periphery (peri-insulitis) or within the islet, with peri-insulitis representing the predominant form. Insulitis in the human pancreas is therefore much less severe than in experimental mouse models, in which a large number of infiltrating cells can be found in the majority of the islets. Insulitis is typically found in ICIs and less commonly in pseudoatrophic islets. Both T and B lymphocytes are present. Cytotoxic $CD8^+$ T cells represent the predominant lymphocyte populations; nPOD studies demonstrated that at least a proportion of the $CD8^+$ T cells are autoreactive and target β-cell autoantigens; the diversity in the antigen specificity of the infiltrating $CD8^+$ T cells was higher in patients with longer disease duration, suggesting that

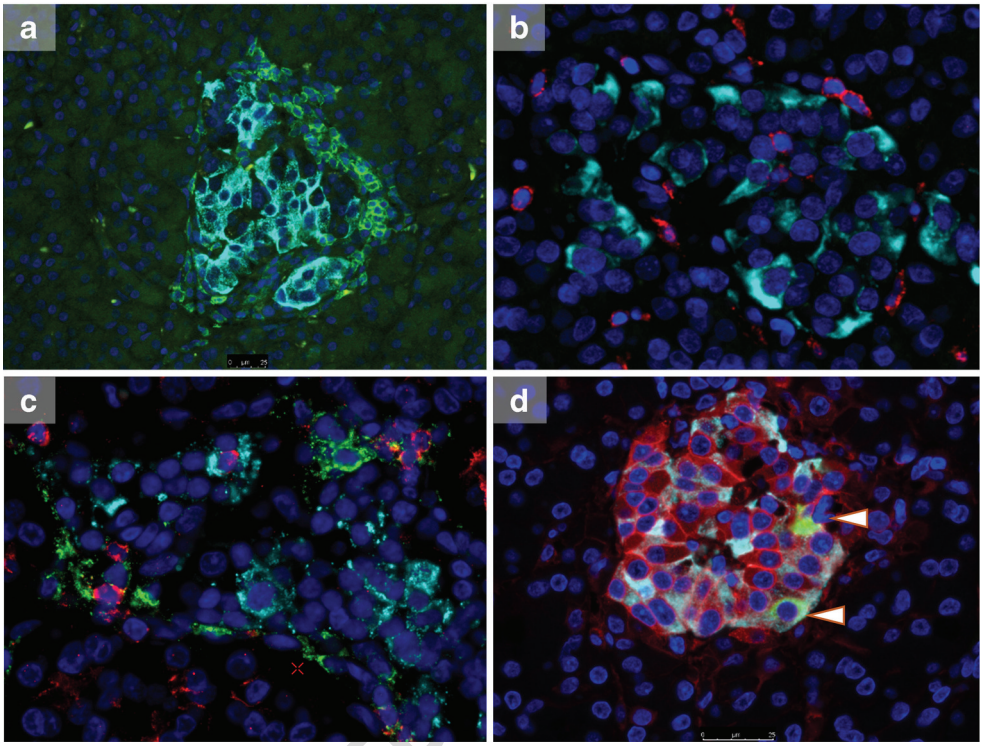

Fig. 2 Key features of islets from type 1 diabetes donors. **a** A representative T1D donor islet with insulitis (DiViD3); insulin (*light blue*), CD45 (*green*), and DAPI (*dark blue*). Representative islet from a CD20Lo case (DiViD2) (**b**) and a CD20Hi donor (nPOD 6209) (**c**); insulin (*light blue*), CD20 (*green*), CD8 (*red*), and DAPI (*dark blue*). Images courtesy of P. Leete (University of Exeter). **d** Expression of HLA class I and Enteroviral VP1 in an ICI from a T1D donor (EADB E560); insulin (*light blue*), VP1 (*green, arrows*), HLAI (*red*), and DAPI (*dark blue*)

autoimmunity evolves even after diagnosis [22••]. Other cell types commonly detected in the insulitis lesion are B lymphocytes, macrophages, and $CD4^+$ T cells [19]. The analysis of 21 patients (1 day–6 months' duration, median age 12 years) demonstrated two distinct patterns of infiltration: one characterized by large numbers of infiltrating cells, especially $CD20^+$ B lymphocytes, defined as CD20 high (CD20hi; Fig. 2b); the second pattern was characterized by infiltrates with fewer cells, including less $CD20^+$, defined as CD20 low (CD20lo; Fig. 2c) [23•]. CD20hi subjects had a lower number of ICIs and they were younger (mean of 7.8 years) when compared to CD20lo subjects (mean of 13 years). The association of insulitis lesions containing higher proportions of B lymphocytes with younger age at diagnosis suggests that these cells may contribute to a more aggressive form of autoimmunity [23•, 24]. Of importance is also the fact that all of the inflamed islets within a given patient display the same insulitic profile but that this profile differed significantly between individuals [23•].

β-Cell Destruction The most striking pathological feature in the T1D pancreas is loss of β cells. Lack of insulin staining is the predominant feature, and it is severe in the pancreas from patients who had T1D for many years. There is also substantial loss by the time of diagnosis, yet the long-held belief that 90% of the β-cell mass is universally lost at diagnosis is no longer supported. Consistent with the findings of Gepts, studies from the EADB, nPOD, DiViD cohorts, and other collections [25, 26] support that younger children have more severe loss of β cells (Table 1); however, patients who develop T1D when teenagers or older may still have 40–60% of their islets containing β cells and staining positive for insulin at diagnosis [23•, 26]. Accordingly, the DiViD biopsies of six adult patients demonstrated insulin staining, on average, in 36% of the islets (range 18–66%) [12•]. Among 80 nPOD donors with T1D, of whom only a few had disease duration less than 1 year [20•], residual β cells were observed in all T1D donors with insulitis, who had a 10-fold higher β-cell mass compared to those without insulitis. By contrast, the analysis by Leete et al. [23•] of 20 young patients (mean age 10.5 years) who died within 3 months of diagnosis showed much more severe β-cell loss; moreover, this varied according to the insulitis pattern, CD20lo and CD20hi; the ratio CD20 to CD4 also varied consistently with the two phenotypes (>1 in CD20hi and <1 in CD20lo). This ratio led to the separation of all individuals into three different groups: 1) <7 years (CD20hi), 2) 7–12 years, and 3) ≥ 13 years (CD20lo). Group 1 retained less ICIs than groups 2 and 3. Strikingly, the proportion of residual ICIs in those diagnosed early in life was around 14% while those diagnosed from their teens or beyond had higher number of insulin-positive islets (39% ICIs). Combination of all the available data for the EADB and nPOD cases with >1 year duration of disease (Fig. 1b) demonstrates that ICIs are preferentially retained in the older onset, group 3 cases. Thus, the emerging evidence suggests that β-cell destruction is quite heterogeneous but

t1.1 **Table 1** Key pathology features of the T1D pancreas in the EADB, nPOD, and DiViD cohorts

t1.2	Type 1 diabetes cohorts	EADB*	nPOD*	DiViD*
t1.3	Tissue source	Postmortem organ donors [2]	Organ donors	Live donor pancreatic biopsy
t1.4	Total number of cases	169	133	6
t1.5	Geographical location	UK	USA	Norway
t1.6	Collection period	1935–1991	2007 onwards	Feb 2011–Dec 2012
t1.7	Sample types collected	FFPE	Multiple [11]	Multiple [9]
t1.8	Median age (years) of onset (IQR) Range of age (years) of onset*	11 (5–16) 0.5–40	11.5 (6.2–18.4) 1–82	28 (24.25–33.25) 24–35
t1.9	Median disease duration (years) (IQR) Range of disease duration (IQR)	0.14 (0.04–3.75) 0–19	12 (5.5–23.0) 0–84	0.1 (0.08–0.1) 0.05–0.17
t1.10	Number of cases with ≤ 1 year duration Median age of onset (years) (IQR)	85 12 (6.0–17.0)	9 17.4 (11.6–23.4)	6 28 (24.25–33.25)
t1.11	Hallmark features of type 1 diabetes: insulitis, loss of insulin-containing islets (ICIs), and hyperexpression of HLA class I			
t1.12	% of cases with residual ICIs at < 1 year duration*	97.6%	100%	100%
t1.13	% of cases with insulitis < 1 year duration (*N*)			
t1.14	< 7 years 7–12 years ≥ 13 years	100% [16] 100% [16] 100% [15] [6] †	100% [1] N/A 100% [2] [20•]	N/A N/A 100% [6] [9]
t1.15	Average % of ICIs with insulitis/case			
t1.16	< 1 year duration (*N*) < 7 years 7–12 years ≥ 13 years	39.6% [16] 33.2% [16] 18.3% [15] [6]†	54% [1] N/A 66.5% [2] [20•]††	N/A N/A 30.5% [6] [12•]‡
t1.17	% of cases with residual ICIs at > 1 year duration (*N*)*			
t1.18	< 7 years 7–12 years ≥ 13 years	10% [20] 28.6% [15] 56.3% [17]	15.6% [27] 25.8% [28] 46.9% [29]	N/A
t1.19	Hyperexpression of HLA class I in residual ICIs	All recent-onset cases [15]	All recent-onset cases, reduces with disease duration [28•]	All cases [28•, 30•]
t1.20	Median % of ICIs with hyperexpression of HLA class I (IQR) (*N*)			
t1.21	< 7 years' duration ≥ 7 years' duration	100% (87–100) [31] 14.3% (0–52) [14] [15, 28•]		N/A

*Onset and duration data available on 128 EADB cases, 133 nPOD, and 6 DiViD cases (*IQR* interquartile range)

†The inclusion of later data from an additional 30 EADB patients confirms the original observation (< 7 years—40.3% [12•]; 7–12 years—27.3% [21]; > 13 years—13.8% [32])

††Data provided by Martha Campbell-Thompson from a published study [20•]

‡The published data [12•] were used to calculate the average % of ICIs with insulitis/case

greater loss is associated with younger onset of disease, the autoimmune process affects only a moderate proportion of islets at any given time, and it continues for several years after diagnosis.

Hyperexpression of HLA-Class I Molecules by Islet Cells The elevated levels of HLA class I molecules (Figs. 2d and 3) in islet cells highlight an inflammatory state and it is often associated with insulitis [33]. Like insulitis, hyperexpression of HLA class I molecules is typically found in ICIs, and it is often associated with $CD8^+$ T-cell infiltrates. It is possible that β cells hyperexpressing HLA class I molecules present their self-antigens to autoreactive T cells. Hyperexpression of class I molecules may result from viral infections associated with T1D [30•, 34], but it is unknown whether infiltrating $CD8^+$ T cells target viral epitopes presented by infected β cells [35]. Like insulitis, this phenomenon continues to be present for several years after diagnosis, and it has been validated with multiple approaches at the protein and RNA levels [28•]. A 2018 study classified patients based on a urinary C-peptide/creatinine ratio regression model and revealed that C-peptide loss continues for the first 7 years post-diagnosis but C-peptide levels stabilize afterwards, suggesting that from then on residual β cells are no longer being actively destroyed [27•]. To ascertain if the decline in hyperexpression of HLA class I correlates with this phenomenon, we combined data

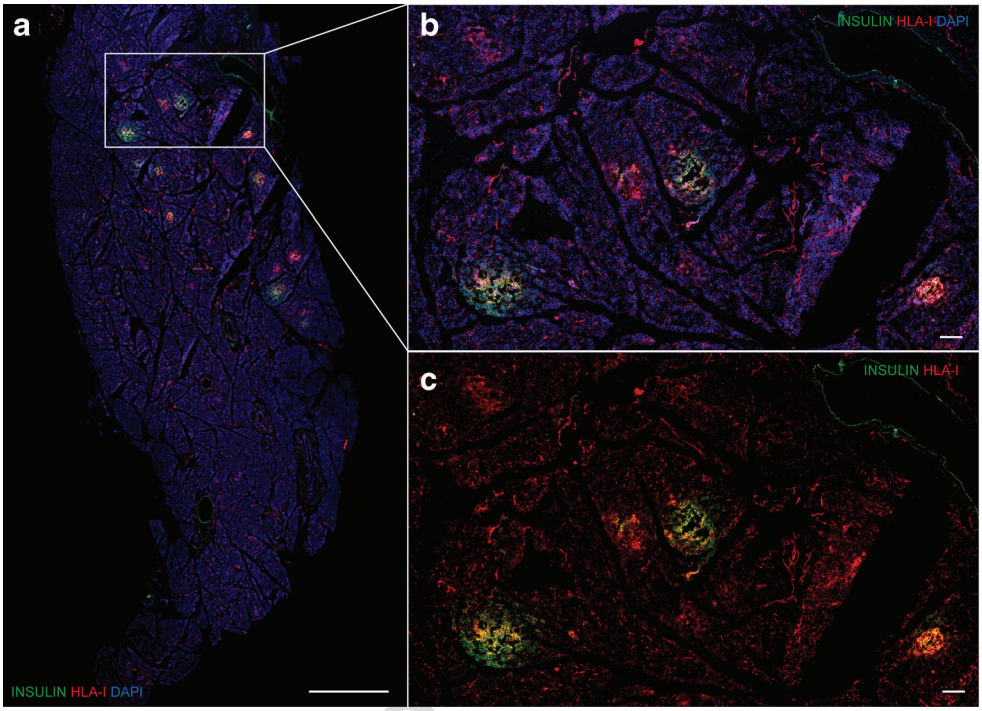

Fig. 3 Immunofluorescence analysis of HLA-I expression in frozen pancreas from a patient with type 1 diabetes (disease duration, 7 years) from the nPOD cohort. **a** Hyperexpression of HLA-I (*red*) can be predominantly seen in ICIs islets (*green*). Scale in whole tissue image = 2000 μm. **b**, **c** Higher magnification of the inset from (**a**), with (**b**) or without DAPI counterstain (**c**); scale in zoomed image = 200 μm

from the EADB [15] and Richardson et al. [28•] and found that HLA class I hyperexpression was not restricted only to recent-onset patients but also in those with longer disease duration who had residual ICIs. However, the proportion of residual ICIs hyperexpressing HLA class I clearly decreased over time. In patients with T1D for < 7 years, almost all ICIs hyperexpressed HLA class I molecules in contrast to only a median of 14% (0.0–52.3) among those who had T1D for > 7 years (Table 1). In summary, HLA class I hyperexpression persists on the majority of residual ICIs within the first 7 years post-diagnosis and this may contribute to β-cell demise by facilitating the presentation of self-peptides to infiltrating autoreactive $CD8^{++}$ T cells. As this hyperexpression declines in long-standing disease, β-cell antigen presentation would be attenuated, potentially leading to a reduction in the rate of destruction, even in the face of low-level, persistent insulitis.

Novel Pathology Findings in the T1D Pancreas

Studies are demonstrating additional pancreatic pathological abnormalities. Among these are changes in extracellular matrix components. Accumulation of hyaluronan (HA), a key constituent of the extracellular matrix, and HA binding proteins is found around islet cells and infiltrating lymphocytes in islets affected by insulitis [36]. HA deposits occur along the edge capillaries of diabetic islets, where leukocyte infiltrates in insulitis are frequently observed, and along intra-islet microvessels. HA deposition is more pronounced in islets from younger donors with T1D and those examined within the first year from diagnosis, confirming a more aggressive pathology in these patients. Conversely, the morphological pattern of HA in insulitis-free pancreas from donors with long-standing diabetes is similar to normal islets [36]. These studies indicate that HA and proteins associated with it form a matrix that interacts with infiltrating cells and it is directly related to pancreatic β-cell loss and insulitis [32]. HA might create a permissive environment that favors autoimmunity by restricting regulatory T-cell differentiation [37, 38], thus favoring effector T cells. Treatment of NOD mice with an inhibitor of HA synthesis, 4-methylumbelliferone (4-MU), inhibited progression to diabetes and increased the ratio of regulatory T cells to T effector cells [38]. Immunohistochemistry for laminin, perlecan, and collagen shows that components of the peri-islet basal membrane are lost at sites of leukocyte infiltration of the islets [39]. This indicates that removal of the basal membrane takes place during leukocyte entry into the islets. Moreover, cathepsins were found in the insulitis lesion near areas of disruption of the peri-islet basement membrane and may favor the penetration of lymphocytes inside the islets [39]. Alterations of extracellular matrix components also impact β-cell function and survival [40], including the loss of heparan sulfate which is associated with β-cell apoptosis [41].

Emerging studies are providing growing support for a viral contribution to the disease pathogenesis, particularly by enteroviruses [42]. Enterovirus proteins, enterovirus RNA, and an active anti-viral host response have been demonstrated in

the pancreata of T1D donors from each of the three biobanks [43]. Viral capsid protein is detected in a small number of β cells, typically only in ICIs, often in association with hyperexpression of HLA class I molecules and insulitis (Fig. 2d) [34, 44]. Enterovirus infections can severely impair insulin secretion [45], impact gene expression and microRNA regulation [46], and induce inflammation.

In turn, inflammation promotes β-cell stress, protein misfolding, dysfunction, and apoptosis [31, 47–49]. Indeed, there is growing evidence, also at the pathology level, that residual β cells in the T1D pancreas exhibit multiple signs of cellular stress, including an increased expression of ER stress markers [29, 50], especially in infiltrated ICIs [29, 51], which may contribute to insulin secretion abnormalities during the prediabetic phase [52]. β-Cell dysfunction may be an important contributor to insulin deficiency also at onset, when, as discussed above, many patients would be likely to have a significant residual β-cell mass [29, 53–56]. Moreover, islet function may be recoverable [53]; in the DiViD study, islets isolated from pancreas biopsies from newly diagnosed patients recovered function in culture. There is also evidence for dysregulated sphingolipid metabolism [57] and altered proteomic profiles that involve inflammatory, immune, and metabolic pathways [58]. β-Cell inflammation and stress may favor the formation of post-translationally modified and hybrid autoantigen peptides which may have a critical role in breaking self-tolerance and triggering islet autoimmunity [59]. For example, endoplasmic reticulum stress alters the endomembrane distribution of GAD65 autoantigen, resulting in accumulation of a more immunogenic, palmitoylated form of this molecule in trans-Golgi membranes, as demonstrated by the pathological examination of nPOD donors [60].

The exocrine pancreas is also impacted in T1D: the pancreas of donors with T1D is only 55% the weight of that of donors without diabetes [61]; this reduction in weight primarily affects the pancreatic dorsal lobe, which includes the majority of the head and the entire body and tail. Such a reduction is observed close to onset, and donors with T1D and long disease duration have almost normal pancreatic weights. There is initial evidence that non-diabetic, autoantibody-positive nPOD donors with insulitis have a small decrease in pancreas weight [62]. Pancreas volume, volume normalized by body weight, volume normalized by body mass index, and volume normalized by body surface area were all lower in patients with T1D compared to controls according to imaging studies [63]. As the islets constitute only 1–2% of the pancreas volume, these findings suggest loss of exocrine tissue during the development of T1D. This is consistent with impaired exocrine function, which is reported at T1D diagnosis (low levels of elastase in stools) but not at the time of seroconversion to islet autoantibody positivity [64]. Morphometric studies show that T1D donors have a higher non-exocrine–non-endocrine tissue area to total pancreas area than non-diabetic controls regardless of age, suggesting that T1D affects the entire pancreas [65]. In addition, large numbers of infiltrating cells have been found in the exocrine pancreas; $CD8^+$ and $CD4^+$ T cells, and $CD11c^+$ cells, were present in high numbers in the exocrine pancreas of AAb+ and recent-onset T1D donors, with a predominance of $CD8^+$ T cells and no reported differences between donors with or without pancreatitis [66]. The phenotype and function of these cells remains unclear. Mohapatra et al. created the term "diabetic exocrine pancreatopathy" to define the moderate-to-severe subclinical pancreatic fibrosis and modest exocrine dysfunction in the absence of clinical or histopathological evidence of chronic pancreatitis that affects individuals with T1D [67]. This includes (1) markedly decreased pancreatic weight, size, and volume; (2) increased inter-acinar fibrosis and acinar atrophy with minimal inflammation and no pancreatic ductal changes; (3) reduced exocrine enzyme output and fecal elastase concentrations; (4) normal to minimal decrease in coefficient of fat absorption; and (5) lack of progression of exocrine dysfunction over time.

Pancreas Pathology During Preclinical Disease Stages

Progress has been made toward understanding the natural history of islet autoimmunity from the longitudinal evaluation of relatives or individuals carrying HLA alleles associated with increased T1D risk. The best predictor of future T1D is the detection of circulating autoantibodies to islet autoantigens. Autoantibodies are found in almost 95% of those who develop clinical symptoms of T1D [68]. Longitudinal studies of large birth cohorts at increased genetic risk of T1D have shown that there is a peak in islet autoimmunity at 2–5 years of age; in these young children, progression to clinical disease is faster than those who convert at older age [69–71]. The highest risk is observed in those with autoantibodies against multiple islet autoantigens, in whom risk of T1D is about 40% at 5 years, 70% at 10 years, and 85% at 15 years [70]; however, those with a single autoantibody have much lower risk, around 5–10%, even with long follow-up. Studies have shown that metabolic abnormalities and defects in insulin secretion (assessed by C-peptide levels during an oral glucose tolerance test) become evident late in the progression to clinical disease, typically 18 to 6 months before diagnosis [72]. Based on the above, the JDRF, the Endocrine Society, and the American Diabetes Association have recognized three different stages in the progression of islet autoimmunity toward clinical T1D [73], which are 1) stage 1, defined by the presence of two or more autoantibodies; 2) stage 2, in which glucose intolerance or dysglycemia are also present; and 3) stage 3, which represents clinically manifest diabetes, when classical symptoms (polyuria, polydipsia, fatigue, and diabetic ketoacidosis) and

laboratory evidence of severe, fasting hyperglycemia are present. However, an earlier stage not formally recognized by this classification is characterized by the presence of a single autoantibody. We have discussed the pathology of stage 3 in the preceding sections; here, we will review what is known about pancreas pathology in the preclinical stages of T1D.

Pathology Findings at Stages 1/2 and in Single Versus Multiple AAb Positivity There is little information as to whether pathological alterations are different at stages 1 and 2 of the clinical classification because too few donors have been studied so far. Despite the autoantibody screening of organ donors instituted by nPOD, the number of autoantibody-positive donors, especially those with multiple autoantibodies, and more so those who also have elevated HbA1c levels, is quite low in the general population. Moreover, only a fraction of the autoantibody-positive donors is recovered, as many of these pancreata are allocated to transplantation instead of research; we advocate that these rare donors should be allocated to research [74••]. Furthermore, not all AAb+ donors, especially those with a single autoantibody, may represent true prediabetic individuals who would have developed T1D. Functional assessment of donor pancreas is just beginning through the study of isolated islets from donors with T1D [75, 76], pioneered by nPOD, which will be applied in the future to the pancreas from autoantibody-positive donors. Functional assessment of islet function in pancreas slices [77], which allows examining islet function in the natural tissue environment, is predicted to reveal novel information in the next few years. Sustained efforts may allow the identification of pathological features that define stage 1 and stage 2 T1D.

However, it is possible to examine pancreas pathology and contrast findings in donors with single versus multiple autoantibodies, with single autoantibody positivity representing the earlier phase in the natural history of islet autoimmunity and those with multiple autoantibodies representing donors at stage 1, or 2, if they had elevated HbA1c. Gianani et al. [78] identified a donor with a single autoantibody with no reduction in β-cell area and no insulitis. One of the largest studies identified AAb+ donors [79] by screening donors whose pancreata were used for islet isolation. A total of 1507 donors (25–60 years old) were identified; 55 of these had a single autoantibody, 4 donors had two, 2 donors had three, and 1 donor had four autoantibodies. Of these, only two of the triple autoantibody-positive donors had insulitis and carried high-risk HLA types; a small percentage of islets (9 and 3%, respectively) had insulitis. In both, at least one insulin-negative islet could be found. However, there was no decrease in β-cell mass. Another screening identified 32 autoantibody-positive donors among 969 tested (3.3%): nine expressed multiple autoantibodies but none carried high-risk HLA types and insulitis was not observed [80]. In both studies, the amount of tissue available in this study was limited to a small tissue block, and thus sampling issues cannot be excluded.

So far, nPOD [20•] has reported 21 donors with a single autoantibody, usually in the absence of T1D-associated HLA genes in whom insulitis was absent. However, 2/6 donors with multiple autoantibodies and 1 donor with a single autoantibody had insulitis and T1D-associated HLA types. Another study of nPOD donors reported the $CD8^+$ T cells trended higher in both islet and exocrine areas in some AAb+ donors than controls; the AAb+ group was the only one in addition to T1D donors with remaining ICIs in which the ratio between endocrine and exocrine infiltration was elevated, suggesting a polarization of $CD8^+$ T cells toward the islets [66]. In these two studies, there were no statistically significant differences in β-cell area or mass between non-diabetic and AAb+ individuals. However, AAb+ individuals with insulitis showed higher β-cell area than their non-insulitic counterparts and a slight increase in islet area compared to non-diabetic donors [81] was also reported. Perhaps these findings are consistent with the enlarged islets and features of hyperactivity originally reported by Gepts [4].

As noted, hyperexpression of HLA class I molecules in ICIs is a feature of T1D and has been observed in the EADB, nPOD, and DiViD cohorts. It was also observed in double AAb+ donors [82]; around 13% of the islets showed HLA class I hyperexpression in head, body, and tail of the pancreas with no particular distribution. Areas of islets with normal HLA class I expression were frequently contiguous to areas with hyperexpression. $CD8^+$ T-cell infiltration, although mild, was on average higher in islets with high HLA class I compared to islets with normal expression, consistent with the hypothesis that HLA class I expression could attract cytotoxic T cells to the islets. Ongoing studies by the nPOD-Virus group are screening non-diabetic, single, double AAb+, and T1D donors for the expression of HLA class I molecules together with other markers of viral infection in an attempt to study a possible association with enterovirus infections.

Despite the low number of AAb+ donors analyzed, these studies demonstrate islet pathological changes at stage 1 since insulitis can be found in less than half of the donors with multiple autoantibodies; however, so far it appears that only a limited proportion of islets may show concomitant β-cell loss. Insulitis does not appear in donors with a single autoantibody. While the number of subjects examined cannot be considered sufficient to draw firm conclusions, it appears that the single autoantibody stage may not be associated with the key features of the T1D pancreas pathology.

Stage 2: Multiple AAb and Impaired Glucose Tolerance As noted, at present there is no published study that specifically examines pancreas pathology in individuals with multiple autoantibodies and elevated HbA1c. We speculate that at this stage insulitis and β-cell loss may become more prominent;

moreover, the increase in insulin demand may exceed the ability of β cells to process newly translated proteins, leading to the accumulation of unfolded proteins [52, 83]. As discussed above, this promotes ER stress and apoptosis [29, 51], which precedes clinical onset. A key sign of β-cell ER dysfunction is the accumulation of unprocessed proinsulin [51], which is released to the circulation. This produces an increase in the proinsulin to C-peptide ratio in the serum of at-risk individuals months prior to diagnosis [52, 84]. At the pancreas pathology level, there is an increase in proinsulin and in the proinsulin/insulin ratio in the pancreas of double AAb+ nPOD donors and, importantly, in some with a single autoantibody [81].

Conclusions

The major features of T1D pancreas pathology highlight the chronicity of the disease, as its key pathological features are demonstrated for several years after diagnosis, and to some extent before diagnosis. Critically, insulitis, β-cell loss, and hyperexpression of HLA class I molecules do not affect all islets at the same time. Metabolic testing of living patients at diagnosis demonstrates severe but not complete impairment of stimulated C-peptide responses [85–89], with further decline in the following years; typically, decline is more severe in younger children. When examining the pancreas, the severity of β-cell loss at diagnosis is variable but not as high as previously believed, and several studies have demonstrated low amount of C-peptide and a response to stimulation in patients who had T1D for decades [90–94]. The persistence of β cells even decades after diagnosis with evidence of low-level replication [91], and the growing evidence for inflammation and ER stress [50, 95] imply that β-cell dysfunction plays a significant role in causing the symptoms at the time of diagnosis and probably for a few years thereafter. Recent pathology studies have shown that β-cell destruction is often incomplete at onset and continues after diagnosis for several years; besides T-cell-mediated autoimmunity, there are additional pathological alterations for which therapeutic manipulation is possible. Overall, the therapeutic time window for intervention may be longer than previously thought, and intervention strategies should have broader scope to simultaneously target multiple disease pathways that pathology studies show to be asynchronously active at any given time.

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Compliance with Ethical Standards

Conflict of Interest T.R.-C., S.J.R., and A.P. declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent Studies reviewed in this article involved organ donors or deceased patients (not considered human subjects from the regulatory point of view), and living patients. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed in the animal studies reviewed in this article.

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