INVITED REVIEW

100 YEARS OF INSULIN

Pancreas pathology in type 1 diabetes: an evolving story

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Abstract

We review the current knowledge of pancreas pathology in type 1 diabetes. During the last two decades, dedicated efforts toward the recovery of pancreas from deceased patients with type 1 diabetes have promoted significant advances in the characterization of the pathological changes associated with this condition. The implementation of autoantibody screening among organ donors has also allowed examining pancreas pathology in the absence of clinical disease, but in the presence of serological markers of autoimmunity. The assessment of key features of pancreas pathology across various disease stages allows drawing parallels with clinical disease stages. The main pathological abnormalities observed in the pancreas with type 1 diabetes are beta-cell loss and insulinitis; more recently, hyperexpression of HLA class I and class II molecules have been reproduced and validated. Additionally, there are changes affecting extracellular matrix components, evidence of viral infections, inflammation, and ER stress, which could contribute to beta-cell dysfunction and the stimulation of apoptosis and autoimmunity. The increasing appreciation that beta-cell loss can be less severe at diagnosis than previously estimated, the coexistence of beta-cell dysfunction, and the persistence of key features of pancreas pathology for years after diagnosis impact the perception of the dynamics of this chronic process. The emerging information is helping the identification of novel therapeutic targets and has implications for the design of clinical trials.

Key Words

- ▶ type 1 diabetes
- ▶ insulinitis
- ▶ beta cell
- ▶ pancreas
- ▶ islet autoimmunity

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Introduction

Type 1 diabetes (T1D) is a chronic disease characterized by severe loss of insulin secretion, requiring insulin replacement therapy for the individual's lifetime. The disease is often diagnosed in children and adolescents, but approximately 40% of patients develop T1D as adults (Maahs *et al.* 2010, Thomas *et al.* 2018).

Symptoms often present acutely, especially in children, and can potentially lead to life-threatening complications. However, longitudinal natural history studies in the relatives of patients and in newborns at increased genetic risk have revealed that critical pathogenic mechanisms are triggered months to years before clinical diagnosis. These

culminate in the progressive dysfunction of pancreatic beta cells and their autoimmune destruction. A variety of mechanisms are believed to cause T1D, which could explain the significant heterogeneity in its clinical severity and pathological features. The mechanisms of disease may not be necessarily active in every patient; even in a single patient, these may vary with disease stage. This article will describe the current state of knowledge about the pancreas pathology observed in T1D across its natural history, and we will integrate the interpretation of pathology findings with clinical data. There has been much progress in both research areas in the last decade, given the activities of clinical consortia, improved access to pancreas specimens obtained from organ donors with T1D at various stages of disease progression, and the parallel implementation of novel methodologies to assess pathology as well as immunological and metabolic parameters in the circulation. While many unknowns remain, significant progress has been made which impacts our understanding of disease pathogenesis and heterogeneity, and guides strategies for prevention and treatment in clinical trials.

Access to the pancreas with type 1 diabetes

A major obstacle to understanding the pathogenesis of T1D is that the pancreas, the target organ, is not easily accessible. However, beginning in the 1950s, scientists began to examine the pancreas from deceased patients. Initially, such pancreases were obtained at autopsy, often near the time of a diagnosis reflecting a higher probability of passing following acute complications of ketoacidosis. This was largely through the effort of individual investigators. Advances in the treatment of diabetes mean that such deaths have progressively declined, and pancreas recoveries have become more sporadic in nature. Later, limited efforts involved the collection of pancreas tissue using percutaneous or laparoscopic biopsies. More recently, a number of well-organized efforts are actively obtaining pancreases from T1D patients who have passed, as organ donors. We describe below the major efforts that have, or are, obtaining pancreas tissue from patients, from which important observations have been and continue to be made (Fig. 1):

Figure 1

Seminal pathology observations and collections from the last 100 years illustrated within a timeline. The key observations and references are described in more detail in Table 2. The timeline of key T1D-relevant biobanks that have been established are represented by the colored circles with biobank description. The T1D-relevant biobank collection periods for each biobank are illustrated in the black box, with the timelines of scientific outputs for each biobank illustrated in the red box.

Collections of autoptotic pancreata

Following initial studies of autoptotic pancreata from the 1950s–1960s (Lecompte 1958, Gepts 1965), in the 1980s, Foulis implemented a systematic collection of autopsy pancreas in the UK. Now known as the Exeter Archival Diabetes Biobank (EADB, <https://foulis.vub.ac.be>), the collection consists of formalin-fixed, paraffin-embedded pancreas blocks. The EADB is the world's largest collection of autopsy pancreas samples recovered near a diagnosis of T1D reflecting the higher probability of patients passing away because of acute complications of ketoacidosis, between 1945 and the 1990s, when these autopsies were performed (Gepts 1965, Gepts & De Mey 1978, Foulis *et al.* 1986). Thankfully, deaths have become progressively rarer with improved therapeutic management of hyperglycemia and ketoacidosis. The EADB holds specimens from about 200 patients, of which 100 are from <20-year-old patients with recent-onset T1D.

Pancreas biopsy collections

In the early 1990s, investigators obtained limited amounts of the pancreas from 18 T1D patients in Japan via percutaneous biopsies (Hanafusa *et al.* 1990, Itoh *et al.* 1993). In the 2010s, the Diabetes Virus Detection study (DiViD, <https://www.oslodiabetes.no/diabetes-virus-detection-study-divid>) obtained larger specimens via laparoscopic pancreatic tail resection from six living adults (24–35 years old) with recent-onset T1D (Krogvold *et al.* 2014). Given the surgical complications experienced in this study, the biopsy approach has not been used since 2014 (Atkinson 2014). However, the samples obtained were shared with many investigators worldwide for collaborative studies and yielded important information.

Organ donors

Significant efforts have since been launched to collect and study pancreata obtained from organ donors more systematically. Starting in 1989, the Islet Transplantation Program in Belgium (In't Veld *et al.* 2007) and the Nordic Network for Islet Transplantation (Sweden 2007–2015) (Wiberg *et al.* 2015) screened organ donors for T1D-associated autoantibodies, to identify those with preclinical disease and to ascertain the possible presence of early prediagnostic pathological alterations. As these pancreata were processed for islet isolation, although only limited amounts of whole pancreas tissue were obtained for histopathology, important information was

nevertheless obtained, as described later in this review. The Belgium program screened 1507 organ donors aged 25–60 years (Wiberg *et al.* 2015) and 556 donors younger than 25 years of age (Smeets *et al.* 2021); autoantibody positivity was found in 62 (4.1%) and in 27 (4.8%) of these donors, respectively. Most donors (7/62 and 25/27) expressed a single autoantibody, and the most prevalent single autoantibody was directed against the GAD65 autoantigen. The Nordic Network for Islet Transplantation screened 969 non-diabetic organ donors and identified 32 (3.3%, age range 21–74 years) autoantibody-positive donors, all expressing GAD autoantibodies, of which three also had antibodies to the autoantigen IA-2.

Beginning in 2007, the Network for Pancreatic Organ Donors with Diabetes (nPOD, www.JDRFnPOD.org) (Pugliese *et al.* 2014), launched an autoantibody screening of organ donors in the United States; at the time of writing, pancreata from 48 autoantibody-positive donors have been obtained, of whom 14 had multiple autoantibodies. nPOD actively recovers pancreata from organ donors with T1D, as well as autoantibody-negative organ donors without diabetes (control). nPOD has so far identified 188 donors with T1D and 233 control donors. The T1D cohort includes donors with disease duration <1 year ($n = 13$), 1–5 years ($n = 35$), 5–10 years ($n = 37$), and >10 years ($n = 99$). In addition to frozen and fixed pancreas specimens, nPOD obtains other disease-relevant tissues (lymph nodes, spleen, thymus, duodenum, blood, and more recently, kidney). Specimens are made available to scientists worldwide and include fixed/frozen tissues and live cells. nPOD has also recently developed means for distributing live cells and tissues, including isolated islets and pancreas slices (Panzer *et al.* 2020, Qadir *et al.* 2020).

Partnering with nPOD, the Human Islet Research Network has launched the Human Pancreas Analysis Program (<https://hpap.pcmacs.upenn.edu>), which since inception has studied pancreas from 22 organ donors with T1D and 16 autoantibody-positive donors, 14 with a single autoantibody (13/14 with GAD autoantibodies) and 2 with multiple autoantibodies. The INNODIA consortium in Europe (<https://www.innodia.eu>) and the Quality in Organ Donation (<https://quod.org.uk>) in the UK have recently begun collecting pancreas from organ donors with T1D.

The increasing access to T1D pancreas from a broader spectrum of disease stages, age and disease duration ranges, the availability of additional tissues besides pancreas, the study of live tissues and cells, and the application of the latest technologies are all contributing to dramatic progress. This has evolved our understanding of pancreas pathology, and in turn, the pathogenesis of T1D, well

beyond what we had learned from the initial studies limited to histopathology analyses.

Key features of pancreas pathology in T1D

The major features of pancreas pathology have been traditionally considered to be a marked loss of beta cells and the presence of lymphocytic infiltrates in the pancreatic islets, or insulinitis, which were initially reported in the mid-1960s. Much progress has been made in the characterization and quantification of these phenomena throughout the natural history of the disease. It has also been recognized that age-at-onset, and disease stage and time are major determinants of these features. The hyperexpression of HLA class I molecules in islets and the aberrant expression of HLA class II molecules by beta cells were initially reported in the mid-1980s, yet were not fully accepted until very recently, when, taking advantage of novel technologies, multiple biobanks, and islet cells isolated from donors with T1D, these findings were confirmed and further expanded. The additional features that were reported in recent years include critical alterations of extracellular matrix components, evidence of exocrine abnormalities and inflammation (Campbell-Thompson *et al.* 2015), rare donors with evidence of amyloid deposition (Westermarck *et al.* 2017, Denroche & Verchere 2018, Beery *et al.* 2019), and multidisciplinary evidence for viral infections (Dunne *et al.* 2019, Apaolaza *et al.* 2021, Geravandi *et al.* 2021). Thus, our understanding of pancreas pathology and associated molecular changes has evolved over time, and our knowledge is now more comprehensive. We will now describe the traditional pancreas pathology features and the more recently established alterations and discuss their significance in the disease pathogenesis, with attention to the disease stage at which they are observed.

As noted, the characterization of pancreas pathology in patients with T1D began in the late 1950s, with reports from LeCompte (1958) and Gepts (1965), who first described the two main pathology features, beta-cell loss and insulinitis. Gepts evaluated the pancreas from 22 young children who had passed soon after diagnosis (<3 months for 21) and observed severe loss of beta cells. Residual beta cells exhibited cytological signs of marked activity, and large islets and signs of new islet formation were noted. There were peri- and intra-islet inflammatory infiltrates, or insulinitis lesions. Insulinitis was not observed in the pancreas of 18 donors with a longer disease duration, in whom all beta cells were virtually lost. Another major observation was the expression of elevated levels of HLA

class I molecules by islet cells, first reported by Bottazzo and Foulis in the mid-1980s (Bottazzo *et al.* 1985, Pujol-Borrell *et al.* 1986, Foulis *et al.* 1987).

Insulinitis

This lesion consists of immune cells infiltrating the pancreatic islets (In't Veld 2011) and it represents pathological evidence of the immune-mediated destruction of pancreatic beta cells. Islet-infiltrating immune cells are more commonly detected in the islet periphery (peri-insulinitis), and the presence of inflammatory cells within the islets is considered an expression of increased disease severity in that islet. In the pancreas with T1D, the prevalence of insulinitis is affected by age and time since diagnosis. In the mid-1980s, Foulis *et al.* (1986) observed that most (78%) young patients with recent-onset disease (<1 year) had insulinitis. In 2011, In't Veld (2011) reported a meta-analysis of studies published since 1902, which overall described insulinitis in 213 cases. In this meta-analysis, most young (<14 years) T1D patients within one month of diagnosis had insulinitis, but insulinitis was observed in only 4% of patients who had had T1D for more than one year. Among patients diagnosed between 15 and 40 years of age, insulinitis was reported in only 29% of those with T1D duration <1 month. Thus, insulinitis appears to be more evident in younger patients, possibly representing an age at which the disease has greater severity, a concept also supported by studies in clinical cohorts (Krischer *et al.* 2021).

Another important notion is that insulinitis is predominantly found in islets that contain beta cells; insulin-negative, or 'pseudo-atrophic' islets rarely show insulinitis. In the meta-analysis by In't Veld (2011) involving young patients with recent-onset disease (<1 month), on average 34% of the islets stained for insulin, but only 33.6% of these islets had insulinitis; in older patients with recent-onset disease, an increased proportion of islets contained beta cells (63%), with only 18% of these insulin-containing islets (ICIs) showing insulinitis. Foulis (Willcox *et al.* 2009) reported insulinitis in 23% of ICIs and only 1% in insulin-deficient islets (IDIs) in young patients with <1 year duration of T1D. Willcox *et al.* reported 23.8% of ICIs, of which 34.8% had insulinitis, among 29 young patients diagnosed within the preceding 18 months. In comparison, insulinitis was seen in only 5% of the IDIs. In the DiViD study, which obtained pancreas tail biopsies from six adult patients near diagnosis, an average of 11% of the islets had insulinitis, and a single patient had insulinitis in more than 50% of the islets (Krogvold *et al.* 2014, 2016).

An nPOD study of organ donors with variable T1D duration (0–12 years) reported 17 donors with insulinitis in whom there was a limited inverse correlation with disease duration and no correlation with age, whether age at diagnosis or age at passing. This may reflect the limited size of the cohort and the broad range of age and disease duration. Taken as a whole, these studies suggest that the proportion of islets with insulinitis at any given time is moderate to low, particularly in those diagnosed in their teens and beyond, and it is impacted by age and disease duration. Moreover, more recent studies investigated insulinitis in pancreata obtained years after diagnosis (Campbell-Thompson *et al.* 2016a), and this can be observed usually within the first decade, supporting the chronic nature of islet autoimmunity.

A variety of immune cells are observed in insulinitis lesions, including T and B lymphocytes, macrophages, and less frequently, natural killer cells (Dotta *et al.* 2007, Willcox *et al.* 2009). A consensus definition of insulinitis was published in 2013 (Campbell-Thompson *et al.* 2013), and describes insulinitis as a predominantly lymphocytic infiltration of the islets with minimum 15 CD45-positive cells/islet, to be present in at least 3 islets, along with the presence of pseudo-atrophic islets. It has been debated whether a revised definition of insulinitis should be based exclusively on CD3-positive T cells (Campbell-Thompson *et al.* 2017, Lundberg *et al.* 2017a,b). Cytotoxic CD8 T cells are the predominant population; using class I tetramers, nPOD investigators showed that islet-infiltrating CD8+ T cells are autoreactive toward known beta-cell autoantigens (Coppieters *et al.* 2012, Bender *et al.* 2020). Increasing disease duration was associated with higher diversity in the antigen specificity of the infiltrating CD8 T cells, suggesting epitope spreading even after the clinical diagnosis.

Differences in the cellular composition of the insulinitis lesions have been described. Lesions which are more extensive and enriched in B lymphocytes are more often observed in children younger than 12 years of age, and those pancreata show more severe beta-cell loss than older children with insulinitis lesions containing fewer infiltrating cells and fewer B lymphocytes (Leete *et al.* 2016). These findings suggest that in younger children, B lymphocytes may contribute to more aggressive islet autoimmunity (Leete *et al.* 2016, Morgan 2017) and represent an example of disease endotypes that define disease heterogeneity and possibly guide therapeutic choices (Battaglia *et al.* 2020), considering that that inflamed islets within a given patient have the same insulinitis profile in terms of B-lymphocyte abundance (Leete *et al.* 2016).

Beta-cell loss and dysfunction

Once T1D has been clinically diagnosed, the predominant pathological feature in the T1D pancreas is severe loss of beta cells. However, the long-held belief that 90% of the beta-cell mass is lost by the time of clinical diagnosis does not appear correct for every patient, based on review of earlier data (Klinke 2008, 2011) and on more recent studies (Campbell-Thompson *et al.* 2016a, Krogvold *et al.* 2016, Leete *et al.* 2016). It is now emerging that the severity of beta-cell loss varies in relationship with age and disease duration. The pancreas of younger children typically displays the more severe beta-cell loss, but those diagnosed when in their teenage years or later may have 40–60% of their islets staining positive for insulin (Klinke 2008, Leete *et al.* 2016), based on recent analyses from the DiViD, EADB, and nPOD cohorts (Campbell-Thompson *et al.* 2016a, Krogvold *et al.* 2016, Leete *et al.* 2016). The six DiViD biopsies obtained from adult patients at diagnosis demonstrated insulin staining on average in 36% (18–66%) of the islets examined (Krogvold *et al.* 2016).

Because pathology studies show significant variability in actual beta cell loss, there appears to be an important role for beta cell dysfunction. The relative proportions of beta-cell loss and dysfunction may vary by age group and other individual factors. The functional assessments of insulin secretion in living patients concur with these pathology findings to a reasonable extent. At diagnosis, insulin secretion is severely impaired, and the severity of impaired insulin secretion is greater in younger age groups (Sherry *et al.* 2005, Tsai *et al.* 2006, Greenbaum *et al.* 2009, 2012, Sherr *et al.* 2014); however, many patients have clinically meaningful residual insulin secretion (Sherry *et al.* 2005, Tsai *et al.* 2006, Greenbaum *et al.* 2009, 2012, Sherr *et al.* 2014, Hao *et al.* 2016) exceeding a stimulated C-peptide level of 0.60 ng/mL (0.2 pmol/mL), which is associated with better metabolic control and reduced risk of complications (Steffes *et al.* 2003, Flatt *et al.* 2021, Jeyam *et al.* 2021). Longitudinal studies that included both pre- and post-diagnostic evaluation of insulin secretion revealed that the progressive loss of insulin secretion is more pronounced during the 6-month intervals that precede and follow diagnosis (Bogun *et al.* 2020). This may suggest heightened activity of the underlying disease mechanisms, which is consistent with the more pronounced prevalence of insulinitis around the time of diagnosis. Beta-cell mass becomes more severe with increasing disease duration. The progression of beta-cell destruction varies among individuals, it does not occur simultaneously for all islets, and chronic autoimmune destruction may continue for

a few years after clinical diagnosis. Moreover, at least in some patients, insulin-positive beta cells may persist even decades after diagnosis (Meier *et al.* 2005a,b, Gianani *et al.* 2010, Keenan *et al.* 2010). Several studies have reported that patients with long disease duration may secrete low amounts of C-peptide and also respond with increased levels to stimulation (Keenan *et al.* 2010, Wang *et al.* 2012, Oram *et al.* 2014, 2015). However, the clinical impact of these residual beta cells may be minimal, and they may exhibit apoptosis (Meier *et al.* 2005a,b, Keenan *et al.* 2010) and signs of inflammation (insulinitis, hyperexpression of HLA class I molecules (Morgan *et al.* 2014)). Overall, pathology and clinical studies suggest that T1D is caused by a chronic disease process which affects islets asynchronously, and continues its course over a long period of time after diagnosis (Table 1).

Hyperexpression of HLA class I molecules by islet cells

Another key feature of pancreas pathology in T1D is the presence of elevated levels of HLA class I molecules in islet cells; this finding supports the concept that islets are inflamed (Morgan *et al.* 2014). Hyperexpression of HLA class I molecules was reported initially in the mid-1980s (Foulis *et al.* 1987); it has recently been replicated and extended beyond its demonstration by immunohistochemistry (Richardson *et al.* 2016). HLA class I hyperexpression is typically associated with ICIs and the presence of CD8 T-cell infiltrates, suggesting that beta cells may be presenting autoantigens to autoreactive CD8 T cells. However, ICIs can hyperexpress HLA class I with no detectable immune cell infiltration present (Foulis *et al.* 1987b), suggesting that factors independent of the immune cells can drive hyperexpression. It is also plausible that viral infections associated with T1D may induce hyperexpression of class I molecules (Richardson *et al.* 2009, Krogvold *et al.* 2015a), but it remains to be determined

whether islet-infiltrating CD8 T cells may also target viral epitopes presented by infected beta-cells (Kundu *et al.* 2018). Like insulinitis, class I HLA hyperexpression can be observed in the pancreas even years after diagnosis (Coppieters *et al.* 2012, Richardson *et al.* 2016) in islets that contain residual beta cells. HLA class I hyperexpression has been correlated with the rate of beta-cell destruction (Shields *et al.* 2018). There is evidence that HLA I hyperexpression is also present in pancreatic islets of nPOD donors without diabetes, who however expressed T1D-associated autoantibodies even in the absence of insulinitis (Benkahla *et al.* 2021). HLA class I molecules are mostly expressed by alpha cells, but also beta cells, on the cell surface, and also accumulate intracellularly in the Golgi apparatus. Thus, while hyperexpression of HLA class I may represent an inflammatory response and may facilitate presentation of self/viral epitopes to CD8 T cells, it may also contribute to islet dysfunction by disrupting intracellular trafficking and may affect both alpha and beta cells.

Endotypes

Differences in pathology and clinical features among patients support the concept of the disease endotypes in which these phenotypes may reflect disease heterogeneity in the pathogenesis and clinical course, which may also be relevant to treatment (Arif *et al.* 2014, Battaglia *et al.* 2020, Leete *et al.* 2020). In pancreas pathology, the major features distinguishing endotypes are the intensity of the immune infiltration, the cellular composition of islet infiltrates, the degree of beta cell destruction, and the processing/localization of proinsulin. Pancreas pathology studies involving specimens from the EADB led to the proposal of two T1D endotypes in children and young adults, named T1D endotype 1 and 2, or T1DE1 and T1DE2, respectively. T1DE1 is associated with younger age of onset (typically <7 years), a more aggressive immune attack with an increased frequency of CD20+ B lymphocytes within islet infiltrates,

Table 1 Presence of key pathology features in the T1D pancreas across the natural history of the disease.

	Single AAb	Multiple AAb (Stage 1 T1D)*	T1D onset	0-10 years T1D duration	>10 years T1D duration
Beta-cell loss	—	+	++	+++	++++
Insulinitis	—	+	+++	++	—
Increased HLA I	Rare	+	+++	++	—
Viral Infection Markers	+/-	+	++	++	—

The table illustrates how selected features of pancreatic pathology are more or less represented, depending on disease stage, using a simple scale. It appears that features such as insulinitis, beta-cell loss, hyperexpression of HLA class I molecules, and markers of viral infection are rare at the stage of single autoantibody positivity. All features become detectable at stage 1 (T1D), and are more prominent at onset and afterward for several years after onset. Beta-cell loss is the only feature that remains beyond a decade after onset. Note that these are exemplifications and there is significant individual variation, determined by a variety of factors, including age, genetic and environmental determinants.

*There are no reported studies of the pancreas from donors with stage 2 T1D.

fewer residual beta cells, and evidence of impaired proinsulin hormone processing within the remaining beta cells. In these patients, proinsulin is found in mature granules, colocalizing with the processed insulin. Similar observations in terms of proinsulin were made in the nPOD cohort (Wasserfall *et al.* 2017, Sims *et al.* 2019). This is also consistent with elevated proinsulin:C-peptide ratio described in children during progression to clinical T1D (Sims *et al.* 2016). One might assume, therefore, that following stimulation, increased levels of proinsulin will be released in combination with insulin in the T1DE1 individuals, a phenomenon that appears to bear out when also examining the proinsulin:C-peptide ratio in the blood of individuals <10 years of T1D duration. In contrast, T1DE2 is associated with an older age-at-onset (≥ 13 years), a less aggressive immune attack with very few or no CD20+ B lymphocytes within the infiltrate, and the retention of a significant number of beta cells. Although insulin appears to be processed normally in these beta cells, it may be that it is not being released appropriately. Individuals diagnosed between 7 and 12 years can be defined as either T1DE1 or T1DE2 through the examination of the immune profile and localization of proinsulin. The field is advancing the characterization of endotypes through a combination of integrated assessments, including genetic, immunological and metabolic, at both the pathology and the clinical levels.

Additional pathology features of the T1D pancreas

Additional pancreatic pathological abnormalities have been described and appear to contribute to the disease pathogenesis.

Inflammation, beta cell stress, and dysfunction

Islet inflammation induces stress, protein misfolding, dysfunction, and apoptosis in beta cells (Fu *et al.* 2013, Marroqui *et al.* 2015, Brozzi & Eizirik 2016, de Beeck & Eizirik 2016). Residual beta cells in the T1D pancreas show evidence of stress, including increased expression of endoplasmic reticulum stress markers (Marhfour *et al.* 2012, Eizirik & Coomans de Brachène 2017, Marroqui *et al.* 2017, Eizirik & Colli 2020); these are particularly evident in infiltrated ICIs (Marhfour *et al.* 2012, Eizirik *et al.* 2013). Beta-cell stress may lead to the generation of post-translationally modified and hybrid autoantigens which could represent critical targets of islet autoimmune responses (Marre *et al.* 2015). For example, endoplasmic reticulum stress leads to the accumulation of an immunogenic, palmitoylated form of this GAD65 autoantigen in trans-Golgi membranes of beta cells (Phelps *et al.* 2016). Chronic inflammation and endoplasmic reticulum stress could all be important mediators of beta-cell dysfunction (Marhfour *et al.* 2012,

Table 2 Key observations and references.

Key observations	Reference
1 The term 'Insulinitis' first coined	von Meyenberg 1940
2 Demonstration that there is a reduction in extractable insulin from the pancreas of young donors with diabetes	Wrenshall <i>et al.</i> 1952
3 First description of the anatomy of juvenile diabetes pancreas	Lecompte 1958
4 Dramatic reduction in beta cells in juvenile diabetes and peri and intra-insular inflammatory infiltrates (termed 'inflaminatory') in 68% of donors.	Gepts 1965
5 Insulin-deficient islets are shrunken but contain normal numbers of alpha and delta cells	Gepts and De Mey 1978
6 First report of acinar cell atrophy in the vicinity of insulin-deficient islets	Foulis and Stewart 1984
7 Aberrant expression of HLA I and II in pancreatic islets of type 1 diabetes donors	Bottazzo <i>et al.</i> 1985
8 Histopathology of type 1 diabetes in UK donors diagnosed <20 years. <i>Autopsy cases. Describes histopathology of 119 donors; 47 of 60 patients with recent onset disease have insulinitis and confirm previous reports that insulinitis is more prevalent in donors <15 years. Suggests beta cells in PP lobe targeted very early in disease process.</i>	Foulis <i>et al.</i> 1986
9 Aberrant expression of HLA I and II restricted to ICIs in T1D donors	Foulis <i>et al.</i> 1987
10 Histopathology of 7 T1D donors using pancreatic punch biopsies (<i>living donors</i>). Confirms loss of beta cells and hyperexpression of HLA I, but no insulinitis. Later expanded to 35 donors (Imagawa <i>et al.</i> 2001), where insulinitis was observed.	Hanafusa <i>et al.</i> 1990
11 Insulinitis is rare and beta-cell loss not apparent in 65 donors with islet autoantibodies (AABs) but no diabetes.	Imagawa <i>et al.</i> 2001 In't Veld <i>et al.</i> 2007
12 Meta-analysis on the presence of insulinitis in 213 T1D donors. Insulinitis shown to be more frequent in donors <14 years	In't Veld 2011
13 Collection of larger samples of pancreas tissue by pancreatic tail resection from six live adult patients newly diagnosed with type 1 diabetes. Study halted due to complication.	Krogvold <i>et al.</i> 2014
14 Pancreas tissue slices from organ donors to perform in situ analysis of T1D pathogenesis	Panzer <i>et al.</i> 2020

Burch *et al.* 2015, Grzesik *et al.* 2015, Krogvold *et al.* 2015b, Imai *et al.* 2016), which contributes to insulin secretion abnormalities during the prediabetic phase and after diagnosis (Ludvigsson & Heding 1982, Heaton *et al.* 1988, Røder *et al.* 1994, Truyen *et al.* 2005, Van Dalem *et al.* 2016, Sims *et al.* 2016, 2018, 2019, Evans-Molina *et al.* 2018). As noted above, evidence of dysfunction is exemplified by abnormalities in the processing of the proinsulin prohormone to mature insulin, which has been observed in pancreas pathology studies of nPOD donors (Wasserfall *et al.* 2017, Rodriguez-Calvo *et al.* 2021): compared to controls, immunostaining of about 50% of insulin-positive T1D donors revealed increased proportions of proinsulin-enriched, insulin-poor beta cells with reduced mRNA levels of the proinsulin processing enzymes prohormone convertase 1/3 (PC1/3) and carboxypeptidase E (CPE). Experimentally, inflammatory cytokines reduced mRNA expression of PC1/3, PC2, and CPE in human islet cells. Of note, proinsulin processing was also inhibited by reduction of PC2 following persistent infection of the Ins-1 beta-cell line with Coxsackie B virus (Nekoua *et al.* 2021). Taken together, alterations of proinsulin processing appear linked to multiple disease mechanisms and represent a critical aspect of beta-cell dysfunction in T1D. It is possible that islet dysfunction may be corrected, as shown *in vitro* by the DiViD study (Krogvold *et al.* 2015b), where islets isolated from the biopsied pancreases recovered function in culture. The recognition of beta-cell dysfunction, especially at diagnosis, implies that therapeutic strategies should include interventions that restore and support beta-cell function. The overall modest prevalence of insulinitis at any given time suggests that the benefit of immunotherapy may be limited to those islets with insulinitis and limit overall treatment efficacy. Combinatorial therapies should therefore address pathogenic cofactors, attempt to mitigate inflammation, and correct beta-cell dysfunction (Skyler 2015).

Aberrant expression of HLA class II molecules by beta cells

Another early observation is the aberrant expression of HLA-II molecules by beta cells, initially reported in 22/26 and in 6/12 pancreata from patients with recent-onset and long-standing disease, respectively (Bottazzo *et al.* 1985, Foulis *et al.* 1987, Lernmark *et al.* 1995). Like HLA class I molecules, the class II molecules may be upregulated by viruses linked to T1D (Krogvold *et al.* 2015a, Hyoty 2016) and by inflammatory cytokines such as IFN- γ plus TNF- α or lymphotoxin, which were shown to induce

HLA I/II molecules on beta cells experimentally (Pujol-Borrell *et al.* 1987, Campbell *et al.* 1988). More recently, the observation of aberrant expression of HLA class II molecules has been reproduced using a variety of advanced methodologies such as transcriptome analysis of bulk-sorted and single beta cells (Russell *et al.* 2019): transcripts for HLA class II and other molecules in the class II antigen presentation pathway were demonstrated in cells lacking the macrophage marker CD68; this is important because macrophages that had captured dying beta cells had been considered as the explanation for this phenomenon. Immunohistology of pancreas sections from donors with recent-onset T1D from three independent cohorts demonstrated HLA class II antigens and the class II trans-activator protein in CD68-negative, insulin-positive beta cells, and were found in islets with insulinitis. Finally, beta cells were shown to express HLA class II molecules on their surface, by flow cytometry. Thus, the expression of HLA class II molecules by beta cells has now been validated. This suggests that beta cells may interact directly with islet-infiltrating CD4+ T cells, as originally proposed by Bottazzo in the mid-1980s (Bottazzo 1986).

PDL1 expression by beta cells

Another example of interactions between beta cells and infiltrating T cells derives from studies demonstrating that the programmed death-ligand 1 (PDL1) can be expressed in insulin-positive beta cells in the T1D pancreas, but not in IDIs or in control pancreas (Colli *et al.* 2018, Osum *et al.* 2018). PDL1 expression in beta cells has been linked to interferon alpha and gamma, in the context of alterations induced by islet inflammation, and is likely to represent a defense mechanism against autoreactive T cells expressing the PD1 receptor (Colli *et al.* 2020).

Alterations of extracellular matrix components

Hyaluronan (HA), a key constituent of the extracellular matrix, and HA-binding proteins, deposit around islet cells and infiltrating lymphocytes in islets affected by insulinitis (Bogdani *et al.* 2014a). Hyaluronan deposition is found along intra-islet micro-vessels and the edge capillaries of the islets, near infiltrating lymphocytes; it is more evident in younger donors with T1D and within the first year from diagnosis, suggesting a link to the disease pathogenesis (Bogdani *et al.* 2014a). Similar alterations were reported among nPOD donors with autoantibodies (Bogdani *et al.* 2020): abundant HA deposits were observed in pancreatic islets from donors with single and multiple

autoantibodies, even in the absence of insulinitis. Thus, HA may form a matrix that may favor lymphocytic infiltration of the islets (Bogdani 2016) and promote an inflammatory milieu (Nagy *et al.* 2015, Kuipers *et al.* 2016). Other studies have noted loss of laminin, perlecan, and collagen, critical components of the peri-islet basal membrane, in islets with insulinitis (Korpos *et al.* 2013), suggesting that the removal of the basal membrane is associated with islet infiltration by inflammatory leukocytes, where its degradation appears to be mediated by cathepsins (Korpos *et al.* 2013). Moreover, islet cell loss of heparan sulfate has been associated with impaired beta-cell function and survival, as it promotes beta-cell apoptosis (Bogdani *et al.* 2014b, Simeonovic *et al.* 2018).

Tertiary lymphoid organs (TLOs)

Recent studies of extracellular matrix components have led to the observation that another feature of pancreas pathology is the formation of tertiary lymphoid organs (TLOs). First described in NOD mice pancreas (Penaranda *et al.* 2010), TLOs were demonstrated in the human pancreas among donors without diabetes expressing multiple autoantibodies, among patients with T1D, and in the transplanted pancreas with demonstrated recurrence of islet autoimmunity (Korpos *et al.* 2021). Several features of the TLOs in the human pancreas were shared with NOD mice, such as the cellular and ECM composition of reticular fibers, the presence of high endothelial venules, and immune cell subtypes detected. Importantly, compartmentalized TLOs with distinct T-cell and B-cell zones were detected in younger donors with shorter disease duration, and TLOs were mainly associated with ICIs. The frequency of TLOs was inverse to the severity of beta-cell loss, suggesting that TLOs contribute to the progression of islet autoimmunity.

Viral infections

There is extensive support for a viral contribution to the disease pathogenesis, particularly by enteroviruses (Richardson & Morgan 2018, Dunne *et al.* 2019). At the level of the pancreas, 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 (Morgan & Richardson 2014, Geravandi *et al.* 2021). Viral capsid protein is detected in a small number of beta cells, typically only in ICIs, often in association with hyperexpression of HLA class I molecules and insulinitis (Richardson *et al.* 2009, 2013). There is

evidence that enterovirus infections impair beta-cell function (Gallagher *et al.* 2015), their gene expression, and microRNA regulation (Kim *et al.* 2016), and may promote or induce the deleterious inflammatory and autoreactive immune responses observed in T1D (Colli *et al.* 2020).

Abnormalities of the exocrine pancreas

The exocrine pancreas is also impacted in T1D. Atrophy of acinar cells was originally reported in the mid-1980s (Foulis & Stewart 1984). By morphometry, T1D pancreata show higher non-exocrine-non-endocrine tissue area to total pancreas area than non-diabetic controls, regardless of age (Bonnet-Serrano *et al.* 2018). The term 'diabetic exocrine pancreatopathy' (Mohapatra *et al.* 2016) has been proposed to describe the moderate-to-severe subclinical pancreatic fibrosis and modest exocrine dysfunction that occurs in T1D in the absence of clinical or histopathological evidence of chronic pancreatitis. This pancreatopathy is characterized by (i) decreased pancreatic weight, size, and volume; (ii) increased inter-acinar fibrosis and acinar atrophy with minimal inflammation and no pancreatic ductal changes; (iii) reduced exocrine enzyme output and fecal elastase concentrations; (iv) normal to minimal decrease in coefficient of fat absorption, and (v) lack of progression of exocrine dysfunction over time. Indeed, the T1D pancreas is reduced in weight (Campbell-Thompson *et al.* 2016b). Of note, this reduction is observed near disease diagnosis, while pancreata from donors with long disease duration had almost normal pancreatic weights. As the islets constitute only 1–2% of the pancreas volume, these findings suggest loss of exocrine tissue during the development of T1D. Imaging studies revealed that pancreas volume, volume normalized by body weight, volume normalized by BMI, and volume normalized by body surface area were all lower in patients with recent-onset T1D compared to controls (Virostko *et al.* 2016). Moreover, non-diabetic, autoantibody-positive nPOD donors with insulinitis have decreased pancreas weight (Campbell-Thompson *et al.* 2012). Reduced pancreas volume was recently demonstrated in two imaging studies of living at-risk relatives and newly diagnosed patients; it was more pronounced in newly diagnosed patients than relatives, suggesting a progressive loss of exocrine tissue with disease progression (Campbell-Thompson *et al.* 2019, Virostko *et al.* 2019). A loss of volume was observed to further advance in patients that were assessed longitudinally, during the first year after diagnosis (Virostko *et al.* 2019). Further analysis of nPOD donors revealed that T1D pancreata displayed lower frequency of amylase-negative cell clusters, suggesting a contribution

of the islet–acinar axis in pancreatic development and a possible abnormality linked to the disease pathogenesis (Kusmartseva *et al.* 2020). Clinical cohorts of at-risk relatives and patients revealed that trypsinogen and lipase levels were significantly reduced in individuals with multiple autoantibodies, recent-onset, and established T1D compared to control subjects and those with a single autoantibody; amylase levels were lower only in established T1D (Li *et al.* 2017, Ross *et al.* 2021). These measurements could serve as circulating biomarkers of pancreas volume, as measured by MRI (Campbell-Thompson *et al.* 2019). Moreover, lower elastase levels are reported in the stools at T1D diagnosis (Kondrashova *et al.* 2018). Finally, immune cells infiltrate the exocrine pancreas, including CD8+ and CD4+ T cells, as well CD11c+ cells, which were found in the exocrine pancreas of autoantibody-positive and recent-onset T1D donors. There is a predominance of CD8+ T cells and these findings were not linked to pancreatitis (Rodriguez-Calvo *et al.* 2014); the CD8+ T cells in the exocrine pancreas also include pre-proinsulin reactive cells which then accumulate in the islets (Bender *et al.* 2020).

Pancreas pathology during preclinical disease stages

Three different stages have been defined to classify the progression of islet autoimmunity toward clinical T1D (Insel *et al.* 2015): stage 1, characterized by positivity for at least two autoantibodies; stage 2, at which glucose intolerance or dysglycemia are demonstrated in those with multiple autoantibodies; and stage 3, corresponding to the time of diagnosis, when clinical symptoms become apparent. Of note, this classification does not include the stage at which an individual may have single autoantibody positivity, reportedly associated with low risk of future progression, although individuals may progress from single autoantibody positivity to multiple autoantibody positivity and later clinical disease. A critical question in the field is how pancreas pathology evolves and what pathogenetic mechanisms are active during the natural history of the disease. Describing the relationship between pathological change and autoantibodies in relation to disease stages has important therapeutic implications. As noted earlier, the most systematic effort was launched by nPOD across the U.S. and has so far obtained pancreas specimens from 48 organ donors with autoantibodies. However, the identification of autoantibody-positive donors from the general population remains a challenge, and a significant proportion of pancreata

from autoantibody-positive donors is transplanted instead of being recovered for research (Burke *et al.* 2017). The number of donors that can be identified with multiple autoantibodies is limited; most donors with autoantibodies have a single autoantibody.

Single autoantibody positivity likely represents the earlier phase in the natural history of islet autoimmunity. Multiple autoantibody positivity among organ donors may be considered equivalent to the clinical stage 1 or stage 2, in the rare event that a donor also has an elevated HbA1c; however, one must consider that impaired glucose tolerance and dysglycemia cannot be assessed in organ donors. An earlier study from the Nordic Network for Islet Transplantation identified autoantibody-positive donors (In't Veld *et al.* 2007) among donors whose pancreas was used for islet isolation. Of the 1507 donors (25–60 years old) screened, 55 had a single autoantibody and 7 had multiple autoantibodies. However, insulinitis affecting a minority of islets, was observed only in two donors with multiple autoantibodies, but there was no obvious decrease in β -cell mass. Another study screened 969 donors and identified 32 with autoantibodies: 9 had multiple autoantibodies but insulinitis was not observed (Wiberg *et al.* 2015). One must consider that for these studies, only small amounts of pancreas tissue were available, and thus lack of insulinitis may reflect sampling limitations. However, the examination of larger amounts of tissue in nPOD studies has returned similar observations: nPOD (Campbell-Thompson *et al.* 2016a) published the analysis of 21 donors with a single autoantibody, usually in the absence of T1D-associated HLA genes, in whom insulinitis was absent. However, two/six donors with multiple autoantibodies and one donor with a single autoantibody had insulinitis and T1D-associated HLA types. Another nPOD study observed that CD8+ T cells trended higher in both islet and exocrine areas in some autoantibody-positive donors than controls. Among donors with autoantibodies, and donors with T1D with residual beta cells, there was a polarization of the CD8+ T cells toward the islets (Rodriguez-Calvo *et al.* 2014). As noted, TLOs were also observed in the pancreas of donors with multiple autoantibodies and insulinitis, but not among donors with a single autoantibody (Korpos *et al.* 2021). This is also consistent with TLOs having a role in promoting islet autoimmunity.

Collectively, these studies demonstrate insulinitis and other islet pathological changes in about 40–50% of donors with multiple autoantibodies. In general, beta-cell loss was limited (Table 1). Given that most of these donors had normal HbA1c levels, these are more likely to correspond to individuals at stage 1 of the clinical

Figure 2

Representative histologically stained islets from type 1 diabetes pancreas. (A) An insulin-containing islet (ICI) with no evidence of immune cell infiltration (Insulin – brown; glucagon – pink, nPOD donor 6211). (B) An ICI with evidence of a mild insulitic infiltrate at one pole of the islet (Insulin – brown; glucagon – pink, nPOD donor 6211). (C) An ICI with evidence of aggressive insulitis (Insulin – brown; glucagon – black; CD20 brown; CD8 – blue, nPOD 6533). (D) Enteroviral VP1 positive cells within an ICI (serial section in B & E; VP1 – brown, nPOD 6211). (E) HLA I hyperexpression in an ICI (serial section in B & C; HLA I – brown, nPOD 6211). (F) An insulin-deficient islet (IDI).

classification. Consistently, the hyperexpression of HLA class I molecules in pancreatic islets reported in the EADB, nPOD, and DiViD cohorts has been also described in donors with multiple autoantibodies (Rodriguez-Calvo *et al.* 2015); CD8+ T-cell infiltration appeared more prominent in islets with increased HLA class I expression compared to islets with normal expression. This is consistent with the hypothesis that HLA class I expression could attract cytotoxic T cells to the islets. It has also been shown that autoantibody-positive donors with insulitis may have an increased islet area (Rodriguez-Calvo *et al.* 2017), possibly a sign of hyperactivity, which was originally proposed by Gepts (Gepts 1965). As the disease progresses and beta-cell dysfunction/loss begins to occur, the demand on beta cells may disrupt their physiology with accumulation of unfolded proteins (Mirmira *et al.* 2016, Sims *et al.* 2016), ER stress, and apoptosis (Marhfour *et al.* 2012, Eizirik *et al.* 2013), which have all been shown to precede clinical onset. As noted, the accumulation of unprocessed proinsulin (Eizirik *et al.* 2013)

which is released to the circulation is a key sign of ER stress and beta-cell dysfunction (Røder *et al.* 1994, Sims *et al.* 2016); this finds correspondence in pancreas pathology in the increased proinsulin:insulin ratio in the pancreas of double autoantibody-positive nPOD donors and, importantly, in some donors with a single autoantibody (Rodriguez-Calvo *et al.* 2017). Thus, although not all the known expressions of the disease pathogenesis have been found in the pancreas of donors with a single autoantibody, there is evidence for abnormalities at this stage as well. Table 1 illustrates the presence of key pathology features across the natural history of T1D, with examples of these shown in Fig. 2.

Conclusions

In the last two decades, the implementation of dedicated efforts for the collection and analysis of pancreas tissue from patients with T1D diabetes, largely post-mortem, has

supported afforded much progress in the characterization of pancreas pathology. The classical features, insulinitis and beta-cell loss, are now better understood and characterized in relation to age and disease duration. There is mounting evidence that beta-cell dysfunction is a critical factor in the disease pathogenesis, and increased recognition that the true extent of beta-cell loss may be overestimated as there is evidence that beta cells may persist in many patients for years after diagnosis. Moreover, the autoantibody screening programs launched to identify donors with preclinical T1D provide information about the evolution of the disease process in the pancreas, and it is possible to drive parallels to the clinical stages of disease. It appears that beta-cell loss and insulinitis may be absent/limited in the early stages of T1D (single AAb, stage 1 T1D), and become more prominent at later stages. This is consistent with more severe decline of insulin secretion during the 6 months preceding and the 6 months following diagnosis (Bogun *et al.* 2020). Beta-cell destruction is not complete by the time of diagnosis in many patients, but it continues over time, roughly for about 10 years after onset. Another consideration is that insulinitis, islet autoimmunity and inflammation appear chronic, but more prevalent and perhaps severe (particularly in young individuals) closer or even after diagnosis, but can persist for years after diagnosis together with other signs of inflammation. Together with increased recognition of pathology and clinical disease endotypes, all of the above findings have implications for advancing therapeutic strategies and clinical trials. One-hundred years after the discovery of insulin, our understanding of the pathology of T1D is still evolving; however, much progress has been made, despite limited access to the target organ, and much more progress is expected in the future, at a faster pace, based on the implementation of well-organized recovery efforts, advancing technologies, and scientific collaboration.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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