

Pancreas Pathology in Type 1 Diabetes: An evolving Story

Short title: Pancreas Pathology in Type 1 Diabetes

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ABSTRACT

We review the current knowledge of pancreas pathology in type 1 diabetes. During the last two decades dedicated efforts towards 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 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, insulinitis, and 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 identifying novel therapeutic targets and have implications for the design of clinical trials.

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 in adult age (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 relatives of patients, and 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. Mechanisms of disease may not be necessarily active in every patient and, even in a single patient, these may also 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 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 diagnosis, reflecting the 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 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):

Collections of autoptic pancreata. Following initial studies of autopsy 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 and 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 years 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 6 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, prediagnosis pathological alterations. As these pancreata were processed for islet isolation, limited amounts of whole pancreas tissue were obtained for histopathology, but important information was nevertheless obtained as described later in this review. The Belgium program screened 1,507 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 3 also had antibodies to the autoantigen IA-2.

Beginning in 2007, the Network for the Pancreatic Organ Donor 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 identified so far 188 donors with T1D and 233 control donors. The T1D cohort include 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 pancreas specimens, frozen and fixed, 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 (HPAP, <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 (QUOD, <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 initial studies limited to histopathology analyses.

KEY FEATURES OF PANCREAS PATHOLOGY IN T1D

The major features of pancreas pathology have been traditionally considered 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, 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. Additional features that were reported in recent years include critical alterations of extra-cellular matrix components, evidence of exocrine abnormalities and inflammation (Campbell-Thompson et al., 2015), rare donors with evidence of amyloid deposition (Westermarck et al., 2017, Beery et al., 2019, Denroche and Verchere, 2018) 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 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 (Lecompte, 1958) and Gepts (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; 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 (Foulis et al., 1987a, Pujol-Borrell et al., 1986, Bottazzo et al., 1985b).

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 (Foulis et al., 1986) observed that most (78%) young patients with recent onset disease (<1 year) had insulinitis. In 2011, In't Veld (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 1 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 (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, in 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 6 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., 2016, Krogvold et al., 2014). 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 ages 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 (Willcox et al., 2009, Dotta et al., 2007). 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 (Lundberg et al., 2017b, Lundberg et al., 2017a, Campbell-Thompson et al., 2017). Cytotoxic CD8 T-cells are the predominant population; using class I tetramers, nPOD investigators showed that islet-infiltrating CD8+ T-cells are autoreactive towards known beta cell autoantigens (Coppieters et al., 2012b, 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., 2016b). These findings suggest that in younger children B-lymphocytes may contribute to more aggressive islet autoimmunity (Leete et al., 2016b, 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., 2016b).

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, Klinke, 2011) and more recent studies (Krogvold et al., 2016, Leete et al., 2016a, Campbell-Thompson et al., 2016a). 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 teenagers or older may have 40%-60% of their islets staining positive for insulin (Klinke, 2008, Leete et al., 2016a), based on recent analyses from the DiViD, EADB, and nPOD cohorts (Krogvold et al., 2016, Leete et al., 2016a, Campbell-Thompson et al., 2016a). The 6 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 assessment 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 (Tsai et al., 2006, Sherry et al., 2005, Greenbaum et al., 2009, Greenbaum et al., 2012, Sherr et al., 2014); however, many patients have clinically meaningful residual insulin secretion (Hao et al., 2016, Greenbaum et al., 2012, Tsai et al., 2006, Sherry et al., 2005, Greenbaum et al., 2009, Sherr et al., 2014) 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 (Flatt et al., 2021, Steffes et al., 2003, Jeyam et al., 2021). Longitudinal studies that included both pre- and post-diagnosis 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, Meier et al., 2005b, Keenan et al., 2010, Gianani et al., 2010). Several studies have reported that patients with long disease duration may secrete low amount of C-peptide and also respond with increased levels to stimulation (Oram et al., 2014, Keenan et al., 2010, Wang et al., 2012, Oram et al., 2015). However, the clinical impact of these residual beta cells may be minimal, and these may exhibit apoptosis (Meier et al., 2005a, Meier et al., 2005b, Keenan et al., 2010) and signs of inflammation (insulinitis, hyper-expression 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., 1987b); it has recently been replicated and extended beyond its demonstration by immunohistochemistry (Richardson et al., 2016). HLA class I hyper-expression 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., 1987), suggesting that factors independent of the immune cells can drive hyperexpression. It is also plausible that viral infections associated with T1D may induce hyper-expression of class I molecules (Krogvold et al., 2015a, Richardson et al., 2009), 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 hyper-expression can be observed in the pancreas even years after diagnosis (Richardson et al., 2016, Coppieters et al., 2012a) in islets that contain residual beta cells. HLA class I hyper-expression has been correlated with the rate of beta cell destruction

(Shields et al., 2018). There is evidence that HLA-I hyper-expression 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 hyper-expression 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 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, Leete et al., 2020, Battaglia et al., 2020). In pancreas pathology, 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 <7y), a more aggressive immune attack with an increased frequency of CD20+ B-lymphocytes within islet infiltrates, fewer residual beta cells and evidence of impaired proinsulin hormone processing within 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 (Sims et al., 2019, Wasserfall et al., 2017). This is also consistent with elevated proinsulin:C-peptide ratio described in children during progression to clinical T1D (Sims et al., 2016b). 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 y), a less aggressive immune attack with very few/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 not be being released appropriately. Individuals diagnosed between 7-12y can be defined as either T1DE1 or T1DE2 through the examination of the immune profile and localisation 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 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, de Beeck and Eizirik, 2016, Brozzi and Eizirik, 2016, Marroqui et al., 2015). Residual beta cells in the T1D pancreas show evidence of stress, including increased expression of endoplasmic reticulum stress markers (Marhfour et al., 2012, Eizirik and Colli, 2020, Eizirik and Coomans de Brachène, 2017, Marroqui et al., 2017); these are particularly evident in infiltrated ICIs (Eizirik et al., 2013, Marhfour et al., 2012). Beta cell stress may lead to the generation of post-translationally-modified and hybrid autoantigens which could represent critical target 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 (Krogvold et al., 2015b, Marhfour

et al., 2012, Burch et al., 2015, Grzesik et al., 2015, Imai et al., 2016) which contributes to insulin secretion abnormalities during the prediabetic phase and after diagnosis (Ludvigsson and Heding, 1982, Heaton et al., 1988, Røder et al., 1994, Truyen et al., 2005, Van Dalem et al., 2016, Sims et al., 2016b, Sims et al., 2018, Evans-Molina et al., 2018, Sims et al., 2019). As noted above, evidence of dysfunction is exemplified by abnormalities in the processing of the proinsulin pro-hormone to mature insulin, which has been observed in pancreas pathology studies of nPOD donors (Rodriguez-Calvo et al., 2021, Wasserfall et al., 2017): 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 in

22/26 and 6/12 pancreata from patients with recent-onset and long-standing disease, respectively (Bottazzo et al., 1985a, Foulis et al., 1987a, Lernmark et al., 1995). Like HLA class I molecules, 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, there has been now validation of the expression of HLA class II molecules by beta cells. 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 by insulin-positive beta cells in the T1D pancreas, but not in insulin-deficient islets 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 likely represent a defense mechanism against autoreactive T cells expressing the PD1 receptor (Colli et al., 2020).

Alterations of extra-cellular matrix components. Hyaluronan (HA), a key constituent of the extra-cellular matrix, and HA binding proteins, deposit around islet cells and infiltrating lymphocytes in islets affected by insulinitis (Bogdani et al., 2014a). HA 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 (Kuipers et al., 2016, Nagy et al., 2015). 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 extra-cellular 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 (RFs), 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 (Dunne et al., 2019, Richardson and Morgan, 2018). 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 and 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, Richardson et al., 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 induce 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 and 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 (1) 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. 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 body mass index, 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), and 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); CD8+ T cell 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 towards clinical T1D (Insel et al., 2015): Stage 1, characterized positivity for at least two autoantibodies; 2) Stage 2, at which glucose intolerance or dysglycemia are demonstrated in those with multiple autoantibodies; 3) 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 changes, autoantibodies and 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., 2016). The number of donors that can be identified with multiple autoantibodies is limited and 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 1,507 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 not 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, 2/6 donors with multiple autoantibodies and 1 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 towards 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 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 insulinitis 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 disease progressed 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., 2016a), ER stress and apoptosis (Marhfour et al., 2012, Eizirik et al., 2013), which have all be shown to precede clinical onset. As noted, a 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., 2016a); 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 are informing about the evolution of the disease process in the pancreas, and it is possible to draw 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, Funding, Author contributions statements

The authors declare no conflict of interests.

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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 afterwards 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 Stage 2 T1D.

FIGURE LEGENDS

Fig.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 the box below. The timeline of key T1D relevant biobanks that have been established are represented by the coloured 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.

	Key Observations	Reference
1	The term 'Insulinitis' first coined	<i>Von Meyenberg, H. Ueber „insulinitis“ bei Diabetes. Schweiz. Med. Wochenschr. 21: 554, 1940.</i>
2	Demonstrated that there is a reduction in extractable insulin from the pancreas of young donors with diabetes	<i>Wrenshall GA, Bogoch A, Ritchie R. Extractable insulin of pancreas. Diabetes 1:87–107, 1952</i>
3	First description of the anatomy of juvenile diabetes pancreas	<i>Lecompte PM. Insulinitis in early juvenile diabetes. AMA Arch Pathol. 1958;66(4):450–7.</i>
4	Dramatic reduction in beta cells in juvenile diabetes and peri and intra-insular inflammatory infiltrates (termed 'inflammatory') in 68% of donors.	<i>Gepts W. Pathological anatomy of the pancreas in juvenile diabetes mellitus. Diabetes 14: 619, 1965</i>
5	Insulin-deficient islets are shrunken but contain normal numbers of alpha and delta cells	<i>Gepts W, De Mey J. Islet cell survival determined by morphology. An immunocytochemical study of the islets of Langerhans in juvenile diabetes mellitus. Diabetes. 1978;27(Supplement 1):251–61.</i>
6	First report of acinar cell atrophy in the vicinity of insulin-deficient islets	<i>Foulis AK, Stewart JA. The pancreas in recent-onset Type 1 (insulin-dependent) diabetes mellitus: insulin content of islets, insulinitis and associated changes in the exocrine acinar tissue. Diabetologia 1984; 26: 456-461.</i>
7	Aberrant expression of HLA I and II in pancreatic islets of Type 1 diabetes donors	<i>Bottazzo GF et al, In situ characterization of autoimmune phenomena and expression of HLA molecules in the pancreas in diabetic insulinitis. New Eng J Med. 1985;313:353-60.</i>
8	Histopathology of Type 1 diabetes in UK donors diagnosed <20y. <u><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 <15y. Suggests beta cells in PP lobe targeted very early in disease process.</i></u>	<i>Foulis AK et al, The histopathology of the pancreas in type 1 (insulin-dependent) diabetes mellitus: a 25-year review of deaths in patients under 20 years of age in the United Kingdom. Diabetologia. 1986;29(5):267–74</i>

9	Aberrant expression of HLA I and II restricted to ICIs in T1D donors	<i>Foulis AK et al</i> , Aberrant expression of class II major histocompatibility complex molecules by B cells and hyperexpression of class I major histocompatibility complex molecules by insulin containing islets in type 1 (insulin-dependent) diabetes mellitus. <i>Diabetologia</i> . 1987;30(5):333-43.
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 et al, 2001), where insulinitis was observed.	<i>Hanafusa et al</i> , Examination of islets in the pancreas biopsy specimens from newly diagnosed Type 1 (insulin-dependent) diabetic patients. <i>Diabetologia</i> 1990. 33:105-111. <i>Imagawa A, et al</i> . Pancreatic biopsy as a procedure for detecting in situ autoimmune phenomena in type 1 diabetes: close correlation between serological markers and histological evidence of cellular autoimmunity. <i>Diabetes</i> 2001. 50(6): 1269-73.
11	Insulinitis is rare and beta cell loss not apparent in 65 donors with islet autoantibodies (AABs) but no diabetes.	<i>In't Veld P et al</i> ,. Screening for insulinitis in adult autoantibody-positive organ donors. <i>Diabetes</i> 2007. 56:2400-2404.
12	Meta-analysis on the presence of insulinitis in 213 T1D donors. Insulinitis shown to be more frequent in donors <14y	<i>In't Veld P</i> . Insulinitis in human type 1 diabetes: The quest for an elusive lesion. <i>Islets</i> . 2011. 3(4):131-8.
13	Collection of larger samples of pancreas tissue by pancreatic tail resection from 6 live adult patients newly diagnosed with Type 1 diabetes. Study halted due to complication.	Krogvold L et al, Pancreatic biopsy by minimal tail resection in live patients at the onset of type 1 diabetes: experiences from the DiViD study. <i>Diabetologia</i> 2014. 57:841-43.
14	Pancreas tissue slices from organ donors to perform in situ analysis of T1D pathogenesis	<i>Panzer J et al</i> , Pancreas tissue slices from organ donors enable in situ analysis of type 1 diabetes pathogenesis. <i>JCI Insight</i> 2020. 23; 5(8):e134525.

Fig. 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 insulinitis (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).



