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List of Abbreviations

ADCC	Antibody Dependent Cellular Cytotoxicity
AIRE	Autoimmune Regulator
APC	Antigen Presenting Cell
BBDP	Bio-Breeding Diabetes-Prone
BMI	Body Mass Index
CALM1	Calmodulin 1
C4d	Complement component 4d
CTRC	Chymotrypsin C
CPE	Carboxypeptidase E
DASP	Diabetes Autoantibody Standardization Program
DKA	Diabetic Keto-Acidosis
ELISA	Enzyme Linked Immuno-Sorbant Assay
FFPE	Formalin Fixed Paraffin Embedded
FOXP3	Forkhead Box P3
GADA	Glutamic Acid Decarboxylase Autoantibodies
GCG	Glucagon
HbA1c	Hemoglobin Alpha 1 fraction c
HLA	Human Leucocyte Antigen
hnRNA	heterogeneous nuclear RNA
IAA	Insulin Autoantibodies
IAPP	Islet Amyloid PolyPeptide
IASP	Islet Autoantibody Standardization Program
IA-2A	Insulinoma Associated -2 Autoantibodies
ICA	Islet Cell Autoantibodies
IF	Immunofluorescence
INS	Insulin
INS-IGF2	Insulin – Insulin Like Growth Factor 2 read through
ISH	In Situ Hybridization
MHC	Major Histocompatibility Complex
mRNA	messenger RNA
NK	Natural Killer cell
NOD	Non Obese Diabetic mouse

nPOD	Network for Pancreatic Organ donors with Diabetes
OPO	Organ Procurement Organizations
PCSK1	Proprotein Convertase Subtilisin/Kexin Type 1
PCSK2	Proprotein Convertase Subtilisin/Kexin Type 2
PPIA	Peptidylprolyl Isomerase A or cyclophilin A
RIA	Radioimmunoassay
RNA	Ribonucleic Acid
RT-qPCR	Real-Time quantitative Polymerase Chain Reaction
ROC	Receiver Operating Characteristic
SOP	Standard Operating Procedure
SST	Somatostatin
SPINK1	Serine Protease Inhibitor Kazal-type 1
TCR	T-Cell Receptor
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
VNTR	Variable Number of Tandem Repeats
ZnT8A	Zinc Transporter-8 Autoantibodies

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Summary

1 Motivation

In ancient times, diabetes was described as literally the flow through of urine and was further stratified as either diabetes insipidus (urine tasted insipid) or mellitus (urine tasted like honey) (Karamanou, et al., 2016; Qureshi, et al., 2014). Moving centuries forward (i.e., 1920s), the landmark discovery by Banting and Best (Banting, et al., 1922) that insulin regulated glucose and was produced in the endocrine pancreas set the stage for the treatment of diabetes mellitus. Today we recognize multiple forms of diabetes mellitus, with type 1 diabetes (T1D) and type 2 diabetes (T2D) forming the majority of cases (Kharroubi and Darwish, 2015). There are also multiple other types, including those that are now termed monogenic forms or where diabetes occurs as part of a syndrome, that are beyond the scope of this thesis (Kharroubi and Darwish, 2015) .

The diabetes nomenclature has undergone several iterations with idiopathic, juvenile, adult, non-insulin requiring and insulin-dependent all being qualifiers in the classification of either what we today call T1D or T2D. We now understand that T1D is autoimmune in nature and our knowledge base has rapidly expanded (Atkinson, et al., 2014). Nonetheless critical knowledge voids exist in research efforts for this disease and we have yet to fully comprehend the etiology let alone find a cure for this disorder. Much of this knowledge has arisen from both human studies and animal models of T1D. The community has been fortunate to have spontaneous models of disease such as the Non Obese Diabetic (NOD) mouse (Anderson and Bluestone, 2005). This is somewhat unique to T1D as most other autoimmune conditions require induced models of disease for experimentation (Yu, et al., 2015). There are, however, many caveats to these models, and a rich literature exists with both detractors and supporters of this particular mouse model (Leiter and von Herrath, 2004; Roep and Atkinson, 2004). Much of this debate centers on the success in preventing and reversing T1D in the NOD mouse (Bowman, et al., 1994; Roep, et al., 2004; Shoda, et al., 2005) while disappointingly, such efforts have not translated into therapeutic efficacy for human T1D (Greenbaum and Atkinson, 2011; Haller, et al., 2010; Herold and Bluestone, 2011; Herold, et al., 2013; Staeva, et al., 2013; van Belle, et al., 2011). This translational inefficiency is not limited to T1D research, with calls for reproducibility and rigor in animal models of human diseases increasing (Landis, et al., 2012). Some of our own efforts have, however, yielded progress in this regard (Gill, et al., 2016), yet it is most important to note that even under the best of circumstances, animal models will obviously not fully replicate all aspects of the human disease.

In terms of human T1D, researchers are to a large extent handicapped by the need for non-invasive research techniques since the pancreas, as the target organ, is not amenable to biopsy. This said, there have been autopsy materials studied (Foulis and Stewart, 1984; Gepts, 1965; Willcox, et al., 2009) and some work on biopsies (Imagawa, et al., 1999; Krogvold, et al., 2014), but neither of these are widely used anymore for logistical and ethical reasons. This then forms the ***motivation of my work*** to study human tissue taking advantage of the JDRF sponsored Network for Pancreatic Organ donors with Diabetes (nPOD) program. This effort was established to obtain transplant grade human tissue for research (Campbell-Thompson, et al., 2012a). Specifically, we contracted with organ procurement organizations (OPO) throughout the United States to obtain tissue from donors with T1D, those that were T1D associated autoantibody positive without diabetes, and those without diabetes. As this program evolved, donors with T2D, cystic fibrosis, pregnancy and other cases were obtained as additional controls or as interests arose (www.jdrfnpod.org). The tissues procured include pancreas, pancreatic draining lymph nodes, mesenteric lymph nodes, spleen, thymus, bone marrow and peripheral blood (for isolation of both leucocytes and serum), wherever possible. These organs are shipped under specialized conditions to maximize viability and integrity. Once received, standard operating procedure (SOP) protocols are applied (Figure1.1-1) (Campbell-Thompson, et al., 2012c; Campbell-Thompson, et al., 2012d).

This ensures high quality samples for live cell analysis, standard histology, immunohistochemistry, immunofluorescence and downstream "omic" analyses (Kusmartseva, et al., 2017; Philips, et al., 2017; Pugliese, et al., 2014; Wasserfall, et al., 2016).

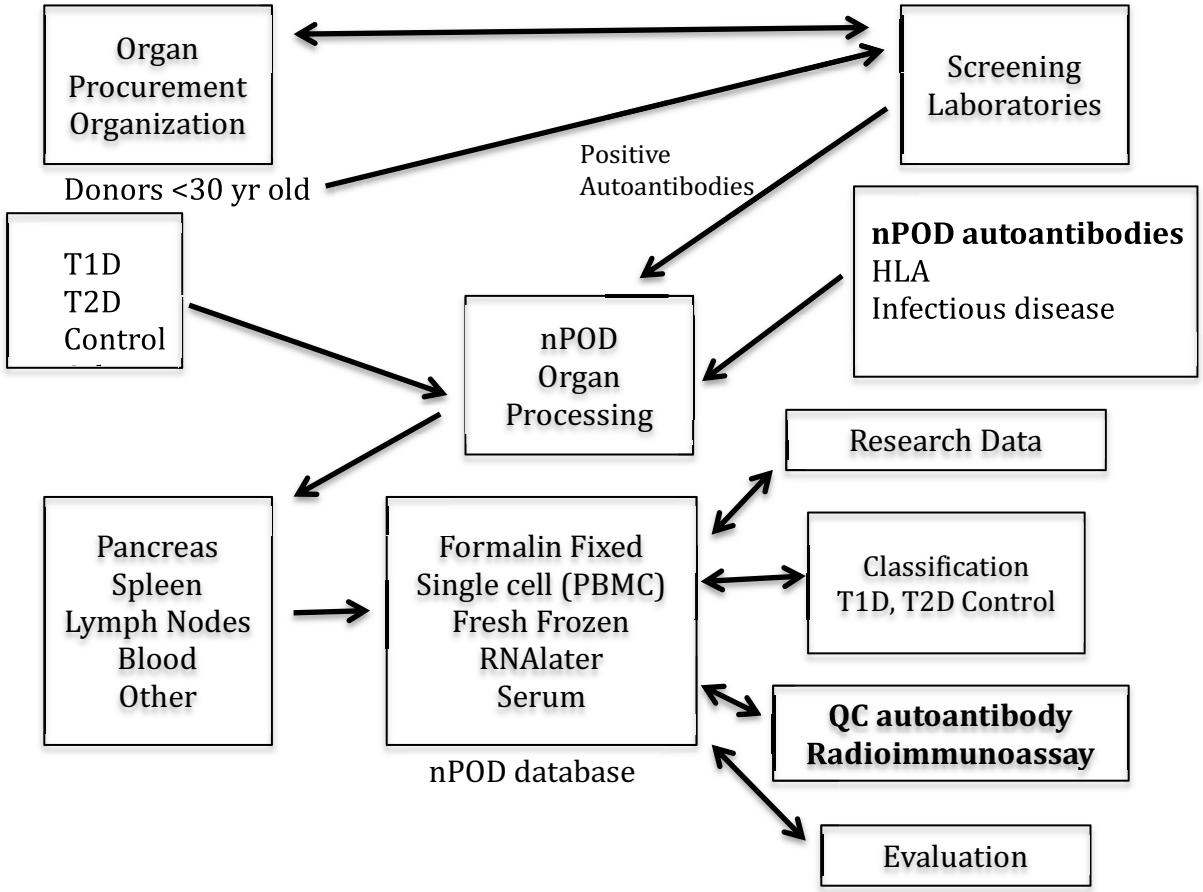

Figure 1.1-1. Organizational chart of nPOD operations. Organ procurement organizations offer the nPOD organ-processing center cases. Screening laboratories around the United States while testing for HLA and infectious diseases also screen under 30 year old donors for T1D associated autoantibodies. Positive autoantibody donors are then offered as cases to the nPOD program. Cases obtained are then processed by nPOD, with standard operating procedures and quality control processes in place as indicated in the chart.

In order to obtain at risk pre-T1D donors, we had to establish a network of screening laboratories to capture this population across the United States. A pilot effort (2005) had been undertaken (Gianani, et al., 2006) using the gold standard radioimmunoassay (RIA) for T1D associated autoantibodies. However, it became clear in 2006 with nationwide roll-out that this would not be feasible in laboratories that are not set up to handle radioactivity. This led me to pursue an enzyme linked immuno-sorbant assay (ELISA) for these autoantibodies; this, for the

purpose of both validation and implementation of an SOP based protocol (Wasserfall, et al., 2016), [CW 1].

To date, we have screened over 6,000 samples from organ donors and procured 32 autoantibody positive cases from individuals without T1D into the nPOD program. nPOD has also acquired 174 controls, 151 T1D, 41 T2D and 41 miscellaneous (pregnancy, monogenic diabetes, cystic fibrosis, etc.) cases. The nPOD group is set up as collaborative project and open to the T1D research community, which has led to approximately 170 publications using this sample resource. With these valuable tissues in hand, we have been able to address a number of long standing dogmas and ideas regarding human T1D. One of these notions is is in regard to insulitis and the immune attack on β-cells. This is well characterized in the NOD mouse model (Anderson and Bluestone, 2005) but remains elusive in human T1D (In't Veld, 2011; In't Veld, 2014). Much of the previous work on this lesion has been derived from autopsy material, pancreatic biopsies or isolated islets (Dotta, et al., 2007; Foulis and Stewart, 1984; Gepts, 1965; Graham, et al., 2012; In't Veld, 2011; In't Veld, 2014; Kent, et al., 2017; Krogvold, et al., 2014; Richardson, et al., 2014). We therefore undertook a more comprehensive study of human insulitis within the nPOD collection. Of note and to our advantage, we have: a) the whole pancreas, b) donors covering the natural history from higher-risk (i.e., autoantibody positive) through onset, and c) into established disease. Thus, we were able to not only evaluate potentially pre-diabetes pancreatic tissue but also, address issues surrounding disease duration in terms of insulitis, as well as characterization of the infiltrates. Furthermore, with immunostaining, we were able to enumerate α- and β-cell mass (Campbell-Thompson, et al., 2016a) [CW 2].

Given the relative differences in T1D infiltrates between humans and rodent models, we questioned afresh the presumed role of autoantibodies in the disorder's pathogenesis. It is well established that autoantibodies are excellent biomarkers of T1D either in diagnosis or in prediction of future disease (Kupila, et al., 2001; LaGasse, et al., 2002; Pinhas-Hamiel, et al., 1998; Schatz, et al., 1994; Siljander, et al., 2007). There are autoantibody mediated diseases such as Grave's disease where transplacental passage of IgG autoantibodies transfer transient hypo- or hyperthyroidism (Brown, 1996) as an example, and while transplacental passage of T1D associated autoantibodies has been documented, this does not appear to impact β-cell function (Hamalainen, et al., 2001; Ziegler, et al., 1993). The traditional role of antibodies in the immune system is in the neutralization of toxins and viruses as well as the opsonization of bacteria and protozoans; however, by activation of: a) the classical complement pathway; or b) natural killer (NK) cell mediated antibody dependent cellular

cytotoxicity (ADCC), cells may be killed by antibody driven events. Taken together, while evidence for autoantibody initiation of T1D is lacking, it may still be possible that participation in ongoing pathology occurs. There is at least one report of NK cell involvement by *in situ* staining for these cells in recent onset disease (Dotta, et al., 2007). We decided, however, to investigate the role that complement may have in T1D as an alternative hypothesis for autoantibody involvement in T1D progression (Rowe, et al., 2013) [CW-3].

Another longstanding dogma of T1D is that the clinical diagnosis of disease is made when hyperglycemia occurs because the functional β-cell mass has declined to a point where about 10-20% is left, and this continues to absolute loss of these cells (Figure 1.1-2). Therefore, the islets are insulin deficient and insulinopenia follows with progressive loss of C-peptide (a marker of endogenous insulin production) (Eisenbarth, 1986). While this has come under challenge and subject to modification in recent years, we were motivated to use the nPOD samples to investigate the endocrine pancreas, in particular the pathways of mature insulin production from the proinsulin molecule using immunostaining, protein extraction and gene expression studies (Wasserfall, et al., 2017) [CW 4].

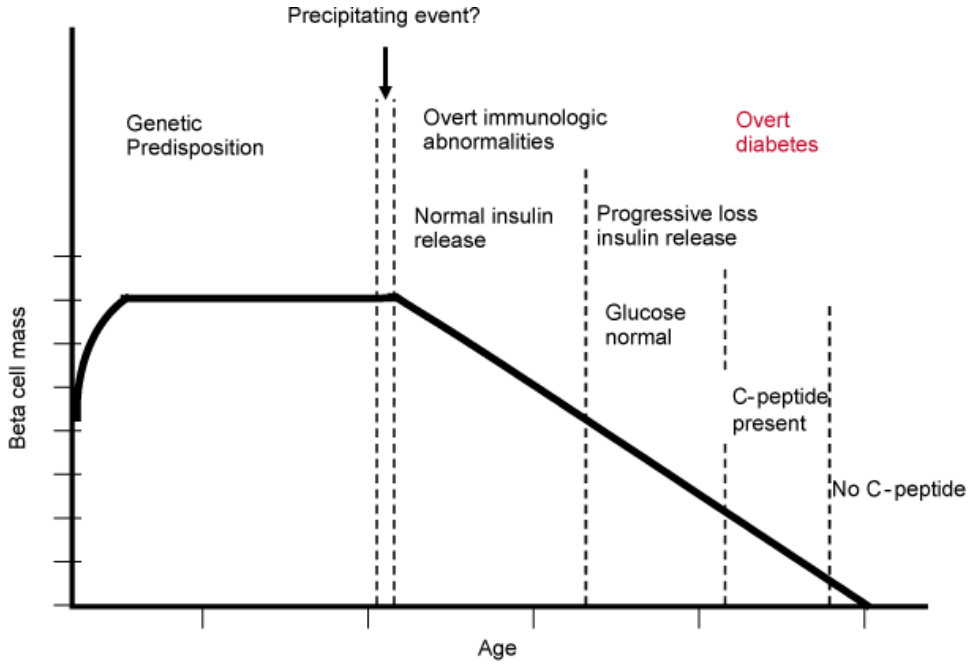

Figure 1.1-2. Model of progression to T1D. This model describes T1D as the progressive loss of beta cells following genetic and environmental events that initiate an autoimmune attack. It proposes the absolute loss of beta cells to occur in T1D. (From Eisenbarth, GS, NEJM 1986)

2 State of the Art

2.1 Type 1 diabetes is an autoimmune disease

The two major forms of diabetes account for about 5-10% (T1D) and 80-90% (T2D) of cases, respectively(Kharroubi and Darwish, 2015) . T1D is absolutely insulin requiring to sustain not only blood glucose levels but also life (Atkinson, et al., 2014). The next major event in the effort to understand etiopathogenesis was the discovery of leukocytic infiltration into the insulin producing β-cells in the islets of Langerhans within the pancreas of individuals who died at the onset of disease (Gepts, 1965). Shortly thereafter, the discovery of islet cell autoantibodies (ICA) (Bottazzo, et al., 1974) and then, the association with the human leucocyte antigen (HLA) complex (Cahill and McDevitt, 1981) set the stage for ultimately ascribing an autoimmune etiology for T1D. Subsequently, the establishment of T1D as an autoimmune disease has been corroborated by a large number of studies through the present and in part, is described in the following sections 2.2 through 2.6. Nonetheless, there is also a growing body of evidence to suggest that the end organ, in this case the β-cells of the islets, may also contribute to their own demise (Atkinson, et al., 2011; Bottazzo, 1986; Bottazzo, et al., 1988), and this is further discussed in section 2.5.

2.2 Models of type 1 diabetes

Contemporaneously to the initial findings in humans, several animal models emerged that allowed for experimentation not possible in humans. Some of these models are induced by β-cell toxicity (streptozotocin or alloxan, for example) (Yoon, et al., 1987); however, these replicate the loss of insulin but not necessarily the autoimmune nature of T1D. Others for example, the viral antigen induced model (von Herrath, 2002), are models of immune attack of the β-cells. Spontaneous models, such as the Bio-Breeding Diabetes-Prone (BBDP) rat (Yale and Marliss, 1984) and the aforementioned NOD mouse (Anderson and Bluestone, 2005), have made up the majority of the knowledge base about animal T1D and have been extensively studied (Lenzen, 2017). As discussed earlier, there are caveats and concerns regarding animal models (Landis, et al., 2012; Leiter and von Herrath, 2004; Roep and Atkinson, 2004); however, the NOD mouse in particular has served as a guide to dissect human T1D. In the NOD, autoantibodies to insulin predominate; T-lymphocytes cause destruction of β-cells; T-lymphocytes can transfer T1D into a recipient immune deficient NOD

animal; the major histocompatibility complex (MHC) is critical; insulitis begins early and leads to complete loss of insulin production; and a number of immune defects in regulation exist in this model (reviewed in (Anderson and Bluestone, 2005)). So collectively and with a critical appraisal of this model, wherein genetic manipulation can test hypotheses, we can also use this as a roadmap to address human T1D. Ultimately though, human studies have to be undertaken to understand clinical T1D.

2.3 Humoral immune responses in human type 1 diabetes

The landmark discovery of ICA (Bottazzo, et al., 1974) led to a search for the nature of the autoantigens that comprise the ICA reaction. This largely followed on from increased understanding of both physiology and technological advancements in assay design. Since radiolabelled insulin existed (Yalow and Berson, 1960) for competitive measurement of insulin, a reagent like this was used to detect autoantibodies in humans with T1D prior to the treatment with insulin (Palmer, et al., 1983). These insulin autoantibodies (IAA) along with ICA were the first to be used in both diagnosis and prediction of T1D and set the stage for some of the largest T1D prevention studies ever undertaken (Schatz and Bingley, 2001) While this was occurring, the search for other autoantigens continued, and these too were subsequently used in prediction studies (discussed in 2.6 below). The use of radioactively labeled methionine in pulse chase experiments with isolated β-cells followed by immunoprecipitation of the radiolabelled extracts with human T1D sera led to identification of autoantibodies initially called by their molecular weights (e.g., 64k, 40k or 37k) on gel electrophoresis (Atkinson and Maclaren, 1988; Christie, et al., 1990; Christie, et al., 1994; Gerling, et al., 1986; LaGasse, et al., 1997). The 64kD autoantigen was subsequently identified as glutamic acid decarboxylase (GAD) (Baekkeskov, et al., 1990), and the autoantibody (GADA) was found to be another effective marker for T1D. Phage display library approaches discovered ICA512 (Rabin, et al., 1994), while an insulinoma mRNA subtraction approach identified insulinoma associated-2 (IA-2) (Lan, et al., 1996) as autoantigens, and the autoantibody is commonly referred to as IA-2A. Upon sequencing these two autoantigens it turned out that not only were these two molecules identical, but as fragments, they were also related to the aforementioned 40kD and 37kD autoantigens (Bonifacio, et al., 1995; Payton, et al., 1995). Using an array coupled with a bioinformatics type approach, zinc transporter 8 autoantibodies (ZnT8A) were found to be present in sera from T1D subjects (Wenzlau, et al., 2007). The aforementioned molecules are considered the major targets of autoantibodies and are currently the ones used in

epidemiological studies. The specificities for these when comparing new onset T1D to control subjects are typically in the range of 95-99% while sensitivities are approximately 50% for IAA, 70-80% for GADA, 60-70% for both IA-2A and ZnT8A (Bingley, et al., 2003; Bingley and Williams, 2013; Bingley, et al., 2010; Lampasona, et al., 2011; Miao, et al., 2013; Xie, et al., 1997). However, there certainly have been a number of other autoantigens identified and probably more remain to be discovered (Mansson, et al., 2001; Roep and Peakman, 2012; Winter and Schatz, 2011). These, however, typically have lower than 40% sensitivity and are not usually included in standard screening or diagnostic applications.

Antibody mediated effector functions (Quast, et al., 2017; Valenzuela and Reed, 2017) include neutralization of toxins and viruses, opsonization of bacteria and protozoans for phagocytosis (Lu, et al., 2017), complement fixation for killing of cells (Shokal and Eleftherianos, 2017) and NK cell mediated ADCC. Another possibility is for antibodies to behave as either an agonist or antagonist as for anti-thyroid stimulating receptor autoantibodies (Brown, 1996). The potential pathogenic role of autoantibodies in T1D is unresolved, and the question remains: are they simply markers of ongoing autoimmunity or do they have a pathological significance beyond the potential role of B-lymphocytes, which not only produce antibodies but also can present antigen (Ilonen, et al., 2007; Knip and Siljander, 2016)?

These humoral responses to insulin, GAD and IA-2 have been shown to be of the IgM or IgG classes, with additional subclasses of IgG (Fuchtenbusch, et al., 2000; Hawa, et al., 2000; Hillman, et al., 2009; Hoppu, et al., 2004a; Hoppu, et al., 2004b; Ng, et al., 2002; Oak, et al., 2011; Omar, et al., 1987; Piquer, et al., 2005; Ronkainen, et al., 2006). This argues for B-lymphocyte class switching and hence, for a thymic dependent antigen (den Haan, et al., 2014). Collectively, this is a demonstration of an immune response to these autoantigens. In order for this response to occur, these autoantigens have to be presented to the effector cells of the immune response by antigen presenting cells (APC). Furthermore, $CD4^+$ T cell help is required for the class switching aspect, and this cell-mediated response is discussed in section 2.4 (den Haan, et al., 2014).

2.4 Genetics and cell mediated immunity in human type 1 diabetes

Similar to the autoantibody discoveries, the role of cellular immune responses followed technological innovations along with insights and progress into unraveling mechanisms of the overall immune response. In terms of T1D, firstly, the descriptions of cellular infiltrates into the

islets of langerhans essentially laid the foundation of cellular immune responses being involved in T1D (Foulis and Stewart, 1984; Gepts, 1965; Karamanou, et al., 2016). Secondly, the association of the MHC or HLA with T1D (Cahill and McDevitt, 1981) and the discovery that this set of molecules dictates the presentation of antigen peptides to the immune system firmly cemented the relationship of cell-mediated immunity with this disorder (Doherty and Zinkernagel, 1975; Zinkernagel and Doherty, 1974a; Zinkernagel and Doherty, 1974b). It is known that in the thymus HLA class I restricts the immune response of CD8 positive T-lymphocytes while HLA class II restricts the response of CD4 positive T-lymphocytes (Kondo, et al., 2017). Following this process of positive selection, the $CD4^+$ or $CD8^+$ T cells undergo a round of negative selection. This entails promiscuous self antigen presentation to these emerging T cells and the subsequent deletion of cells reacting to self, or in another pathway the development of T regulatory cells (Treg) (Kondo, et al., 2017). Outside of this central tolerance program there exist multiple ways in which tolerance occurs in the periphery. This so-called peripheral tolerance involves checkpoints in the immune response, cytokines and soluble mediators, peripherally induced Treg, apoptosis mechanisms and others (Danikowski, et al., 2017; Frydenlund and Mahalingam, 2017; Theofilopoulos, et al., 2017).

How then collectively do T cells arise that recognize β-cell specific antigens? Part of the answer lies in decades of work implicating failures in both central and peripheral tolerance mechanisms, yet the complete story has not been revealed. Why particular MHC alleles associate with risk from a functional standpoint is largely unknown and mostly speculative; however, a genetic region known as the insulin variable number of tandem repeats (VNTR) has been shown to modulate proinsulin expression in the thymus, potentially pointing to a failure in central tolerance as well as explaining some antigenic specificity for T1D (Pugliese, et al., 1997). Approximately 50 loci in the human genome appear to be associated with T1D (Cooper, et al., 2012; Onengut-Gumuscu, et al., 2015; Rich, et al., 2012), and these are largely found to be immune-centric, implying perhaps, amongst other things, peripheral tolerance defects in immune responses. However, the functional link of these putative loci to causation of T1D remains elusive.

The immune response culminates in an effector phase, and we classify these according to the pathway used. The entire immune response is usually involved in all responses, but it is the effector phase that leads to elimination of a foreign invader or in some instances, pathology to the host. For example Type 1 hypersensitivity is the IgE mediated release of mast cell contents leading to allergic responses (Reber, et al., 2017). In T1D, it is thought that Type IV (delayed hypersensitivity) effector $CD8^+$ T-lymphocytes mediate responses that lead the death

of β-cells (Knight, et al., 2015; Zaldumbide, et al., 2013). Unlike B-lymphocytes, which produce antibodies that recognize whole protein in native configuration, T-lymphocytes recognize small peptides presented by MHC. As described earlier, $CD8^+$ T-lymphocytes are restricted by MHC class I, while the major risk allele in T1D is MHC class II which implicates $CD4^+$ T-lymphocytes. The role of $CD4^+$ T-lymphocytes in providing signals to B-lymphocytes to effect class switching (den Haan, et al., 2014) is one possibility, or it is possible that $CD4^+$ T-lymphocytes produce cytokines (Amrani, et al., 2000; Calderon, et al., 2006; Padgett, et al., 2013) that are toxic to β-cells. The evidence for these as well as $CD8^+$ T-lymphocytes is discussed in the following section 2.5.

The vast majority of studies on human cell mediated immunity in T1D have been conducted on peripheral blood leukocytes. This has proven much more challenging than the autoantibody assays. The parallels to these efforts include development of blinded workshops for T-lymphocyte assays (Brooks-Worrell, et al., 2011; James, et al., 2011; Mallone, et al., 2011a; Mallone, et al., 2011b), and while this has helped standardize aspects of these assays, they are still not as robust as the autoantibody assays. This limitation has been attributed to several reasons, one of which is that serum can be stored and shared much more readily than T-lymphocytes for workshops. However, more compelling is that a) the nature of the antigen/peptide is not known, and b) the frequency of these cells in the periphery is very low (Roep, 1996). Nonetheless, progress has been made in these directions, and with more modern approaches in assay technology, such as dye dilution assays, tetramer analyses, and cytokine secretion in response to autoantigen(s), have revealed specific T-lymphocytes in the peripheral blood of T1D patients (Mannering, et al., 2010). More recently, notions of altered peptide processing for presentation by APC have come to the fore, including post-translational modification (Mannering, et al., 2005), hybrid peptide formation (Delong, et al., 2016), and other means (Kracht, et al., 2017; McLaughlin, et al., 2016).

To date, several peptide antigens including hybrid peptides have been shown to either, stimulate or by tetramer staining, enumerate T-lymphocytes from T1D subjects, and they indeed are in low frequency in the periphery. The list includes but is not limited to, for $CD4^+$ cells: preproinsulin, GAD65, GAD67, IA-2, phogrin, islet cell antigen 69, 38k antigen and heat shock protein 60; for $CD8^+$ cells: GAD65, IA-2, islet amyloid polypeptide (IAPP) and islet-specific glucose-6-phosphatase catalytic subunit-related protein (Roep and Peakman, 2012).

On the other side of these effector mechanisms is immunoregulation. This similarly has been the subject of intense research with some conflicting results. In particular, $CD4^+CD25^+$ Treg cells have either been found to be in reduced frequency (Brusko and Atkinson, 2007;

Tree, et al., 2006), reduced function (Garg, et al., 2012; Lindley, et al., 2005; McClymont, et al., 2011; Visperas and Vignali, 2016) or not different at all (Brusko, et al., 2007; Hamari, et al., 2016), while others have found increased resistance of effector cells to immunoregulation (Schneider, et al., 2008), when comparing blood from controls and T1D subjects. Yet other studies have found B-lymphocyte regulation (Kleffel, et al., 2015), myeloid derived suppressor cell regulation (Whitfield-Larry, et al., 2014), checkpoint type defects (Andre, et al., 1996; Dai, et al., 2014; Paterson, et al., 2011) and other immunoregulatory issues (Fuhrman, et al., 2015; Roep and Tree, 2014) in T1D subjects.

There are, as described, multiple lines of evidence for humoral and cellular immune events in the progression to T1D. Some of these have been documented or confirmed by using islets, biopsies, autopsies and organ donor tissue of pancreas and pancreatic draining lymph nodes, which will be discussed in the following section 2.5.

2.5 The lesion in human type 1 diabetes

Ultimately, the effector phase of this autoimmune response must take place in the pancreas with the killing of islet cells and in particular, β-cells. In fact, the remarkable precision of this process with α- and δ- cells being largely left unscathed is further evidence of a specific autoimmune event. While animal models have informed us of some of the events in the demise of β-cells, it has been controversial and perhaps sometimes misleading, as has been described earlier. The number of T1D human pancreas cases examined is astonishingly small, relatively speaking. However, between earlier autopsy (Foulis and Stewart, 1984; Gepts, 1965), limited biopsy (Imagawa, et al., 1999; Krogvold, et al., 2014), islet isolation (Kent, et al., 2017) and organ donor (Campbell-Thompson, et al., 2012a; Gianani, et al., 2006) efforts, there is sufficient number to begin addressing events in the actual lesion of T1D.

Collectively, these results have yielded that in general, insulitis occurs in younger donors, closer to onset of disease, and in those with multiple autoantibodies (Foulis, et al., 1986; Gianani, et al., 2006; In't Veld, 2011; In't Veld, et al., 2007; Krogvold, et al., 2014; Wiberg, et al., 2015). The nature of this insulitis has been addressed a few different ways. By tetramer staining of pancreas from nPOD cases (Coppieters, et al., 2012) at the inception of the program, it was discovered that CD8 positive cells specific for insulin, IGRP and IA-2 could be found in distinct islets from individuals with T1D. Along with the CD8 positivity, this group also demonstrated MHC class I as being highly expressed on islets in T1D pancreata, which suggests that these infiltrates are in all likelihood, effector cells killing β-cells. This finding of

MHC class I increased expression in T1D pancreas has been replicated in other studies (Richardson, et al., 2016) while yet others dispute this finding (Skog, et al., 2015). The reason for increased expression of MHC class I on islets is unresolved, and it is speculated that a virus (Rodriguez-Calvo, et al., 2016) or elements of the viral response pathway may initiate this response (Newby, et al., 2017; Newby and Mathews, 2017). Another way this has been interrogated is by cloning T cells out of islets isolated from T1D donors (Kent, et al., 2017; Pathiraja, et al., 2015). The advantage of this approach is the ability to then perform live cell assays (Newby, et al., 2017) by growing these clones of cells. The antigen specific receptors on these cells can also be sequenced (Michels, et al., 2017) and then, for example, the T cell receptor (TCR) can transgenically be recreated for additional experiments (Babad, et al., 2015; Sprouse, et al., 2016). APCs, $CD4^+$ or $CD8^+$ T cells may also produce cytotoxic cytokines and contributing to β-cell dysfunction/loss in this manner (Eizirik, et al., 2012).

The presence of insulitis, while not near the intensity of that found in animal models, begs the question of what functional/effector pressure is exerted on the β-cell? Included in this equation then also is the question of how the milieu and β-cell contribute to the overall outcome of loss of insulin leading to loss of glucose homeostasis?

The primary function of β-cells is to react to an increase in blood glucose (Jouvet and Estall, 2017). This is achieved by integrating actual glucose levels by receptor-mediated events along with incretin signals from the gut and neuronal signals from the brain (Hussain, et al., 2016). An increase in ATP from glucose metabolism in the cytosol of the β-cell allows potassium channels to open, and along with calcium influx, this leads to exocytosis of granules with stored insulin, C-peptide and IAPP secreted into circulation (Yang, et al., 2014). This allows rapid release of insulin followed by a period of replenishing these molecules. Proinsulin and pro-IAPP, once translated from mRNA on ribosomes, are passaged through the endoplasmic reticulum and are then both processed by the Proprotein Convertase Subtilisin/Kexin Type 1 (PCSK1) and Proprotein Convertase Subtilisin/Kexin Type 2 (PCSK2) enzymes into insulin and C-peptide from proinsuilin and IAPP from ProIAPP (Figure 2.5-1 (A)). The enzyme carboxypeptidase E (CPE) then cleans up lysine and arginine residues on the ends of the mature insulin and IAPP molecules post convertase cleavage (Alarcon, et al., 2012; Hutton, 1994; Smeekens, et al., 1992; Steiner, 2011; Steiner, et al., 1969). Insulin and C-peptide then are logically in equivalent molar amounts while IAPP is independently co-secreted with insulin. This is useful for us in that C-peptide is often measured as a surrogate for insulin in β-cell function assays, especially in T1D subjects, since the exogenous therapeutic recombinant insulin does not contain C-peptide (Steiner, 2011). In α-cells, proglucagon is

processed by only the PCSK2 and CPE enzymes to form mature glucagon (Figure 2.5-1 (B)). If PCSK1 is present, as in the L-cells of the intestine, proglucagon will be processed into glucagon like peptide 1, glicentin and oxyntomodulin (Figure 2.5-1 (C)) (Tucker, et al., 1996).

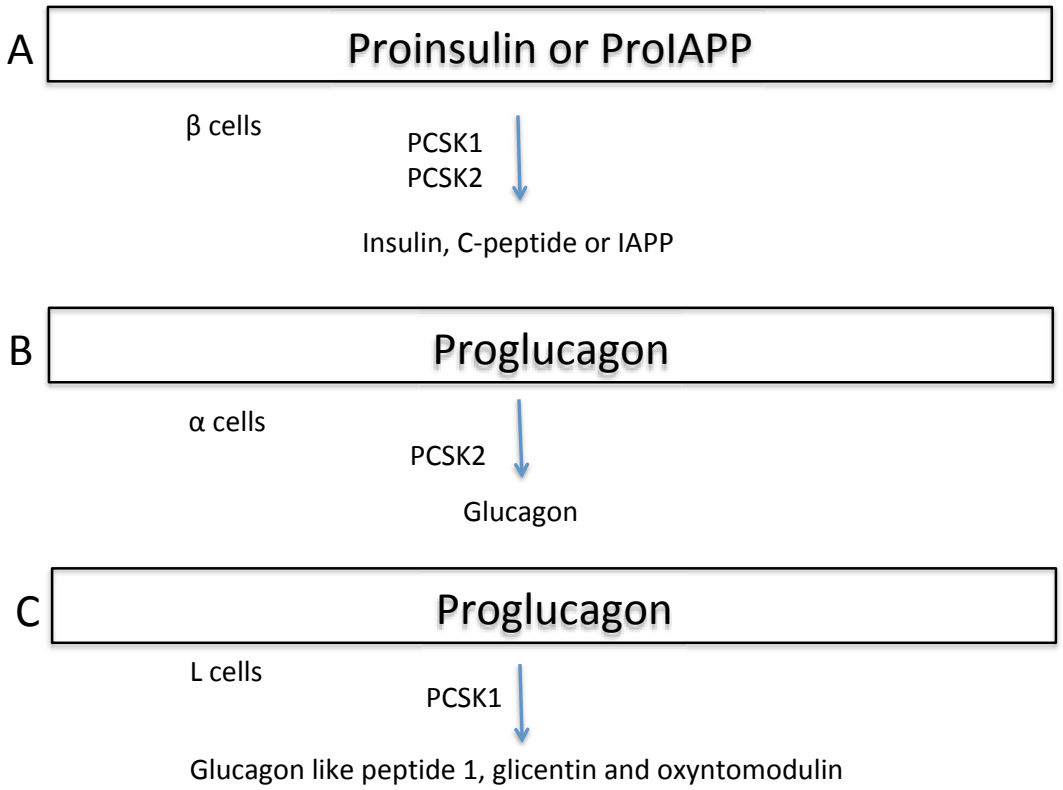

Figure 2.5-1 Prohormone processing of (A) Proinsulin or ProIAPP to either insulin and C-peptide or IAPP respectively in β cells. (B) Proglucagon into glucagon in α cells. (C) Proglugagon into glucagon like peptide 1, glicentin and oxyntomodulin in L cells.

In T2D, it is thought with increasing insulin resistance the output of the β-cells is greater than processing and this leads to increased proIAPP and proinsulin in circulation. ProIAPP acts as a nidus to attract IAPP with tangles of this protein then forming amyloid leading to further β-cell failure (Jaikaran and Clark, 2001; Montane, et al., 2012; Raleigh, et al., 2017). Stress in the endoplasmic reticulum has also been noted as a feature in T2D (Herbert and Laybutt, 2016; Liu, et al., 2007; Ozcan and Tabas, 2016; Wang, et al., 2016). Whether this is something that occurs in T1D is unclear but certainly is a concept getting increasing attention (Brozzi and Eizirik, 2016; Engin, 2016; Zhong, et al., 2012). This potentially creates a complex milieu of β-cell stress/dysfunction and autoimmunity collaborating to result in T1D

2.6 Prediction of type 1 diabetes in the general human population

Since the discovery of autoantibodies (Bottazzo, et al., 1974) in T1D, decades of work have gone into using these markers as a means to predicting this disorder (Bonifacio, 2015). Starting with ICA (Bottazzo, et al., 1974) and IAA (Palmer, et al., 1983), these have been used to predict T1D in first-degree relatives as well as in the general population. As GADA (Baekkeskov, et al., 1990), IA-2A (Rabin, et al., 1994) and ZnT8A (Wenzlau, et al., 2007) were discovered, they too were used in prediction. As a number of cohorts have now been followed from birth to onset of T1D, various prediction strategies can be both prospectively and retroactively tested (Bonifacio, et al., 2004; Krischer, et al., 2017; Rewers, et al., 1996a; Rewers, et al., 1996b; Vehik, et al., 2016; Ziegler and Bonifacio, 2012). In general, seroconversion to autoantibody positivity usually occurs after maternal antibodies have waned at around 6 months of age (Stanley, et al., 2004; Ziegler, et al., 1993). The order in which these autoantibodies appear is IAA, then GADA, while IA-2A and ZnT8A are very specific and increase closer to onset (Bonifacio, 2015). However, this is a generalization and not always the case. The affinity titer and number of different autoantibodies also matter in terms of predicting future T1D. Here, increased titer (Bonifacio, et al., 1990), higher affinity (Achenbach, et al., 2004) and greater number (Ziegler, et al., 2013) of different specificities of autoantibodies serve to enhance the specificity of prediction at the cost of decreased sensitivity (Giannopoulou, et al., 2015). Using orthogonal assays usually also improves specificity (Bonifacio, 2015).

While 80-90% of individuals with an incident diagnosis of T1D do not have a family history of the disease, having a family member does elevate risk for disease (Bonifacio, 2015). This has led to the use of family cohorts to enrich study cohorts to find pre-diabetes subjects. Clearly there is genetic involvement, but this is of a polygenic nature and not that of simple Mendelian inheritance. As mentioned earlier, HLA has the highest predictive value genetically for T1D, and around 50 or more genomic polymorphisms have been identified to be associated with risk for this disease (Cooper, et al., 2012). While HLA is predominant in these risk assessments, it has been possible to mathematically weight the other genetic polymorphisms to devise and improve a risk score (Frohnert, et al., 2017; Oram, et al., 2016; Patel, et al., 2016; Winkler, et al., 2014; Winkler, et al., 2012a).

It is worth mentioning that mutations in forkhead box P3 (FOXP3) (Verbsky and Chatila, 2013) and autoimmune regulator (AIRE) (Bruserud, et al., 2016) while rare, are involved in tolerance induction and lead to T1D with high certainty along with multiple other

autoimmunities. These particular defects are part of syndromes and not part of this discussion. Secondly, environment clearly plays a part in T1D progression and maybe even initiation, but this has been difficult to pin down let alone use for prediction. The evidence is several fold: for example, concordance for T1D in identical twins was initially thought to be about 34% (Olmos, et al., 1988) but may reach 70% with sufficiently long follow-up (Redondo, et al., 2008), implying that environmental factors may effect rates of progression. Another example would be the different prevalence and incidence in neighboring countries such as Finland and Estonia (Tuomilehto, et al., 1991), which are genetically similar but differ in environment and socioeconomics, with the implication that environmental factors modulate T1D initiation and/or progression. Agents such as Vacor (Esposti, et al., 1996) that are β-cell toxic will also lead to insulinopenia although through toxicity without autoimmune involvement. These are all not commonly useful for prediction especially in sporadic T1D. Similarly, the increased use of checkpoint inhibitors in cancer immunotherapy has increased the risk of autoimmunity, including T1D, in subjects receiving this therapy, and this is useful to know in these particular instances but not a factor in prediction of regular autoimmune T1D (Yoest, 2017).

The use of T-lymphocytes in specific predictive assays has yet to yield efficacy in screening assays for future T1D. This is an area that is actively being researched with, for example, direct typing and then imputation of the amino acid sequences of the TCR (Seay, et al., 2016) which may prove predictive of T1D in the future by finding these rare cells in the periphery.

On the metabolic side, factors like rising glycation of proteins such as hemoglobin alpha 1 fraction c (HbA1c) levels and alterations β-cell function are specific but not sensitive for prediction of impending T1D (Vehik, et al., 2012). How best to incorporate these in to predictive scores would seem to be a composite score of oral glucose challenge test results with glucose and C-peptide levels (Greenbaum, et al., 2011; Sosenko, et al., 2015a; Sosenko, et al., 2015b). Ongoing work is suggestive of altered ratios of proinsulin to either insulin or C-peptide may precede the onset of T1D (Sims, et al., 2016; Van Dalem, et al., 2016). Markers of β-cell death such as a so-called liquid biopsy of serum measuring the ratio of demethylated to methylated insulin DNA may offer another marker once validated (Akirav, et al., 2011; Lehmann-Werman, et al., 2016). Additional serum markers are continuously being sought, and again a composite score of multiple serum markers (e.g., metabolomics, proteomics, etc.) may be beneficial in improving the prediction of future T1D (Overgaard, et al., 2016; von Toerne, et al., 2017).

The importance of predicting T1D lies in the eventual goal of preventing this disorder. Secondly it has also been shown that an awareness of potentially impending T1D has

improved timely diagnosis if and when the disease declares itself (Chan, et al., 2015; Winkler, et al., 2012b). While most of the work described above is within families with a T1D proband, for the most part, the same findings hold true for general population screening. The difference being the population prevalence is even lower than in families, and this adds to the difficulty in terms of positive predictive values. However, with a multiplicity of factors we can predict future T1D in the general population (Batstra, et al., 1997; Batstra, et al., 2001; Bingley, et al., 1993; Gianani, et al., 2006; Knip, et al., 1998; Knip, et al., 2010; Kupila, et al., 2001; LaGasse, et al., 2002; Maclaren, et al., 2003; Schatz, et al., 1994; Ziegler, et al., 2013). We have used this collective understanding of prediction to attempt to find pre-diabetes in the general ***organ donor*** population. This is in order to obtain and study the pancreas of individuals who may be truly pre-T1D. This represents a challenge on several fronts but primarily, because the prevalence of T1D in the USA general population is 1:250 and since these are organ donors, we are looking at a cross-section without being able to monitor conversion to additional autoantibodies or metabolic changes to know if T1D would ever occur (Atkinson, et al., 2014). So we rely on the natural history studies, including those from the general population, in assessing risk. Currently, logistically the best we can use is HLA (used for tissue matching in transplant and hence available for organ donors), a screen for multiple autoantibodies and sometimes HbA1c (if available) to recover pancreata from organ donors at presumptive increased risk for T1D. *Post hoc* additional genetic scoring, perhaps histology and orthogonal autoantibody assays can be applied to confirm risk for T1D. Ultimately, we also hope that pre-diabetes, T1D and control tissues obtained by the nPOD program can not only find targets for therapeutic intervention but also, provide information relevant to future risk assessments.

3 Objectives

The study of human pancreas at various stages of the progression to T1D is the overall objective of this study. In order to achieve that, access to relevant tissues is required, as well as having appropriate protocols established to evaluate the *hypothesis that T1D is autoimmune in nature but that the β-cells may contribute to their own demise*. The following specific aims were used to achieve that goal.

Specific Aims

1. Establish a screening program to acquire human tissue from organ donors with type 1 diabetes associated autoantibodies.

The overall structure of the nPOD program (Fig 1-1), as originally planned, included the establishment of autoantibody screening in order to acquire pancreas from individual donors at risk for T1D, who do not have a diagnosis of hyperglycemia or diabetes.

(1) I modified a commercially available ELISA to measure GADA, IA-2A and ZnT8A in three hours so that results would be available in a time frame suitable for decisions to be made about organ placement.
(2) I set up and trained laboratories around the USA to cover a large pool of potential organ donors.
(3) I implemented screening and quality control programs which we used to fine-tune the reporting and performance of the assay.
(4) We acquired pancreas samples from donors that were controls without diabetes, autoantibody positive donors without diabetes, and those who had T1D or T2D.

2. Evaluate the role of insulitis and complement in human type 1 diabetes.

While the progression of insulitis is well characterized using animal models of disease this is less so in the case in human T1D. This aim addressed both the frequency/extent of insulitis throughout the natural history of T1D and the potential role of humoral immune responses in T1D.

(1) We set up staining protocols to evaluate immune cell infiltrates in control, autoantibody positive without diabetes, and T1D organ donor pancreas samples.
(2) We used standardized criteria from a pathology consensus manuscript.
(3) Imunohistochemistry stains were used to quantify α-cell (glucagon) and β-cell (insulin) mass.

(4) Metadata associated with each case, such as age of disease onset, disease duration, number and type of autoantibodies, HLA and diabetic ketoacidosis (DKA) were correlated with insulitis or α- or β-cell mass.

(5) We stained pancreas cryosections for complement component 4d (C4d) from control, autoantibody positive without diabetes, T1D and T2D donors.

(6) We evaluated the C4d staining in the context of disease, HLA and autoantibody type and frequency.

3. Address the loss of endocrine hormones in the pancreas of humans with type 1 diabetes.

(1) We stained human pancreas by both immunofluorescence and immunohistochemistry techniques for proinsulin, insulin and glucagon.

(2) We performed in situ hybridization for insulin mRNA on cryosections of human pancreas.

(3) We measured total protein, proinsulin, insulin, C-peptide, glucagon and IAPP following acid ethanol extractions of thick (3x50μm) pancreas cryosections.

(4) We designed primers and performed real-time quantitative polymerase chain reaction (RT-qPCR) on extracted Ribonucleic Acid (RNA) from flash frozen pancreas blocks of tissue.

(5) We analyzed and annotated immunostained pancreas tissue slides to semi- quantify the number of β-cells in islets and in isolated clusters or single cells in the whole section.

4 Own Research

4.1 Screening organ donors for type 1 diabetes autoantibodies is feasible.

The nPOD program, as previously described (Campbell-Thompson, et al., 2012a; Pugliese, et al., 2014), was set up to obtain human tissue from organ donors with T1D, those at risk for T1D, and matched controls. The goals of my effort were to specifically optimize and implement the autoantibody-screening (Wasserfall, et al., 2016) [CW1] program to find organ donors at risk for T1D. The logistics of this program entailed a) finding screening partners and b) choosing an assay format that could be widely used. The screening partners that made most sense were laboratories already screening serological samples from OPOs; however, this decision eliminated the gold standard radioimmunoassay for autoantibodies (Bingley, et al., 2003; Bingley and Williams, 2013; Bonifacio, et al., 1988; Brooking, et al., 2003; Gianani, et al., 1995). I therefore tested at that time a newly available ELISA for GADA (Brooking, et al., 2003) and IA-2A (Chen, et al., 2005) autoantibodies and then, subsequently ZnT8A (Petruzelkova, et al., 2014). We used Receiver Operating Characteristic (ROC) analysis to set cutoffs for each ELISA that were highly specific (Fig 1 [CW1]). This was done for reasons of reducing false positives so as to maximize nPOD resources and to avoid potentially removing organs from the transplant pool.

With these assays established, we transferred the technology to OPO screening laboratories across the United States of America. As the program grew, we made adjustments to these assays and training procedures, and as shown in (Fig 2 [CW1]), we improved concordance with the ELISA and the gold standard RIA along with increasing the number of individuals screened. We reported this effort in 2016, but the program continues and thus far as of 2017, we have screened approximately 6000 samples and have 32 confirmed autoantibody positive donors.

An important aspect of this effort is one of quality control. We have entered these ELISA assays into the Diabetes Autoantibody Standardization program (DASP) (Bingley, et al., 2003), subsequently renamed Islet Autoantibody Standardization Program (IASP) (Amoroso, et al., 2016), over the entire course of the nPOD project. The ELISA assays have maintained a high level of specificity 99-100% for each analyte as well as good sensitivity 62-82% (Table 1 [CW1]). Additionally, the screening assay is confirmed with an orthogonal RIA, which is also the gold standard. Direct comparison of these two formats in autoantibody positive subjects with or without T1D reveals a majority concordance, yet there remain some differences (Fig 3

[CW1]). This is attributed speculatively to differences in epitopes or simply false positives. Nonetheless, the nPOD program has decided to maintain the RIA as the gold standard and as the confirmatory assay.

Finally, we have access to the screening assay serum sample as well as the serum sample obtained at the time of recovery of the organs for research (Campbell-Thompson, et al., 2012a). The amount of time that has elapsed between screening and recovery varies form hours to days. We found in two cases that the screening serum sample was positive but the recovery sample was negative in both the ELISA and RIA autoantibody assays (Fig 4 [CW1]). This we attributed to management of the patient in the ICU with drugs, fluids and transfusions and concluded that these parameters could potentially impact results of the autoantibody analyses. In the majority of cases we have confirmed autoantibody screening results and have obtained high quality human pancreas tissue for study by the wider diabetes community. The ELISA format is suitable for these screening efforts.

4.2 The insulitis lesion in humans reveals the heterogeneity of type 1 diabetes.

The nPOD project opens up studies of the human T1D condition in the modern era; however, not to be overlooked are earlier efforts (Foulis and Stewart, 1984; Gepts, 1965; Richardson, et al., 2014). These efforts aided in establishing T1D as a condition of infiltrating leukocytes and together with the discovery of autoantibodies (Bottazzo, et al., 1974) and HLA (Cahill and McDevitt, 1981) associations, suggested an autoimmune origin for this disease. These earlier efforts were performed on cadaveric post mortem subjects whereas nPOD obtains transplant grade tissues (Campbell-Thompson, et al., 2012a). Additionally, nPOD enables study of tissues available from autoantibody positive subjects without T1D, who may have been pre-diabetic. Some pressing questions surround the extent of insulitis throughout the natural history of the disease; therefore, our aims for this project were to conduct an extensive survey of the nPOD tissues for insulitis and β-cell mass ((Campbell-Thompson, et al., 2016a) [CW 2]).

We first determined insulitis frequency (islets with insulitis / total islets) as described elsewhere (Campbell-Thompson, et al., 2013). A total of 80 organ donors with T1D, 18 autoantibody positive donors without diabetes and 61 autoantibody negative control donors were evaluated (Supplementary Table 1 [CW2]). Of these 23% of T1D and 11% of the autoantibody positive without T1D had evidence of insulitis while none of the autoantibody negative donors had insulitis (Table 1 [CW2]).

We noted variability in the lobular distribution of both insulin positive islets and insulitic islets within the T1D cohort (Fig 1 [CW 2]); furthermore, the number of $CD3^+$ cells per islet varied in a given donor (Fig 2 and Supplementary Fig 2 [CW 2]). Of interest in the autoantibody positive subjects, insulitis was restricted to primarily insulin containing islets (Supplementary Fig 1 [CW2]), while all 18 T1D donors with insulitis had insulin containing islets in at least one section.

Not unexpectedly prevalence of insulitis correlated with T1D duration in those with the disease, but not with age of onset or age at demise (Fig 3 A-C [CW2]). Additionally, no correlations of insulitis with body mass index (BMI), ethnicity or sex could be found (Supplementary Table 3 [CW 2]. As far as hospital management, length of stay did not correlate with insulitis, but there was a significantly higher proportion of donors with insulitis who had DKA during the course of their management (47% vs. 7%, p=0.04) [CW2]. The high risk HLA genotypes DR3/DR4 were noted to occur with higher frequency in the T1D donors compared to both autoantibody negative and positive donors without diabetes. This is not unexpected, and no relationship could be found with insulitis (Supplementary Table 3 [CW2]). Interestingly, one T1D subject had the protective HLA DQ06 allele and had insulitis. The two autoantibody positive donors without diabetes with insulitis had intermediate to higher risk HLA DR7/18 DQ02/04 and DR4/- DQ8/-, respectively.

In evaluating the characteristics of the infiltrating leukocytes by multi-color immunofluorescence (Fig 4 [CW2]), we found that the lymphoid series of T and B cells ($CD45^+$, $CD3^+$, $CD4^+$, $CD8^+$ and $CD20^+$) were proportionate to each other and proportionate to the insulitis frequency (Fig 5 [CW2]). There was, however, variability when comparing islet to islet and donor to donor, highlighting the heterogeneity of these infiltrates. Additionally, the myeloid series of cells ($CD68^+$ and $CD11c^+$) did not correlate with insulitis frequency (Supplementary Fig 4 and Supplementary Table 4 [CW2]).

Since pancreatic weight data were available and we had stained for insulin and glucagon, we could estimate α- and β- cell mass. Similar to our previous findings (Campbell-Thompson, et al., 2016b), the weight of the pancreas was decreased in T1D subjects relative to growth (age) and compared to those without T1D (Fig 6 and supplementary Fig 5 [CW2]). Not unexpectedly, β-cell mass was decreased in donors with T1D compared to those without T1D; however, within the T1D donor group those with insulitis had higher β-cell mass than those without insulitis (Fig 6 [CW 2]). We found no differences in α-cell mass across all donor groups tested (Fig 6D and E [CW2]).

4.3 Complement deposition in the human pancreas is not specific to islet vasculature.

While the general consensus is that autoantibodies are excellent biomarkers of T1D, they are not considered pathogenic (Ziegler, et al., 1993). However, given the observed relative paucity of insulitis we decided to investigate aspects of humoral immunity in human pancreas of T1D donors. We took the approach used by the transplant community (Jen, et al., 2012) with the goal of investigating antibody-mediated complement activation ((Rowe, et al., 2013) [CW 3]). Specifically, we immunostained C4d in pancreatic cryosections from 11 T1D, 16 autoantibody positive without T1D, 11 autoantibody negative without diabetes, and 7 T2D subjects.

In all of these groups if C4d staining was detected, it was either on the endothelium of small blood vessels or on the endothelium/extracellular matrix of larger vessels or adventitia of ductal structures (Fig 1A [CW 3]). Total C4d was expressed as percentage pixels positive using image analysis software. This was independently confirmed by an expert pathologist, and the Spearman rank correlation between the automated pixel count versus the pathologist rank was $r=0.86$, $p<0.001$. A significant difference between the groups was found, with T1D subjects having more C4d deposition (Fig 1B [CW 3]). Furthermore, ROC analysis yielded a sensitivity of 81.8% and specificity of 94.4% in discriminating autoimmune diabetes from T2D and no diabetes (Fig 1B [CW 3]).

We observed lower mean C4d deposition in T1D subjects with insulitis (17.8 ± 4.3%, n=5) than those without insulitis (31.4 ± 5.7%, n=6); however, this did not reach statistical significance ($p=0.08$). While as a group the autoantibody positive without diabetes did not differ from either the T2D or autoantibody negative without diabetes, (Fig 1B [CW 3]) there were two autoantibody positive subjects with higher C4d deposition one of whom also had insulitis. There were no correlations with either a) frequency or b) type of autoantibodies for any of the studied groups nor any hospital management metadata (ICU time, downtime or cause of death) [CW 3].

4.4 The pancreas of individuals with type 1 diabetes shows progressive loss of insulin, but proinsulin persists

The standard model of T1D (Eisenbarth, 1986) posits that a steady decline in β-cell mass over time eventually leads to clinical diagnosis of diabetes with loss of glucose homeostasis. Our aims in this project were to investigate the endocrine hormones and processing pathways

throughout the natural history of T1D ((Wasserfall, et al., 2017) [CW 4]). A total of 106 pancreas samples from organ donors comprising 50 controls without diabetes, 16 autoantibody positive without diabetes, and 40 T1D subjects were used in this study (Table 1, [CW 4]).

We performed multicolor immunofluorescence (IF) for proinsulin, insulin and glucagon (Fig 1 [CW 4]) on representative formalin fixed paraffin embedded (FFPE) pancreas sections from control and T1D subjects with 1, 2 and 7 years of disease duration. The control sections not only revealed abundant islets with proinsulin, insulin and glucagon staining (Fig 1A, F, K and P [CW4]) but also, random single cells in the exocrine regions also staining for these hormones (Fig 1A inset [CW 4]). In the case of T1D with varying durations, these sections reveal heterogeneity in progression of disease with glucagon dominant islets along with single cells positive for each hormone individually (Fig 1 [CW4]).

We followed this with acid-ethanol extractions of thick (3x50µm) cryopreserved pancreatic sections from 31 controls, 16 autoantibody positive without diabetes, and 24 T1D subjects. We measured proinsulin, insulin, C-peptide and glucagon by ELISA and islet amyloid polypeptide (IAPP) by Luminex magnetic bead based assays. Within the autoantibody positive group, there were 12 single and 4 multiple autoantibody positive subjects (Table S2 [CW 4]). The extracted proinsulin, C-peptide and insulin levels were not related to frequency of autoantibodies and therefore, these subjects were grouped together and analyzed as an autoantibody positive cohort.

In the T1D group 5/24 individuals had extracted insulin levels similar to control levels, but 17/24 samples were at the lower end of the standard curve, and a further 2/24 had no detectable insulin. By comparison, the controls and autoantibody positive without diabetes tissues had significantly higher levels of insulin (Fig 2A [CW4]). Proinsulin levels, however, were detectable in all extracts from each group including the T1D subjects. The median proinsulin levels were significantly lower in the T1D group relative to controls but still measurable (Fig 2B [CW]). C-peptide levels were significantly lower in T1D pancreas extractions compared to control and autoantibody positive samples (Fig 2C, [CW 4]). Taken together, when analyzing a ratio of proinsulin to insulin or proinsulin to C-peptide, a significant difference was revealed between T1D and controls or autoantibody positive subjects (Fig S1B-C [CW 4]).

IAPP is co-secreted with insulin from the β-cells. The levels of this peptide hormone were undetectable in 13/18 T1D samples and dramatically reduced in the remaining 5 subjects relative to both control and autoantibody positive samples (Fig 2D [CW4]). In contrast glucagon was present in similar levels between T1D and control subjects (Fig 2E [CW4]). An analysis of

proinsulin with insulin showed a correlation in controls but not T1D or autoantibody positive without diabetes subjects for these two analytes (Fig 2F-H [CW4]).

We next assayed by RT-qPCR expression levels of heterogeneous nuclear RNA (hnRNA) for insulin (INS), calmodulin 1 (CALM1), serine protease inhibitor Kazal-type 1 (SPINK1), cyclophilin A (PPIA) and chymotrypsin C (CTRC). Similarly, mRNA for INS, insulin-insulin like growth factor 2 read through (INS-IGF2), IAPP, glucagon (GCG) and somatostatin (SST) were measured. Consistent with the protein levels of proinsulin, we found low but reproducible levels of insulin mRNA in T1D pancreas (Fig 2I [CW 4]); however, the hnRNA for INS was not detectable in most T1D samples (Fig 2J [CW4]). The hnRNA for CALM1, SPINK1, PPIA and CTRC were present and comparable between control and T1D pancreas samples (Fig S2 [CW4]). The INS-IGF2 read through mRNA was largely undetectable in T1D subjects (Fig 2K [CW 4]). Taken together, this suggests the insulin promoter is largely silent in T1D. The mRNA expression levels for IAPP were, for the most part, very low or undetectable in T1D subjects while control pancreas had detectable IAPP in all cases, which corroborates the IAPP protein data. (Fig 2D and L [CW4]). Data for GCG and SST mRNA show that the levels for these two analytes are similar between control and T1D samples (Fig 2M-N [CW 4]).

To better understand the location of and distribution of insulin and glucagon positive cells and based on previous observations, we performed a semi-quantitative analysis of these hormones in a subset of controls (n=5), short duration T1D (0-7 years, n=5) and longer duration T1D (8-35 years, n=6).

Representative images of a control and two T1D subjects (one with 7 years of duration and one with 35 years of duration) double stained for insulin and glucagon (Fig 3A-E and insets [CW 4]) showed the distribution of islets and hormone positive single cells in control pancreas and through the progression of disease. Of note, T1D subjects have a lobular distribution of islets that are termed pseudoatrophic, meaning glucagon positive in the absence of insulin, as also shown in ((Campbell-Thompson, et al., 2016a) [CW3]). Both control and T1D subjects had single cells staining for insulin protein and also INS mRNA as exemplified by INS in situ hybridization (ISH) stained sections (Fig 3F-J [CW4]).

The presence of insulin within islet cells was diminished in T1D samples from short duration compared to controls and absent in longer duration T1D subjects (Fig 3K [CW 4]). This was additionally supported by increasing counts of pseudo-atrophic (insulin+glucagon-) islets, with a concomitant decrease in the counts of double positive (insulin+glucagon+) islets (Fig 3L-M [CW4]) with increasing duration of disease. We also observed insulin positive cells in small clusters (2-5 cells) or as single cells in the acinar areas. These were abundant in the

control pancreata but also present, albeit in reduced numbers, in both short and long duration T1D samples (Fig 3N-O [CW4]).

We next investigated whether the enzymes involved in the processing of proinsulin were altered in T1D pancreata. To that end mRNA for PSCK1 was reduced in T1D samples relative to controls, while mRNA for PCSK2 and CPE were not different between the groups (Fig 3P-R [CW4]). Finally, we measured IFNγ, IL-1β and TNFα expression by RT-qPCR in control and T1D pancreata (Fig S4 [CW 4]). We found that IFNγ was undetectable in all cases, but IL-1β was present in similar levels between the two groups (Fig S4A [CW4]). TNFα was not detectable in approximately one third of the T1D and half of the control samples (Fig S4B [CW 4]).

5 Discussion

5.1 Even with a low prevalence disease such as T1D, it is possible to screen organ donors for the presence of disease specific autoantibodies.

Our experience has been that with practice and refining the cutoffs for positivity, adjustments to the assay preparations and training of screening laboratories, we have been able to detect the frequency of autoantibodies in the published literature. Meaning for any given single autoantibody, we have about 3% of the screens being positive and approximately 0.2% of samples are positive for multiple (i.e., two or more) autoantibodies [CW 1]. This push for a higher threshold also means we have a higher specificity for nPOD borne out in the IASP proficiency challenge testing, where we have consistently scored at around 100% specificity. While not usual for a screening program to emphasize specificity, we have done so for ethical (not removing false positive organs from transplant pool) and logistical reasons in terms of manpower and cost to recover organs. We currently have no way of knowing for sure that these organs were truly from a subject that would ultimately progress to T1D, but together with high-risk HLA and multiple autoantibody positive status, we are reasonably certain that these are true pre-T1D cases. This is consistent with multiple studies using these tissues showing differences between the autoantibody positive cases and control cases. For example changes in proinsulin to insulin ratios in autoantibody positive cases (Rodriguez-Calvo, et al., 2017), differential proteomic changes in autoantibody positive pancreas (Burch, et al., 2015), and others (Kaddis, et al., 2015). It is therefore reasonable to speculate that future studies and increase in knowledge will allow us to better stratify these subjects.

The use of an orthogonal RIA for confirmation of these ELISA screening results has shown that the two assay formats were for the most part in agreement. The few discordant results are speculated to be epitope specific differences or format differences, but this remains unknown. Nonetheless, we have confidence in the overall ability to obtain true autoantibody positive organ donor samples for the nPOD program.

5.2 Insulitis in the natural history of T1D

The ability to analyze pancreas samples across the spectrum of pre-T1D, new onset T1D and established T1D is unique and an invaluable aspect of nPOD. Our study using standardized protocols, high quality tissue and access to the whole organ has allowed us to address insulitis

frequency, leukocyte subtypes, pancreatic weights and hence α- and β-cell mass. This we believe adds much to the collective understanding of cellular infiltrates in human T1D.

Firstly, we have agreement that insulitis occurs in 23% of our cases [CW 2] versus 21% in other studies (In't Veld, 2011). However, we found 100% insulitis in subjects with duration of less than one year and 19% in those with a duration greater than one year, while the published literature suggests 51% and 3%, respectively (In't Veld, 2011). We would note that given the marked heterogeneity in the insulitic lesion, perhaps one reason for this discrepancy is that nPOD has the whole pancreas with multiple blocks available and screened, while others have had smaller pieces of tissue to examine. We are also in agreement regarding the proportion of insulin-containing islets with insulitis (33%) versus 2% of those that do not contain insulin, when compared with the Foulis et al. study (Foulis, et al., 1986) where they found insulitis in 28-35% of insulin-containing versus 1-5% of insulin-absent islets. The notion of insulitis being present in an antigen specific manner and the presence of insulitis in insulin negative islets could imply other autoantigens (which have not been stained for in this case) such as GAD or IA-2 or in ongoing bystander type reactions, which will need to be addressed in future studies.

Secondly, we also found insulitis in 2 out of 5 autoantibody positive subjects without T1D and none of the single autoantibody positive subjects. The two subjects with insulitis both had the combination of GADA and IA-2A. This is consistent with a published study demonstrating insulitis in 2 out of 7 multiple autoantibody positive subjects (In't Veld, et al., 2007). This is also further demonstration that the nPOD screening process is potentially finding true pre-T1D subjects.

Another finding of note was the similarities in leukocyte subtypes in both pre-T1D and diagnosed cases. We found that each insulitic islet was heterogeneous in ratios of various leukocyte subsets, but when compared to overall frequency of insulitis, each subtype in the lymphoid compartment increases linearly. This implies a broad immune response to both insulin containing and insulin deficient islets. This is similar to the findings of others (Bottazzo, et al., 1985; Coppieters, et al., 2012; Dotta, et al., 2007; Foulis, et al., 1986; Hanafusa and Imagawa, 2008; Hanninen, et al., 1992; In't Veld, et al., 2007; Itoh, et al., 1993; Leete, et al., 2016; Richardson, et al., 2011; Somoza, et al., 1994); however, some have an emphasis on finding predominantly $CD8^+$ and macrophage infiltrates. Whether this reflects differences in protocols and/or donor demographics remains to be seen. Once again our study has looked at this question with a greater number of sectional areas of the pancreas studied and at different stages of the disease process. More recently, studies have highlighted potential for

higher $CD20^+$ B-lymphocyte infiltrate frequencies to potentially differentiate those with early onset T1D (Leete, et al., 2016).

Pancreatic mass was found to be significantly smaller in individuals with T1D independent of age or duration. While autoantibody positive individuals did not show this difference when compared to controls, this was in contrast to our previous smaller study of autoantibody positive subjects, where we did see a difference in pancreas size compared to controls; however, the notion of a smaller pancreas in T1D subjects continues to be upheld (Campbell-Thompson, et al., 2012b; Campbell-Thompson, et al., 2016b). Indeed, this has been noted before (Gaglia, et al., 2011; Goda, et al., 2001; Philippe, et al., 2011; Williams, et al., 2012) in living subjects. We hypothesize that since we don't exactly know where autoantibody positive donors are in their potential progression/staging to T1D, that the question of pancreas volume will be best resolved by either an appropriate increase in the number of donors studied or in longitudinal radiological assessments of those at risk for and through onset of disease.

Since we had the whole organ along with pancreatic weights, we were able to provide a metric of α- and β-cell area and mass. Not surprisingly, β-cell area and mass are significantly reduced in T1D subjects. Also of note, the β-cell mass was higher in those with insulitis compared to those without, potentially implying an association of autoantigenic load or these sample reflecting an earlier stage in the process of infiltration.

5.3 Complement deposition in the natural history of T1D

In our study of C4d staining in the pancreas, the initial goal was to test an alternative potential mechanism of β-cell death [CW 3]. This was partly in response to the initial findings of lower than expected frequency of insulitis in T1D cases, but also in response to an earlier literature that had defined complement fixing ICA autoantibodies (Bottazzo, et al., 1980; Mustonen, et al., 1983). However, we found that C4d deposition occurred throughout the pancreas in T1D subjects and in 2 autoantibody positive subjects, 1 of which also had insulitis. The distribution of C4d on mostly exocrine vasculature and extra-cellular matrix components does not implicate islets directly. We therefore cannot conclude that the observed C4d deposition was due to complement fixing ICAs. Future efforts should not only look directly at other aspects of complement biology but also must address what may be occurring in the exocrine pancreas, in terms of inflammation and the aforementioned smaller pancreas in T1D.

5.4 Presence of endocrine hormones in the natural history of T1D

Our analysis of the endocrine parameters throughout the natural history of T1D led to several observations [CW 4]. Collectively, we noted the presence of proinsulin as protein in a large proportion of T1D pancreata, by both immunostaining and specific ELISA methodologies on cryopreserved samples and extracts. We also found mRNA for proinsulin by ISH and by RT-qPCR, but mature insulin and C-peptide proteins were mostly undetectable. We interpret the finding of lower PCSK1 in T1D as one possible reason that we detect proinsulin in the near absence of insulin and C-peptide, which may imply partial processing in the maturation of these analytes. We also found near absence of IAPP and would note that a recently developed assay to measure pro-IAPP revealed a higher pro-IAPP to IAPP ratio in T1D with the authors concluding that the processing machinery may be impaired (Courtade, et al., 2017). Together these two observations are consistent with there being a defect in processing of both proinsulin and pro-IAPP since these utilize the same proconvertases and pathways (Alarcon, et al., 2012). Another potential explanation could be rapid secretion of both pro-hormones, as has been described in T2D (Alarcon, et al., 2016), leaving minimal time to process, this would have to be tested functionally on live tissue.

Isolated islets from control and T1D organ donors have been tested for glucose responsiveness, so functional assays have been possible. However, we would note our other findings herein, namely that of single cells in the acinar regions testing positive for insulin. These single cells exist in abundance in control pancreata and progressively decrease in number with duration of T1D. Importantly, in longer duration T1D, islets are mostly depleted of insulin, but some of these single cells remain. In that light, isolating islets and testing them, will not capture the functionality of these single cells. Also, we do not have sufficient information on these hormone positive single cells to conclude whether these are de-differentiated cells (Accili, et al., 2016; Talchai, et al., 2012) or potentially stem cells (Moin, et al., 2017; van der Meulen, et al., 2017), and this will be pursued in future studies.

We also found similar levels by extraction and mRNA for glucagon consistent with our earlier [CW 2] observations on α-cell mass. The same was also true for levels of mRNA for somatastatin, but we have not formally quantified δ-cells. We did not find any significant differences in any of these endocrine compartments in autoantibody positive pancreas samples; this is in contrast to a recent observation of higher proinsulin by immunofluorescence staining in subjects at risk for T1D (Rodriguez-Calvo, et al., 2017). The immunostaining reagent antibodies used in these two studies may not be able to distinguish the various intermediate

forms of proinsulin, and this would have to be finally resolved by alternative assays (radiolabelling or mass spectrometry) to identify the various partially processed proinsulin molecules (Rhodes, et al., 1992).

Finally, we also found that hnRNA (unspliced nuclear RNA) for insulin was undetectable as was mRNA for the INS-IGF2 read-through product, and this coupled with INS mRNA being present we interpret as the insulin promoter being inactive with long lived INS mRNA explaining the persistence of proinsulin. It is possible that there is insufficient hnRNA for our detection especially if this is mostly in single cells rather than in islets. This remains unresolved and would be subject of future studies with functional access to live cells from the pancreas, as had been pioneered in mouse pancreas (Marciniak, et al., 2014; Speier, et al., 2013).

6 Resume

The study of human T1D as an autoimmune disease of the pancreas started, it could be argued, with the discovery of insulin using pancreatectomized animals. This followed a period of studies of insulin replacement refinements, improvements in technologies and studies in blood samples of metabolic and autoimmune phenomena. While animal models allowed end organ analysis, the study of human pancreas was severely limited in comparison to studies of peripheral blood. It is therefore fitting that what was learned from these peripheral blood studies allowed us to assemble, via nPOD, a human pancreas bank including samples from those deemed at higher risk for T1D by peripheral blood autoantibody analysis (Wasserfall, et al., 2016) [CW 1].

The main goal of my thesis was, with these samples in hand and still continuing to accrue, to address several fundamental questions in human T1D biology. Firstly my colleagues and I were able to study insulitis in autoantibody positive subjects and in those with a diagnosis of T1D (Campbell-Thompson, et al., 2016a) [CW 2]. Herein we found insulitis in 2 of 5 autoantibody positive subjects' pancreas samples, both having the combination of GADA and IA-2A. In pancreas samples from T1D donors with less than a year of disease duration, 100% had insulitis. While, in those with greater than one year of disease duration, 23% had insulitis. This implies that insulitis occurs closer to onset of disease and then wanes thereafter. This is in stark contrast to the NOD mouse model where insulitis occurs very early and in 100% of these inbred mice whether they progress to T1D or not. Also in stark contrast to the NOD, while β-cell mass was reduced in T1D pancreas samples it was not completely absent. We also confirmed findings of reduced overall mass of human T1D pancreas samples that are not explained by missing β-cells, which make up only a small fraction of the organ. This implicates loss of exocrine tissue in some manner that remains unresolved.

Next, we tested whether complement plays a potential role in T1D using C4d deposition as a marker of (auto)antibody mediated events (Rowe, et al., 2013) [CW 3]. Unexpectedly, we found that while there was increased C4d staining in T1D pancreas samples, the distribution implied potential exocrine inflammation and not necessarily islet specific events. Whether this is tied into the findings of smaller pancreata in T1D is an obvious question for future study.

In then compiling these notions together, I used a technique to extract proteins from whole frozen sections of pancreas and found persistence of proinsulin while insulin and C-peptide levels were low to undetectable by ELISA in T1D samples (Wasserfall, et al., 2017) [CW 4]. In further teasing this apart, we discovered INS mRNA was present by ISH and RT-

qPCR along with both proinsulin and insulin by immunohistochemistry. We also were able to semi-quantify the progression of T1D, by the loss of insulin in islets but the persistence of insulin positive single cells with longer disease duration. The PCSK1 enzyme was also reduced in T1D samples by RT-qPCR, suggesting a possible mechanism for incomplete processing of proinsulin into mature insulin and C-peptide. Resolving this pathway and addressing whether the single insulin positive cells can be rescued as a therapeutic option or whether they are dedifferentiating β-cells will be pursued in future endeavors.

In conclusion, my thesis work has demonstrated that insulitis appears closer to T1D onset, is evident at onset, and wanes after a year of duration. It is also true that not every islet is infiltrated, but those that are have T-, B-, dendritic cells and macrophages in a heterogeneous distribution. The loss of β-cells is lobular, and proinsulin can be found in T1D pancreata. Proinsulin, insulin and glucagon can be found in single cells long after the disappearance of β-cells within islet structures in T1D pancreas samples. The overall pancreas is smaller, and by deduction, this must be a loss of exocrine tissue since β-cells make up a tiny fraction of the overall mass. C4d is deposited in exocrine areas and others have found exocrine leukocytic infiltrates (Campbell-Thompson, et al., 2015; Rodriguez-Calvo, et al., 2014). There is an older literature as well as emerging studies, including some from our group, corroborating findings of diminished exocrine secreted products and function (Frier, et al., 1976; Kondrashova, et al., 2017; Lankisch, et al., 1982; Li, et al., 2017). Taken together, there appears to be involvement of the exocrine pancreas in T1D and it remains to be seen if this is part of the autoimmune process or in response to the loss of insulin (Campbell-Thompson, et al., 2015).

Summary [German]

Die Studie des menschlichen T1D (Type 1 Diabetes), als eine Autoimmunerkrankung der Bauchspeicheldrüse, könnte man mit der Entdeckung von Insulin unter Verwendung von pankreatectomisierten Tieren argumentieren. Es folgten Studien zur Verfeinerung des Insulinersatzes, Verbesserung der Technologien und zur Untersuchung von Stoffwechsel- und Autoimmunphänomenen in Blutproben. Während Tiermodelle eine Endorgananalyse erlaubten, war die Untersuchung der menschlichen Bauchspeicheldrüse im Vergleich zu Studien mit peripherem Blut stark eingeschränkt. Anhand der so genannten peripheren Blutstudien, die nPOD (Network for Pancreatic Organ Donors with Diabetes), konnte eine menschliche Bauchspeicheldrüsenbank mit Proben von Personen zusammenzustellt werden, die durch die Analyse peripherer Blut-Autoantikörper (Wasserfall, et al., 2016) für T1D als risikoreich eingestuft wurde [CW 1].

Das Hauptziel meiner Dissertation bestand darin, mit diesen noch immer ansteigenden Proben, einige grundlegende Fragen der menschlichen T1D-Biologie zu beantworten. Zuerst konnten meine Kollegen und ich Insulitis bei Autoantikörper-positiven Probanden und bei Patienten mit T1D-Diagnose (Campbell-Thompson, et al., 2016a) studieren [CW 2]. Dabei fanden wir in 2 von 5 Pankreasproben Insulitis von Autoantikörper-positiven Probanden, beide mit der Kombination GADA und IA-2A. In Pankreasproben von T1D-Spendern mit weniger als einem Jahr Krankheitsdauer hatten 100% der Patienten eine Insulitis. Während, in denen mit mehr als einem Jahr Krankheitsdauer, 23% der Insulitis aufzeigten. Dies impliziert, dass die Insulitis vorwiegend zum Krankheitsbeginn auftritt und danach abnimmt. Das steht im extremen Gegensatz zum NOD-Mausmodell, bei dem Insulitis sehr früh auftritt und bei 100% der Inzuchtmäuse, unabhängig davon, ob sie T1D entwickeln oder nicht. Auch im Gegensatz zum NOD, während β-Zellmasse in T1D-Pankreasproben reduziert wurde, fehlte es nicht ganz. Wir bestätigten ebenfalls die Ergebnisse einer reduzierten Gesamtmasse menschlicher T1D-Pankreasproben, die nicht durch fehlende β-Zellen erklärt werden, da die nur einen kleinen Teil des Organs ausmachen. Dies impliziert den Verlust von exokrinem Gewebe in einer Art und Weise, die bis heute ungeklärt bleibt.

Weiterhin haben wir getestet, ob Komplement eine potenzielle Rolle in T1D spielt, indem die C4d-Deposition als Marker für (Auto) Antikörpervermittelte Ereignisse verwendet wurde (Rowe, et al., 2013) [CW 3]. Unerwarteterweise fanden wir heraus, dass es erhöhte C4d Antikörperfärbung in T1D Pankreasproben gab. Dies implizierte die Verteilung potentieller exokriner Entzündungen und nicht unbedingt Insel-spezifische Ereignisse. Ob dies mit den

Befunden der kleineren Bauchspeicheldrüse in T1D zusammenhängt, ist eine naheliegende Frage für zukünftige Studien.

Bei der Zusammenstellung dieser Begriffe verwendete ich eine Technik, um Proteine aus gefrorenen Abschnitten der Bauchspeicheldrüse zu extrahieren und fand die Persistenz von Proinsulin, während die Insulin- und C-Peptid-Konzentrationen niedrig bis nicht nachweisbar durch ELISA in T1D-Proben waren (Wasserfall, et al., 2017) [CW 4]. Weiterhin konnten wir zeigen, dass INS mRNA durch ISH und RT-qPCR zusammen mit Proinsulin und Insulin durch Immunhistochemie anwesend war. Wir waren ebenfalls in der Lage, die Progression von T1D semi-quantifizieren, durch den Verlust von Insulin in Inselchen, sondern die Persistenz von Insulin-positiven Einzelzellen mit längerer Krankheitsdauer. Das Enzym PCSK1 wurde außerdem in T1D-Proben durch RT-qPCR reduziert, was auf einen möglichen Mechanismus für die unvollständige Verarbeitung von Proinsulin zu reifem Insulin und C-Peptid hindeutet. Die Aufklärung dieses Prozesses und die Frage, ob die einzelnen insulinpositiven Zellen als therapeutische Option gerettet werden können oder ob sie die Zellen von β dedifferenzieren, werden in Zukunft weiterverfolgt.

Zusammenfassend hat meine These gezeigt hat, dass Insulitis näher am T1D-Einstieg erscheint, dass sie zu Beginn offensichtlich ist und nach einem Jahr nachlässt. Es ist zudem nachgewiesen, dass nicht jede Insel infiltriert wird, sondern diejenigen, die T-, B-, dendritische Zellen und Makrophagen in einer heterogenen Verteilung haben. Der Verlust von β-Zellen ist lobulär, und Proinsulin kann in T1D Pankreas gefunden werden. Proinsulin, Insulin und Glukagon sind in Einzelzellen zu finden, lange nach dem Verschwinden von β-Zellen innerhalb von Inselstrukturen in T1D-Pankreasproben. Die gesamte Bauchspeicheldrüse ist kleiner, und durch Abzug muss dies ein Verlust an exokrinem Gewebe sein, da β-Zellen einen winzigen Bruchteil der Gesamtmasse ausmachen. C4d wird in exokrinen Bereichen deponiert und andere haben exokrine Leukozyteninfiltrate gefunden (Campbell-Thompson, et al., 2015; Rodriguez-Calvo, et al., 2014). Es gibt zurückliegende sowie aktuelle Studien, darunter einige aus unserer Gruppe, die die Ergebnisse verminderter exokriner Sekretprodukte und Funktionen bestätigen (Frier, et al., 1976; Kondrashova, et al., 2017; Lankisch, et al., 1982; Li, et al., 2017). Hypotetisch ist eine Beteiligung der exokrinen Bauchspeicheldrüse an T1D möglich, und es bleibt abzuwarten, ob dies ein Teil des Autoimmunprozesses ist oder als Reaktion auf den Verlust von Insulin (Campbell-Thompson, et al., 2015).

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List of Publications

All the publications presented in my thesis are listed below.

[CW1] Validation of a rapid type 1 diabetes screening assay for community-based screening of organ donors to identify subjects at increased risk for the disease.

Supporting information:

http://onlinelibrary.wiley.com/store/10.1111/cei.12797/asset/supinfo/cei12797-sup-0001-suppinfo.pptx?v=1&s=0071ade7b835f3a1fda58d84b94fa7bf0890c5cb

Clive Wasserfall	**Researched the data** **Wrote the manuscript**
Emily Montgomery	Researched the data Reviewed/Edited the manuscript
Liping Yu	Researched the data Reviewed/Edited the manuscript
Aaron Michels	Researched the data Reviewed/Edited the manuscript
Roberto Gianani	Researched the data Reviewed/Edited the manuscript
Alberto Pugliese	Conceived of the study Reviewed/Edited the manuscript
Concepcion Nierras	Contributed to the discssion Reviewed/Edited the manuscript
John S. Kaddis	Analyzed the data Reviewed/Edited the manuscript

Desmond A. Schatz	Contributed to the discssion Reviewed/Edited the manuscript
Ezio Bonifacio	Conceived of the study Reviewed/Edited the manuscript
Mark A. Atkinson	Conceived of the study Reviewed/Edited the manuscript Corresponding author

Select All		Full Journal Title	Total Cites	Impact Factor	Eigenfactor Score
☐	61	CLINICAL AND EXPERIMENTAL IMMUNOLOGY	12,981	3.410	0.01538
☐	62	INTERNATIONAL REVIEWS OF IMMUNOLOGY	1,172	3.279	0.00285
☐	63	Journal of Immunology Research	1,876	3.276	0.00704
☐	64	Expert Review of Clinical Immunology	1,772	3.270	0.00570
☐	65	JOURNAL OF CLINICAL IMMUNOLOGY	4,428	3.253	0.01013
☐	66	AIDS REVIEWS	670	3.244	0.00166
☐	67	MOLECULAR IMMUNOLOGY	10,214	3.236	0.01694
☐	68	VACCINE	36,890	3.235	0.08298
☐	69	MEDIATORS OF INFLAMMATION	7,786	3.232	0.02230
☐	70	DEVELOPMENTAL AND COMPARATIVE IMMUNOLOGY	6,968	3.218	0.01041
☐	71	ALLERGOLOGY INTERNATIONAL	1,487	3.194	0.00327
☐	72	CELLULAR IMMUNOLOGY	4,419	3.172	0.00708
☐	73	FISH & SHELLFISH IMMUNOLOGY	12,261	3.148	0.01412
☐	74	Transplantation Reviews	703	3.113	0.00235
☐	75	JOURNAL OF INVESTIGATIONAL ALLERGOLOGY AND CLINICAL IMMUNOLOGY	2,073	3.094	0.00255
☐	76	MEDICAL MICROBIOLOGY AND IMMUNOLOGY	1,751	3.093	0.00308
☐	77	AMERICAN JOURNAL OF REPRODUCTIVE IMMUNOLOGY	4,317	3.013	0.00739
☐	78	JOURNAL OF MICROBIOLOGY IMMUNOLOGY AND INFECTION	1,814	2.973	0.00365
☐	79	Allergy Asthma & Immunology			

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Validation of a rapid type 1 diabetes autoantibody screening assay for community-based screening of organ donors to identify subjects at increased risk for the disease

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Summary

The Network for Pancreatic Organ donors with Diabetes (nPOD) programme was developed in response to an unmet research need for human pancreatic tissue obtained from individuals with type 1 diabetes mellitus and people at increased risk [i.e. autoantibody (AAb)-positive] for the disease. This necessitated the establishment of a type 1 diabetes-specific AAb screening platform for organ procurement organizations (OPOs). Assay protocols for commercially available enzyme-linked immunosorbent assays (ELISAs) determining AAb against glutamic acid decarboxylase (GADA), insulinoma-associated protein-2 (IA-2A) and zinc transporter-8 (ZnT8A) were modified to identify AAb-positive donors within strict time requirements associated with organ donation programmes. These rapid ELISAs were evaluated by the international islet AAb standardization programme (IASP) and used by OPO laboratories as an adjunct to routine serological tests evaluating donors for organ transplantation. The rapid ELISAs performed well in three IASPs (2011, 2013, 2015) with 98-100% specificity for all three assays, including sensitivities of 64–82% (GADA), 60–64% (IA-2A) and 62–68% (ZnT8A). Since 2009, nPOD has screened 4442 organ donors by rapid ELISA; 250 (5·6%) were identified as positive for one AAb and 14 (0.3%) for multiple AAb with 20 of these cases received by nPOD for follow-up studies (14 GADA+, two IA-2A$^+$, four multiple AAb-positive). Rapid screening for type 1 diabetes-associated AAb in organ donors is feasible, allowing for identification of non-diabetic, high-risk individuals and procurement of valuable tissues for natural history studies of this disease.

Keywords: autoantibodies, nPOD, organ donor, screening, type 1 diabetes

Introduction

The JDRF Network for Pancreatic Organ donors with Diabetes (nPOD) programme was established to investigate the human pancreas in the context of type 1 diabetes mellitus, wherein the insulin-producing beta cells in the islets of Langerhans are destroyed [1]. While the onset of the disease may be acute, in the vast majority of cases it is preceded by a variable period of months to decades where non-diabetic individuals possess autoantibodies (AAb) against one or more of a series of type 1 diabetes-associated autoantigens. Over recent decades, these AAb have been utilized for a variety of purposes, including identifying those at high risk for developing type 1 diabetes mellitus [2,3]. Indeed, investigators within the type 1 diabetes research community have developed and sought actively to standardize assays to detect these AAb [4–11], the majority in the format of radiobinding assays (RBA) for AAb against glutamic acid decarboxylase (GADA) [12], insulinoma-associated protein-2 (IA-2A) [13,14], zinc transporter-8 (ZnT8A) [15] and insulin (IAA) [16]. However, emerging commercially available enzyme-linked immunosorbent assays (ELISAs) have provided alternative means for type 1 diabetes AAb screening [17–19] and possess logistical benefits compared to RBA.

The logistics and biobanking efforts for nPOD have been described previously [1,20]. Herein, we convey information regarding nPOD's novel AAb-screening programme, an effort designed to identify non-diabetic organ donors at risk for type 1 diabetes mellitus. This effort, perhaps

deemed simple, in actuality required performance in situations unlike those undertaken in any setting to date. Specifically, screening needed to be performed within the general population (i.e. low prevalence of the disease), in rapid fashion (< 3 h) and in environments outside typical research laboratories (i.e. clinical laboratories). To achieve this, nPOD modified a series of commercial ELISA-based assays and monitored their performance during a period of 6 years. This report conveys those developmental efforts and describes an effective means for rapid AAb screening of organ donors for the purpose of identifying highly valuable pancreata for addressing key research questions pertaining to the natural history of type 1 diabetes mellitus.

Materials and methods

ELISA core laboratory

Implementing nPOD AAb screening. The nPOD ELISA core laboratory (University of Florida, Gainesville, FL, USA) served as the central quality assessment laboratory for the commercial ELISAs developed by RSR Ltd (Cardiff, Wales, UK), which was chosen as the screening platform. The nPOD ELISA core also developed the modified assays. ELISAs for GADA and IA-2A were modified from the manufacturer's protocol to ensure results in a total of 3 h by shortening the primary incubation step to 1 h, along with shaking at 500 rpm. The nPOD ELISA core laboratory provided training and equipment (spectrophotometer with 450 nm capability and plate washer) to the relevant organ procurement organization (OPO) screening laboratories, with follow-up consultation as necessary.

Beginning in 2009, ELISA kits were distributed directly from the US distributor, Kronus Inc. (Boise, ID, USA) to OPO screening laboratories to measure GADA and IA-2A using the modified assay protocol. In addition to a standard curve with five calibrators, positive and negative controls were supplied by the manufacturer. While six unknown samples could be run in duplicate from each of the 96-well GADA and IA-2A kits, samples were run one at a time because the nature of organ donation processes does not allow for batching of test material. Based on receiver operating characteristic (ROC) curves and testing of 270 healthy controls and 102 new-onset type 1 diabetes subjects, cut-offs defining AAb positivity were assigned for GADA and IA-2A (Fig. 1a,b). This initial protocol was refined further, as described below.

Updates to the AAb screening programme. In 2011, after 2 years of experience and an evaluation by the international Islet AAb Standardization Program (IASP), the cut-off for AAb positivity was raised to increase specificity. The result of this action was to increase concordance between the OPO screening laboratories and the nPOD ELISA core laboratory, as well as to improve concordance between the ELISA assays and confirmatory RBA assays conducted at the nPOD central AAb core (Denver, CO, USA), described below (Fig. 2). In 2013, an ELISA assay for ZnT8A became available from the same manufacturer. Again using the modified protocol described above (shortened primary

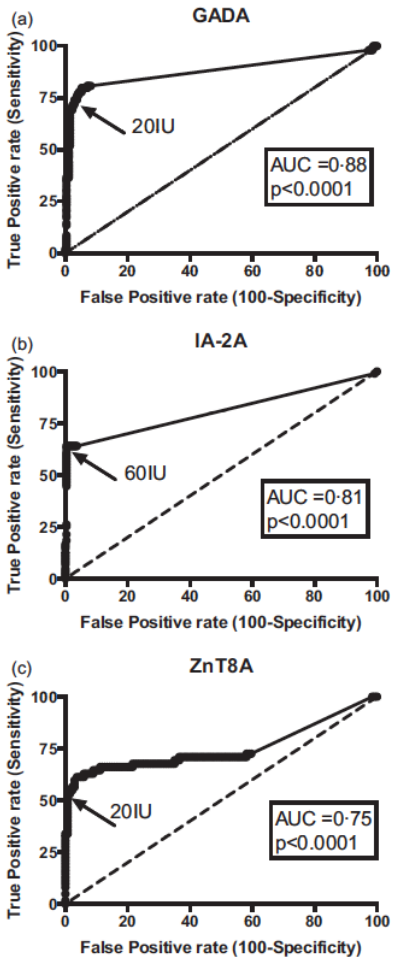

Fig. 1. Receiver operating characteristic (ROC) curve analyses are shown for (a) glutamic acid decarboxylase (GADA), (b) insulinoma-associated protein-2 (IA-2A) and (c) zinc transporter 8 (ZnT8A). Plots are 100 – specificity % (*x*-axis) *versus* sensitivity % (*y*-axis). The following cut-offs were selected to maximize specificity: (a) 20 IU, 98% specificity and 70% sensitivity [area under the curve (AUC) = 0·88], (b) 60 IU, 99% specificity and 63% sensitivity (AUC = 0·80 and (c) 20 IU, 97% specificity with 57% sensitivity (AUC = 0·75).

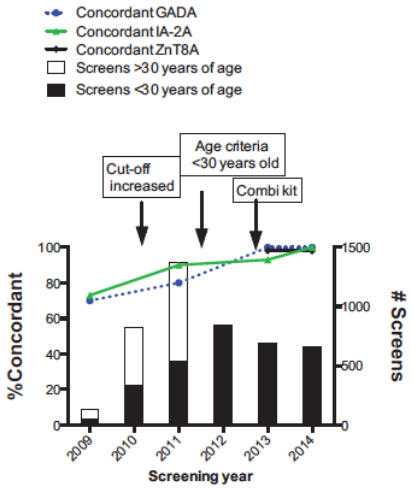

Fig. 2. Overview of the Network for Pancreatic Organ donors with Diabetes (nPOD) autoantibody against insulin (AAb) screening programme over time. The overall concordance of the screening enzyme-linked immunosorbent assay (ELISA) with nPOD ELISA core laboratory is shown for glutamic acid decarboxylase (GADA) (blue line), IA-2A (green line) and zinc transporter 8 (ZnT8A) (black line; left *y*-axis). The number of total subjects screened (bars) and those screened under 30 years of age (filled bars) are shown (right *y*-axis). Changes to the programme are indicated with text box and arrows for each new event above the graph.

incubation with 500 rpm shaking), the nPOD ELISA core laboratory validated this assay with 134 controls and 62 new-onset type 1 diabetes samples with ROC curve analysis (Fig. 1c), and chose a positivity cut-off favouring specificity over sensitivity to minimize the likelihood of procuring tissues from donors yielding a false positive result. The ZnT8A assay was then added to the OPO screening laboratories' nPOD portfolio.

Reconfiguring the assay. In 2013, all ELISA kits were delivered to the ELISA core laboratory, where they were reconfigured into what is now termed the combination nPOD kit for AAb. Design changes were implemented to obtain 12 individual runs per purchased kit, compared to the six runs per kit allowed previously. Calibration curves were eliminated, and instead calibrators were combined from each kit (GADA, IA-2A and ZnT8A) to create a single calibrator/cut-off solution with a value equal to the previous positivity cut-offs favouring specificity. A single-negative control solution for all three analytes and a single-positive control containing known AAb to all three autoantigens were also included. A strip well from the GADA kit was placed into column 1 of a strip well holder; then a strip from the IA-2A kit was placed into column 2; and finally, a strip from the ZnT8A kit was placed into column 3. The entire holder was placed in a sealed foil pouch with a desiccating packet and labelled as a 'single-run kit', suitable for all samples, calibrator and controls to be tested in duplicate. The streptavidin–horseradish peroxidase (HRP) (SAV) from each of the three original kits was combined and diluted to working concentration, and aliquots were prepared for use as a single run. The calibrator, controls and SAV aliquots were stored at 4°C. Biotinylated detection reagents for GADA, IA-2A and ZnT8A were prepared using buffers of varying colour designations, and aliquots sufficient for a single-run kit were stored at −20°C. Similarly, ready-to-use tetramethylbenzidine (TMB) and stop solution (1N H_2SO_4) single-use aliquots were prepared and stored at 4°C. Thus, for each run, the calibrator solution, controls and unknown(s) were common to all three strips while the biotinylated detection reagents were specific, with the buffer colour-coded to match each strip of GADA, IA-2A and ZnT8A. The SAV, TMB and stop solutions were also common to all wells. Taken together, these kits containing the plate and all reagents diluted to working-strength (in amounts sufficient for testing a single unknown sample) were distributed to the OPO screening laboratories. The combination kit expiration date was determined to be 20 weeks from the date of reconstituting the reagents.

Following each run, the results are read from a spectrophotometer at 450 nm, and pasted into an Excel (Microsoft, Redmond, WA, USA) template worksheet that calculates AAb titres based on an index relative to the calibrator/cut-off [sample optical density (OD) divided by calibrator OD], coefficients of variation and pass/fail of the controls. AAb positivity is defined as an index > 1. Results are e-mailed to the nPOD ELISA core laboratory and reported to the relevant OPO along with any other serological results that are usually sent with a transplant organ case. If the results are positive for any of the tested AAb, the OPO may then offer this case to nPOD, unless the organ is accepted for transplant or fails to meet nPOD inclusion criteria [1,20]. From 2009 to 2012, nPOD screened all research-consented cases, and from 2012 to the present, all consented cases that were under the age of 30 years (Fig. 2: open bars, all subjects; filled bars, those under the age of 30).

Screening *versus* recovery serum samples

When possible, the screening laboratories send an aliquot of each AAb-positive serum sample to the nPOD ELISA core laboratory. If a case is referred to nPOD and accepted, the pancreas and other tissues are recovered as described previously [1,20]. An additional serum sample is obtained at the time of organ recovery for both quality control and research purposes. Thus, for the majority of cases, the nPOD ELISA core laboratory can compare AAb results

from the screening and recovery samples. It is important to note that nPOD works with a large number of OPOs, but not all of them are equipped to screen for AAb and, for some cases, only recovery samples are available. Therefore, some nPOD cases (type 1 diabetes mellitus, type 2 diabetes mellitus, controls and other categories) are not screened for AAb prior to organ procurement but may have a recovery serum sample for AAb testing after the fact. All available samples are tested in the ELISA core laboratory, and confirmed by RBA at the nPOD central AAb core laboratory.

nPOD central AAb core laboratory

The nPOD central AAb core laboratory has a long history of excellence in the type 1 diabetes AAb field [10,13,15,21,22], participating routinely in the Diabetes AAb Standardization Program (DASP), now renamed IASP. The nPOD central AAb core began testing for ZnT8A in nPOD cases using RBA prior to the introduction of the ZnT8A ELISA. This core also tests for IAA as there is currently no reliable ELISA for this analyte. Every case with available serum that is referred to nPOD is tested via RBA for GADA, IA-2A, ZnT8A and IAA for either confirmation of the ELISA screening results, or for determination of final AAb status. In cases of discrepancy, the RBA supersedes the ELISA as the result reported on the nPOD website (www.jdrfnpod.org).

Statistical analysis

ROC curve analysis was performed using GraphPad Prism software (San Diego, CA, USA).

Results

Modified ELISA performance in screening and standardization programmes

Using in-house samples (i.e. University of Florida) from healthy controls and subjects with new-onset type 1 diabetes mellitus (Fig. 1 shows ROC curves; Supporting information, Fig. S1 shows the portion of the ROC curves for 95–100% specificity), the modified GADA ELISA has a sensitivity of 76% and a specificity of 95% at a cut-off of 5 IU and a sensitivity of 70% and specificity of 98%, with a cut-off of 20 IU. For the modified IA-2A ELISA, the sensitivity was 64% and specificity of 98% with a cut-off of 15 IU; raising the cut-off to 60 IU revealed a sensitivity of 63% with a specificity of 99%. The modified ZnT8A ELISA showed sensitivity of 64% and specificity of 90% at a cut-off of 10 IU; with an increased cut-off of 20 IU, the sensitivity and specificity was 57 and 97%, respectively. The overall coefficients of variation (CV) were median 2·8% (range 0·2–12·2%) for GADA, 1·8% (0·1–12%) for IA-2A and 3·1% (0·1–15%) for ZnT8A. Assay runs yielding CV $>$ 15% were repeated unless the available serum volume was insufficient, in which case the run was rejected and the sample excluded from the study. Importantly, the CVs for low titre samples near the positivity cut-off (5–100 units for each analyte) were comparatively lower than the entire cohort: 1·7% (0·1–5·4%) for GADA, 1·6% (0·1–11·4%) for IA-2A and 4·1% (0·1–15%) for ZnT8.

The modified ELISAs were enrolled into IASP workshops (2011, 2013 and 2015), and under blinded conditions the assays performed with similar sensitivity and specificity profiles to the in-house validation samples. Specifically, with nPOD ELISA core laboratory-defined cut-offs, IASP 2011 results revealed GADA sensitivity of 64% with 100% specificity; IA-2A sensitivity of 64% with 100% specificity; and ZnT8A had 68% sensitivity and 98·9% specificity. Similarly, with nPOD-defined cut-off for GADA, IA-2A and ZnT8A, IASP 2013 resulted in sensitivities of 64, 60 and 68% and specificities of 98·9, 100 and 98·9%, respectively. The most recent IASP 2015 with nPOD defined cut-offs yielded sensitivities of 82, 64 and 62% with specificities of 98·9, 98·9 and 100% for GADA, IA-2A and Znt8A, respectively (Table 1).

The modified ELISA has identified successfully AAb-positive donors without diabetes and type 1 diabetes organ donors

To date, nPOD has screened a total of 4442 samples from 17 OPOs (Fig. 2). In total, 5·6% were positive for single AAb and 0·3% were positive for multiple AAb. In 2009–12, when two AAb were used in the screen, frequencies were 6·7% for single and 0·2% for multiple AAb, and when thresholds were raised and three AAb were used post-2012, 3·1% single and 0.5% multiple AAb-positive samples were detected (Table 2). Of the 46 offered and accepted cases that were AAb-positive at screening, 20 confirmed as AAb-positive without diabetes, four of whom were multiple AAb-positive, while 15 confirmed as AAb-positive and were found to actually have existing T1D. Eleven did not confirm by RBA, with nine of those occurring prior to 2012 (27% of the 33 AAb-positive accepted cases within that time-frame) and two post-2012 (15% of the 13 AAb-positive accepted cases). In one particular case, the screening programme identified a double GADA plus ZnT8A-positive individual who was later determined to have ketoacidosis, and was diagnosed posthumously as an at-onset type 1 diabetes subject.

The lowering of the upper age limit to 30 years reduced dramatically the total number of donors screened per year; however, the total number under the age of 30 has since stabilized at approximately 700 per year (Fig. 2; filled bars, right-hand *y*-axis). Raising the positivity cut-off and the introduction of the combination kits improved the concordance of the screening ELISA assays with the core

Table 1. Results of the autoantibody against insulin (AAb) standardization programmes, now called Islet Autoantibody Standardization Program (IASP)

Standardization year	Analyte	nPOD cut-off sensitivity (%)	nPOD cut-off specificity (%)	Adjusted sensitivity with Specificity 95%	Area under curve-ROC
2010/11	GADA	64	100	70	88·9
	IA-2A	64	100	64	82·0
	ZnT8A	68	99	72	85·8
2012/13	GADA	64	99	72	89·1
	IA-2A	60	100	62	81·0
	ZnT8A	68	99	72	84·7
2014/15	GADA	82	99	88	93·7
	IA-2A	64	99	64	81·8
	ZnT8A	62	100	66	82·8

Each row represents a given analyte for each participating year in the programme. Columns 3–4 contain sensitivity and specificity as calculated by the programme organizers based on Network for Pancreatic Organ donors with Diabetes (nPOD) core enzyme-linked immunosorbent assay (ELISA) laboratory cut-offs. The programme then also provides a sensitivity calculation with specificity fixed at 95% (column 5). The final column reflects the area under the curve of the receiver operating characteristic (ROC) for each analyte. GADA = glutamic acid decarboxylase; IA-2A = insulinoma-associated protein-2; ZnT8A = zinc transporter 8.

laboratory ELISA assays from 70 to 98% (Fig. 2; line graph, left-hand *y*-axis).

Results from the modified ELISA can be confirmed by RBA

The result of the RBA assay ultimately determines the AAb status of each nPOD case, but due to time and technical requirements the RBA is not feasible for initial screening by clinical laboratories. When comparing the results between ELISA and RBA using recovery serum samples, it is apparent that while there is agreement in the majority of cases, there remains some discordance between these orthogonal formats for each of the analytes tested (GADA, IA-2A and ZnT8A; Fig. 3). Over the entire duration of the AAb screening programme, discordant results include seven positive samples screened by ELISA that were not confirmed by RBA (four GADA, two IA-2A and one ZnT8A). For type 1 diabetes cases, RBA identified two GADA, eight IA-2A and four ZnT8A subjects who were negative by ELISA. Conversely, ELISA found five GADA, two IA-2A and eight ZnT8A type 1 diabetes subjects who were negative by the RBA. RBA found one GADA-positive type 2 diabetes case despite a negative ELISA result, while ELISA identified two GADA-positive type 2 diabetes subjects with negative RBA results. ELISA also found one type 2 diabetes subject positive for ZnT8A, with negative RBA. One sample was IA-2A-positive via ELISA at both screening and recovery, while both samples were negative for IA-2A but positive for GADA as assessed by RBA (Fig. 3).

The nPOD ELISA core laboratory also assessed the stability of AAb in the screening sample in comparison to the recovery sample (Fig. 4). While a few subjects showed variations in AAb titre, few demonstrated discordance in classification as positive *versus* negative for GADA or IA-2A by either method (ELISA or RBA). However, two cases were positive for GADA by both ELISA and RBA assays on the screening sample but were subsequently negative by both ELISA and RBA on the recovery sample. This was thought to be attributed to haemodynamic management of the subjects during the intensive care unit (ICU) stay.

Discussion

The nPOD AAb screening programme demonstrates the feasibility of finding pre-type 1 diabetes subjects among the US organ donor population using an ELISA-based platform for three common disease-associated AAb, GADA, IA-2A and ZnT8A. The observed frequency of these AAb among the organ donor population is consistent with published literature for the general population [8,9,21,23–33]. Raising the positivity cut-off simultaneously improved the concordance within ELISAs (OPO screening *versus* nPOD

Table 2. The number of screens performed each year with the number and frequency (%) of single and multiple autoantibody against insulin (AAb)-positive subjects identified over time since the creation of the Network for Pancreatic Organ donors with Diabetes (nPOD) AAb screening programme

Year	2009	2010	2011	2012	2013	2014
Total screened, *n*	130	827	1352	802	674	657
Screened under 30 years of age, *n*	52	330	541	802	674	657
Single AAb^+, *n* (%)	10 (7·7)	65 (7·9)	91 (6·7)	43 (5·4)	22 (3·3)	19 (2·9)
Multiple AAb^+, *n* (%)	0 (0·0)	1 (0·1)	4 (0·3)	2 (0·2)	6 (0·9)	1 (0·2)

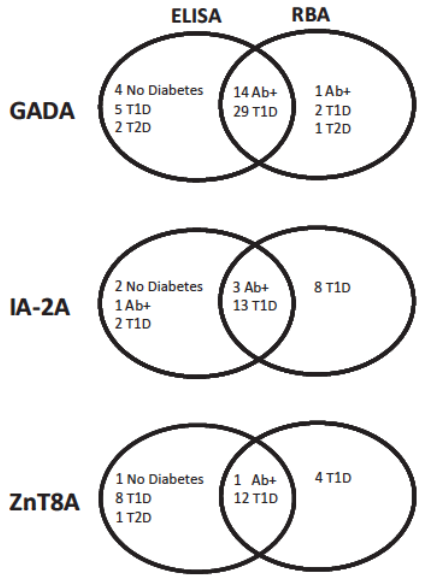

Fig. 3. Venn diagrams are shown comparing the enzyme-linked immunosorbent assay (ELISA) (left) with the radiobinding assay (RBA) (right) results for glutamic acid decarboxylase (GADA) (top), insulinoma-associated protein-2 (IA-2A) (middle) and zinc transporter 8 (ZnT8A) (bottom) within the various categories of organ donors. The RBA is set as the gold standard, upon which the autoantibody against insulin (AAb) status is determined.

ELISA core laboratories) and decreased the frequency of single AAb-positive cases from 7·7 to 2·9%. While it is most common to maximize sensitivity in screening programmes, the nPOD programme has deliberately maximized assay specificity instead, for both ethical and logistical reasons. There is no evidence in the literature supporting or prohibiting transplant of pancreata from AAb-positive organ donors, but we recognize that AAb screening results could influence the decision to transplant. It is our goal to keep organs available for the primary transplant mission and when no matched recipient exists, to procure precious tissue from true AAb-positive individuals without diabetes; thus, we have structured our screening programme to err on the side of assay specificity and minimize the number of false positive samples. Thankfully, nPOD's experience has been that the presence of AAb has not swayed decisions about transplant, and a number of pancreata from our screened AAb-positive individuals went to transplant.

While the prevalence of type 1 diabetes mellitus in the US general population is low (1 : 250), the nPOD AAb screening programme provides the best available means to identify organ donors with subclinical disease pathogenesis, and by confirming with orthogonal assays there is a higher level of confidence that these cases reflect pre-diabetes, particularly with cases that are multiple AAb-positive. Importantly, studies on tissues from these AAb-positive cases are yielding results that suggest these individuals are probably on the path towards the development of type 1 diabetes mellitus [20]. We acknowledge, however, that these observations are indirect, as follow-up for progression to hyperglycaemia is not possible. It is conceivable that some subjects with a single AAb could have been non-progressors; again, without follow-up, we cannot be certain.

The comparison of ELISA and RBA results has led to the following observations. There was agreement between the two assays for the majority of samples; some exceptions were observed, however, including instances where either ELISA or RBA distinctly identified GADA, IA-2A or ZnT8A in individuals diagnosed as having type 1 diabetes mellitus. It is currently not known if these discrepancies are the result of different epitopes that are identified uniquely by the format of the ELISA *versus* RBA [34]. The programme is designed with the RBA as the gold standard and therefore, within the nPOD database, the AAb-positive cases are those confirmed by RBA. In order to draw any conclusion about ELISA-positive RBA-negative results in diabetes-free subjects, one would need to compare larger prospective cohorts of subjects with both the ELISA and RBA, in terms of disease prediction.

Comparing AAb results in screening *versus* recovery samples (separated by only a few days) has revealed that, in a few samples, the titre has either decreased or increased. It is not known definitively whether the management of these individuals in the ICU has resulted in either dilution or concentration of AAb in circulation. Indeed, in two particular cases, the screened sample was positive for GADA in both the ELISA and RBA assay while the recovery sample was found negative by both methods. It is true, however, that most samples were relatively constant in titre between the two samples, but it is important to keep in mind the possible dilution or concentration due to effects of patient management efforts in the hospital. nPOD has implemented a programme that has identified AAb-positive individuals successfully among the general organ donor population in clinical serology laboratories previously inexperienced in type 1 diabetes screening. This was made possible because of the development of a sufficiently sensitive and specific commercial ELISA that was modified for our purposes for the detection of three relevant AAb. The final configuration of these kits has improved the concordance between screening and core laboratories and specificity of the programme. By implementing a conservative approach, favouring specificity in the ELISA and ultimately having the RBA determine final AAb status, we are confident that the programme is able to identify rare AAb-positive cases accurately. At the time of writing, the ElisaRSR 3 Screen

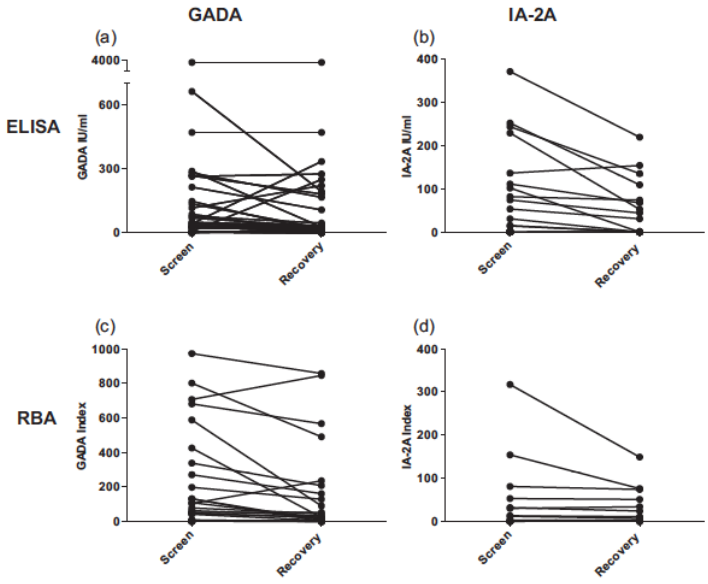

Fig. 4. Screening serum sample *versus* the organ recovery serum sample results by (a,b) enzyme-linked immunosorbent assay (ELISA) and (c,d) radiobinding assay (RBA) are compared for (a,c) glutamic acid decarboxylase (GADA) and (b,d) insulinoma-associated protein-2 (IA-2A).

ICA (Cardiff, Wales, UK) has become newly available for the detection of GADA, IA-2A, and ZnT8A in a single assay, and further testing of this new application is warranted for potential implementation as part of the nPOD OPO AAb screening programme. As these AAb-positive donors are thought to be in the pre-type 1 diabetes phase of disease development, this programme has extended the ability to perform cross-sectional investigation of the target organ in humans, a largely unexplored resource for understanding the disease pathogenesis. Of note is the observation that an AAb-positive donor pancreas, in particular multiple AAb, may already contain compromised islets. This facet warrants additional exploration as to whether these organs should be transplanted. At this time, we have insufficient data to make that proclamation definitively, but given the potential clinical implications, it should be the focus of additional studies.

Author contributions

C. W. researched the data and wrote the manuscript, E. M. researched the data and reviewed/edited the manuscript, L. Y. researched the data and reviewed/edited the manuscript, A. M. researched the data and reviewed/edited the manuscript, R. G. researched the data and reviewed/edited the manuscript, A. P. conceived of the study and reviewed/edited the manuscript, C. N. contributed to discussion and reviewed/edited the manuscript, J. S. K. analysed the data and reviewed/edited the manuscript, D. A. S. contributed to discussion and reviewed/edited the manuscript, E. B. conceived of the study and reviewed/edited the manuscript, and M. A. A. conceived of the study and wrote the manuscript. M. A. A. is the guarantor of his work and, as such, takes responsibility for the integrity of the contents herein.

Disclosure

The authors declare that no disclosures exist.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Receiver operating characteristic (ROC) curve analyses are shown for (a) glutamic acid decarboxylase (GADA), (b) insulinoma-associated protein-2 (IA-2A) and (c) zinc transporter 8 (ZnT8A). Plots, showing only 95–100% specificity, are 100 − specificity % (*x*-axis) *versus* sensitivity % (*y*-axis). The following cut-offs were selected to maximize specificity: (a) 20 IU, 98% specificity and 70% sensitivity [area under the curve (AUC) = 0·88], (b) 60 IU, 99% specificity and 63% sensitivity (AUC = 0·80) and (c) 20 IU, 97% specificity with 57% sensitivity (AUC = 0·75).

[CW2] Insulitis and β-Cell Mass in the Natural History of Type 1 Diabetes

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☐	18	THYROID	10,802	5.515	0.01836
☐	19	INTERNATIONAL JOURNAL OF OBESITY	21,740	5.487	0.03304
☐	20	JOURNAL OF CLINICAL ENDOCRINOLOGY & METABOLISM	76,719	5.455	0.11446
☐	21	ENDOCRINE-RELATED CANCER	6,547	5.267	0.01153

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Insulitis and β-Cell Mass in the Natural History of Type 1 Diabetes

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Descriptions of insulitis in human islets throughout the natural history of type 1 diabetes are limited. We determined insulitis frequency (the percent of islets displaying insulitis to total islets), infiltrating leukocyte subtypes, and β-cell and α-cell mass in pancreata recovered from organ donors with type 1 diabetes (n = 80), as well as from donors without diabetes, both with islet autoantibodies (AAb^+, n = 18) and without islet autoantibodies (AAb^-, n = 61). Insulitis was observed in four of four donors (100%) with type 1 diabetes duration of ≤1 year and two AAb^+ donors (2 of 18 donors, 11%). Insulitis frequency showed a significant but limited inverse correlation with diabetes duration (r = −0.58, P = 0.01) but not with age at disease onset. Residual β-cells were observed in all type 1 diabetes donors with insulitis, while β-cell area and mass were significantly higher in type 1 diabetes donors with insulitis compared with those without insulitis. Insulitis affected 33% of insulin$^+$ islets compared with 2% of insulin$^-$ islets in donors with type 1 diabetes. A significant correlation was observed between insulitis frequency and $CD45^+$, $CD3^+$, $CD4^+$, $CD8^+$, and $CD20^+$ cell numbers within the insulitis (r = 0.53–0.73, P = 0.004–0.04), but not $CD68^+$ or $CD11c^+$ cells. The presence of β-cells as well as insulitis several years after diagnosis in children and young adults suggests that the chronicity of islet autoimmunity extends well into the postdiagnosis period. This information should aid considerations of therapeutic strategies seeking type 1 diabetes prevention and reversal.

Type 1 diabetes (T1D) is a chronic autoimmune disorder resulting from poorly understood combinations of immunologic, genetic, and environmental factors that drive immune responses against multiple β-cell antigens, resulting in the irreversible loss of functional pancreatic β-cells (1). These destructive processes are thought to begin months to years before the clinical symptoms of T1D occur. Ongoing autoimmunity and β-cell destruction are asymptomatic during this prediabetic period, but can be identified serologically by the presence of autoantibodies against one or more of several β-cell autoantigens, including GAD antibody (GADA), islet antigen 2 antibody (IA-2A), insulin autoantibody (IAA), and zinc transporter 8 (ZnT8A) (2). The number, rather than the titer, of these so called "islet autoantibodies" can be used to determine risk for T1D development (reviewed in Brorsson et al. [3]).

Whereas the initial description for inflammation of pancreatic islet cells (i.e., insulitis) in individuals with T1D occurred more than a century ago, a limited number of studies have characterized this lesion in patients with the disease or during the preclinical phase (4). Certain exceptions exist, yet a meta-analysis of the literature would suggest that insulitis is present in young donors (<14 years of age) within 1 year of T1D diagnosis as well as in donors with multiple islet autoantibodies who did not have diabetes (5–9). Difficulties in studying human islets/β-cells in vivo can be ascribed to several factors, including their relative scarcity within the pancreas (1–2%), anatomical inaccessibility, declining patient autopsy rates, and inherent risks associated with pancreatic biopsy (reviewed in Krogvold et al. [10]). This inability to perform pathological evaluations is unfortunate as such evaluations hold the potential to help explain, in part, multiple facets of disease heterogeneity, including age variation at diagnosis and disease progression including

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See accompanying article, p. 545.

rate of C-peptide decline after onset or with experimental therapy (11,12). In an attempt to overcome these limitations, organized efforts have been developed to obtain high-quality pancreas biospecimens from organ donors to study mechanisms of β-cell loss in T1D (e.g., PanFinn, Belgian Diabetes Registry, JDRF Network for Pancreatic Organ Donors with Diabetes [nPOD]) (7,13,14).

In the current study, our major objective was to screen pancreata from nPOD donors with and without T1D, as well as from donors with and without islet autoantibodies but no diabetes, for islets with insulitis followed by leukocyte subtyping of infiltrating cells. Insulitis frequency and leukocyte subtypes in islets still expressing insulin as well as insulin$^-$ islets were correlated with donor clinical attributes (age at onset or demise, diabetes duration, autoantibody numbers, HLA, and diabetic ketoacidosis [DKA]). The β-cell and α-cell area and mass were also determined for each donor group and were tested for correlations to insulitis frequency and diabetes duration.

RESEARCH DESIGN AND METHODS

Study Design

Pancreata were recovered from organ donors during a 7-year period through the JDRF nPOD program according to procedures previously described (14–16). This report provides results from donors with the following: *1*) no history of diabetes or other pancreatic disease and negative for islet cell autoantibodies (AAb$^-$, n = 61); *2*) no history of diabetes or other pancreatic disease but positive for islet autoantibodies (AAb$^+$, n = 18); or *3*) T1D (n = 80). The lower age limit in this study was 4 years because the youngest donor with T1D was 4.4 years of age and estimates of β-cell proliferation were reported to be near adult levels by this age (17,18). Clinical and demographic data are summarized (Supplementary Table 1) and include the proportions of donors by numbers of islet autoantibodies and those with DKA in relation to cause of death. Diabetes durations were known for 79 of 80 donors with T1D; 1 donor with unknown diabetes duration was a 34-year-old Caucasian male with a clinical history of insulin-dependent diabetes, undetectable C-peptide levels, and no insulin$^+$ islets, as determined by histopathology (nPOD case 6032). Ethical permission was obtained from the Institutional Review Board at the University of Florida, and informed consent was obtained from a legal representative of each donor.

Laboratory Assessments

Islet autoantibody assays, serum C-peptide levels, and high-resolution HLA genotyping were performed as previously described (14–16). Islet autoantibodies were measured by radioimmunoassay against all four major T1D-associated autoantigens (GADA, IA-2A, ZnT8A, and IAA) in all but one donor, in whom T1D was diagnosed at 10.7 years of age and who had diabetes for a duration of 6 years (nPOD 6062). Of note, after institution of insulin therapy for T1D, IAAs could not be distinguished from antibodies induced by insulin injections, so IAAs were excluded from autoantibody counts for the donors in the T1D subgroup in Table 1 and Supplementary Tables 1 and 3 (19).

Insulitis Screening and Insulitic Islet Subtyping for Insulitis Frequencies

Pancreata were processed to formalin-fixed paraffin blocks for each pancreas region (head, body, and tail) as previously described (20). For each donor, serial sections (average two blocks per region) were stained by hematoxylin-eosin and two double-immunohistochemistry (IHC) stains (Ki67 and insulin, CD3 and glucagon) (Supplementary Table 2) (21). When insulitic islets were found in a given donor, additional blocks were screened (as detailed below). Stained sections were scanned at ×20 magnification using an Aperio CS scanner (Leica/Aperio, Vista, CA), and all images were stored in an online pathology database (eSLIDE; Leica/Aperio).

Screening for insulitic islets was performed on CD3$^+$ glucagon–stained sections. An islet was defined as ≥10 α-cells. Insulitis was defined as an islet with six or more CD3$^+$ cells immediately adjacent to or within the islet with three or more islets per pancreas section, according to recent criteria (4). Islets with insulitis were marked in an image layer using ImageScope software (Leica/Aperio). The two IHC serial images were aligned using the synchronization tool, and insulin$^+$ islets were also marked on the image layer. All islets/sections from donors with insulitis were subsequently subtyped as follows: *1*) insulin$^+$ CD3$^-$, *2*) insulin$^+$ CD3$^+$, *3*) insulin$^-$ CD3$^+$, and *4*) insulin$^-$ CD3$^-$ (see Table 1 for numbers of islets analyzed). Then, all islets were counted by subtype. The process was reversed for AAb$^+$ donors (i.e., islet subtypes were counted using the Ki67-insulin image after markup for CD3$^+$ insulitic islets and insulin$^-$ islets using the CD3-glucagon image). The number of pancreas sections subtyped for insulitis ranged from 2 to 16 sections/donor (8.1 ± 4.1 sections/donor, n = 162 sections) (Table 1). The lowest number of available sections was due to partial pancreas recovery (tail only in nPOD 6198).

Insulitis frequency (percent) was calculated as the total number of insulitic islets (sum of insulin$^+$ CD3$^+$ and insulin$^-$ CD3$^+$ islets) divided by the total number of islets (sum of four subtypes). The frequency of insulin$^+$ insulitic islets in relation to the total number of insulin$^+$ islets was determined by the ratio of (insulin$^+$ CD3$^+$ islets)/(sum of insulin$^+$ CD3$^-$ and insulin$^+$ CD3$^+$ islets) with similar calculations for the frequency of insulin$^-$ insulitic islets (insulin$^-$ CD3$^+$)/(sum of insulin$^-$ CD3$^+$ and insulin$^-$ CD3$^-$).

Insulitis Leukocyte Phenotyping

Paraffin sections from blocks having the maximum insulitis frequency for each donor were stained, and positive leukocytes/insulitic islets (six or more CD3$^+$ cells) were counted using multi-immunofluorescence. Serial sections (4 μm) were dewaxed and rehydrated with Tris

Table 1—Insulitis frequency in donors

	Case ID	Sections (*n*)	Ins$^+$ CD3$^-$	Ins$^+$ CD3$^+$	Ins$^-$ CD3$^+$	Ins$^-$ CD3$^-$	Total islets*	Insulitis frequency (%)†	Age at onset (years)‡	Diabetes duration (years)	Age at demise (years)	Autoantibody results§‖
T1D	6264	6	7	3	0	1,017	1,027	0.3	3.00	9.00	12.00	Negative
	6212	12	51	13	10	1,601	1,675	1.4	15.00	5.00	20.00	
	6268	5	0	1	13	808	822	1.7	9.00	3.00	12.00	
	6265	6	8	6	5	508	527	2.1	3.00	8.00	11.00	GADA$^+$
	6039	4	0	2	5	324	331	2.1	16.70	12.00	28.70	GADA$^+$ IA-2A$^+$ ZnT8A$^+$
	6046	5	92	2	6	268	368	2.2	10.80	8.00	18.80	IA-2A$^+$ ZnT8A$^+$
	6113	14	21	28	9	1,640	1,698	2.2	11.52	1.58	13.10	
	6224	8	30	24	12	1,486	1,552	2.3	19.50	1.50	21.00	Negative
	6088	7	73	7	6	433	519	2.5	26.20	5.00	31.20	GADA$^+$ IA-2A$^+$ ZnT8A$^+$
	6245	6	131	7	28	1,021	1,187	2.9	15.00	7.00	22.00	GADA$^+$ IA-2A$^+$
	6247	6	77	20	25	1,068	1,190	3.8	23.40	0.60	24.00	
	6195	16	0	2	53	1,385	1,440	3.8	14.20	5.00	19.20	GADA$^+$ IA-2A$^+$ ZnT8A$^+$
	6070	4	56	15	8	333	412	5.6	15.60	7.00	22.60	IA-2A$^+$
	6198	2	16	9	5	181	211	6.6	19.00	3.00	22.00	GADA$^+$ IA-2A$^+$ ZnT8A$^+$
	6052	9	10	27	41	921	999	6.8	11.00	1.00	12.00	IA-2A$^+$
	6209	11	32	42	54	1,247	1,375	7.0	4.75	0.25	5.00	IA-2A$^+$ ZnT8A$^+$
	6243	8	162	55	32	859	1,108	7.9	8.00	5.00	13.00	
	6228	15	250	246	74	2,447	3,017	10.6	13.00	0.00	13.00	GADA$^+$ IA-2A$^+$ ZnT8A$^+$
	Total	144	1,016	509	386	17,547	19,458					
AAb+¶	6197	5	1,981	28	0	4	2,013	1.4			22.00	GADA$^+$ IA-2A$^+$
	6267	13	2,149	139	8	18	2,314	6.4			23.00	GADA$^+$ IA-2A$^+$
	Total	18	4,130	167	8	22	4,327					

Multiple slide sections were reviewed from each donor pancreas, and islets were counted by insulin immunopositivity (Ins$^+$, Ins$^-$) and insulitis status (CD3$^-$, CD3$^+$). The total number of insulitic islets divided by the total number of islet was used to calculate insulitis frequency (%) for each donor. Donors were listed in order of increasing insulitis frequency (%). Demographic information for age at demise and diabetes onset, diabetes duration, and autoantibody types were also shown. *Total islets calculated as the sum of four islet subtypes. †Insulitis frequency (%) calculated as sum insulitic islets: (Ins$^+$ CD3+ and Ins$^-$ CD3$^+$)/total islets. ‡Calculated as age at demise minus diabetes duration. §All donors with insulitis had autoantibody testing by radioimmunoassay. Radioimmunoassay data values were converted to National Institute of Diabetes and Digestive and Kidney Diseases units and defined as positive if one or more of the following applied: GADA if ≥20, IA-2A if ≥5, or ZnT8A if ≥0.020. ‖IAA were not listed for donors with T1D as they could not be distinguished as autoantibodies versus antibodies to parenteral insulin as described in RESEARCH DESIGN AND METHODS. An empty field indicates that only insulin autoantibodies were detected by radioimmunoassay. ¶AAb$^+$ donors without diabetes.

buffer. Heat-induced antigen retrieval was performed using Trilogy (Cell Marque, Rocklin, CA) at 95° for 20 min followed by rinsing in water for 20 min. The staining series was designed to phenotype leukocytes (total leukocytes [CD45], T [CD3] and B [CD20] lymphocytes, T-lymphocyte subsets [CD4 and CD8], and monocytes [dendritic cells (CD11c) and macrophages (CD68)]) (21) in conjunction with subtyping islets for insulin immunopositivity. The staining series was as follows: *1*) $CD45^+$glucagon$^+$insulin, *2*) $CD20^+CD3^+$glucagon, *3*) $CD8^+CD4^+$glucagon, and *4*) $CD11c^+CD68^+$insulin. Chromogranin A staining was also used to delineate endocrine cells. Antigens are listed in order of primary antibody incubation, and the corresponding secondary antibody and conjugated fluorochrome (AF488-AF555-AF647) (Supplementary Table 2). After blocking, sections were sequentially incubated with the primary antibody followed by the appropriate secondary antibody. For anti-CD4, a Cy3 Tyramide Signal Amplification Kit (PerkinElmer, Waltham, MA) was used according to the manufacturer's instructions. All sections were mounted with ProLong Gold Antifade mounting media containing DAPI (Life Technologies, Grand Island, NY). Positive controls included human spleen, tonsil, and donor intrapancreatic lymph nodes, and negative controls included omission of the primary antibody.

The numbers of leukocytes/insulitic islets were determined using multichannel image acquisition software on a Zeiss Axiophot microscope (AxioVision; Carl Zeiss Inc., Thornwood, NY). Fluorescent channels were viewed in combination with DAPI to count the number of positive leukocytes/islet.

β-Cell and α-Cell Area and Mass

Insulin- and glucagon-immunopositive areas were determined using the IHC sections to estimate β-cell and α-cell areas, respectively, in relation to total tissue area using a single Aperio colocalization algorithm (22). An average of six sections was used per donor (two sections/head, body, and tail regions). The β-cell and α-cell areas were expressed as a ratio (percent) to the total sectional area, including acinar and interstitial regions, to permit the use of pancreata weights. The average β-cell and α-cell area per pancreas was calculated from regional area averages. The β-cell or α-cell mass (in milligrams) was calculated by multiplication of the respective average area and pancreas weight (in grams).

Statistical Analysis

Numbers and percents were reported for all categorical factors, the mean (±SD, n = number of donors unless noted in figure legend) was reported for normally distributed continuous variables, and the median (minimum, maximum) was reported for continuous variables with a skewed distribution. Donor characteristics were analyzed using a Satterthwaite-corrected t test if continuous and parametric, a Kruskal-Wallis test if continuous and nonparametric, a Pearson χ^2 test if categorical values in all cells exceeded 5, or a Fisher exact test if categorical values in one or more cells were <5. P values were not calculated if values from two or more cells were equal to 0. Donor characteristics were correlated to insulitis frequency, and leukocyte subtype numbers/islets were correlated to other subtypes by Spearman correlation and plotted with linear regression lines. Data were analyzed with the exclusion of the AAb^+ donors with insulitis due to the small sample size (n = 2).

To obtain a subset of T1D donors and to compare clinical characteristics in the presence or absence of insulitis, a selection of controls for all 18 T1D insulitis cases was attempted using a 1:1 ratio to identify the single best match. A matching method was used to minimize bias in lesion effect estimates from key covariates (i.e., age at diabetes onset, sex, ethnicity, and BMI). A propensity score, given the selected covariates, was calculated for all T1D donors (23). The logit of the propensity score was used to match donors without insulitis to those with insulitis. A randomly sorted nearest-available-neighbor matching method without replacement was used with an optimal caliper width equal to 0.20 SD of the logit value (0.15), as has been shown to be optimal (24), along with an SAS Software (SAS Institute, Cary, NC) macro (25,26). All reported P values are two-tailed and significant if <0.05. All analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA) or SAS Software version 9.3.

RESULTS

Insulitis Screening in Donors With T1D and AAb^+ Donors With No Diabetes

A total of 159 pancreata were reviewed for insulitis (no diabetes [n = 61 AAb^-; n = 18 AAb^+]; n = 80 T1D) (Supplementary Table 1). Insulitis was observed in 18 T1D donors (18 of 80 donors, 23%) and 2 AAb^+ donors (2 of 18 donors, 11%). Variability in the lobular distribution of insulin$^+$ islets and insulitic islets was high in donors with T1D (Fig. 1*A–D*). In AAb^+ donors with insulitis, insulitis was primarily observed in insulin$^+$ islets (Supplementary Fig. 1). The number of $CD3^+$ cells/islet varied in a given donor, irrespective of islet insulin immunopositivity or size, and $CD3^+$ cells were observed diffusely infiltrating islets, in variously sized aggregates, or both (Fig. 2 and Supplementary Fig. 2).

All 18 donors with T1D and insulitis had insulin$^+$ islets detected in at least one section, while only 5 of 62 donors (8%) with T1D and no insulitis had insulin$^+$ islets. Insulitis was found in 509 of 1,525 insulin$^+$ islets (33%) and in 379 of 17,718 insulin$^-$ islets (2%) in donors with T1D (Table 1). In AAb^+ donors with insulitis, the opposite was found; insulitis affected 167 of 4,297 insulin$^+$ islets (4%) and 8 of 30 insulin$^-$ islets (27%). Insulitis was found in T1D donors ranging in age from 3 to 26.2 years (mean age 13.3 ± 6.5 years) at disease onset and with disease durations of 0–12 years (mean duration 4.6 ± 3.4 years) (Table 1). The highest insulitis frequency (10.6%) was observed in a

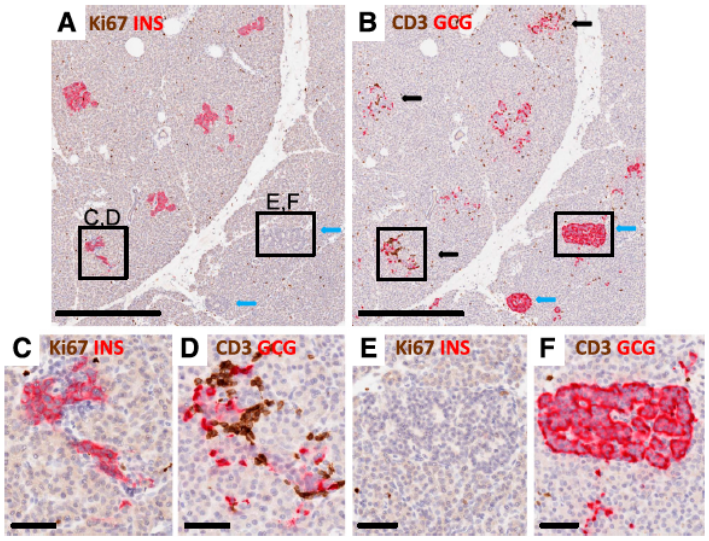

Figure 1—Lobular variability in insulin$^+$ islets and insulitis. Islets were imaged from a 13-year-old patient with T1D for 5 years (nPOD 6243). Serial paraffin sections were stained for Ki67 plus insulin (INS) (*A*, *C*, and *E*) and CD3 plus glucagon (GCG) (*B*, *D*, and *F*), and islets were subtyped as described in RESEARCH DESIGN AND METHODS. Five insulin$^+$ islets (*A*) are seen in a lobule adjacent to a lobule with two insulin$^-$ islets in the patient with T1D (*A* and *B*, blue arrows). Three insulin$^+$ islets had insulitis (*B*, black arrows), and both insulin$^-$ islets did not have insulitis. One of the insulin$^+$ islets with insulitis is shown at higher magnification (*C* and *D*) as well as an insulin$^-$ insulitis$^-$ islet (*E* and *F*). Few islet cells were Ki67$^+$ (*A* and *C*), indicating no effect of insulitis on proliferating cell numbers. Scale bars: *A* and *B*, 500 μm; *C–F*, 50 μm.

13-year-old who presented with DKA at disease onset (nPOD 6228). The lowest insulitis frequency (0.3%) was observed in a 12-year-old who had T1D for 9 years (nPOD 6264). The two AAb$^+$ donors with insulitis had multiple autoantibodies and were 22 and 23 years old (Table 1). The AAb$^+$ donors had insulitis frequencies (1.4%, 6.4%) within the range observed for donors with T1D; few insulin$^-$ islets, with or without insulitis, were observed in these donors.

The percent of islets with insulitis (insulitis frequency) was tested for a correlation with diabetes duration. Insulitis frequency showed a low, yet significant, inverse relationship to diabetes duration, whereas age at onset or demise did not (Fig. 3).

Correlation of Insulitis Prevalence With Diabetes Duration

Because past studies reporting insulitis primarily examined young donors with T1D within a year of onset, the prevalence of donors with insulitis was analyzed in this study using similar criteria (6). All four donors with recent-onset T1D (duration ≤1 year) had insulitis; three of four donors were <15 years of age at disease onset (Table 2). In donors with disease durations >1 year, insulitis was found in 7 of 51 donors (14%) who were <15 years of age at disease onset and in 7 of 24 donors (29%) who were ≥15 years of age at disease onset. Using 12 years of diabetes duration as the cutoff, the prevalence of insulitis was 45% in donors (18 of 40 donors) with T1D.

To further define other potential clinical variables as they relate to insulitis, 15 T1D donors with insulitis were matched for age at onset, ethnicity, sex, and BMI with 15 T1D donors without insulitis (Supplementary Table 3; in three cases, no matches were found and thus were excluded). Diabetes duration was significantly shorter in T1D donors with insulitis versus those without insulitis (5 vs. 14.5 years, respectively). Correspondingly, donors with insulitis were significantly younger at demise compared with matched donors without insulitis (20 vs. 27.6 years, respectively). Interestingly, islet autoantibody numbers were not different between the matched T1D donors. Hospitalization durations were not significantly different in this matched subset and ranged from a mean of 3.2 to 3.7 days across all donor groups (Supplementary Tables 1 and 3). Correlation of DKA during the hospitalization showed a significantly higher proportion of donors with insulitis (7 of 15 donors, 47%) versus those without insulitis (1 of 15 donors, 7%, $P = 0.04$).

Infiltrating Leukocytes Have Similar Proportions of B and T Cells and T-Cell Subsets, and Numbers Were Proportional to Insulitis Frequency

Infiltrating leukocytes in insulitic islets were examined by multi-immunofluorescence on serial sections (Fig. 4).

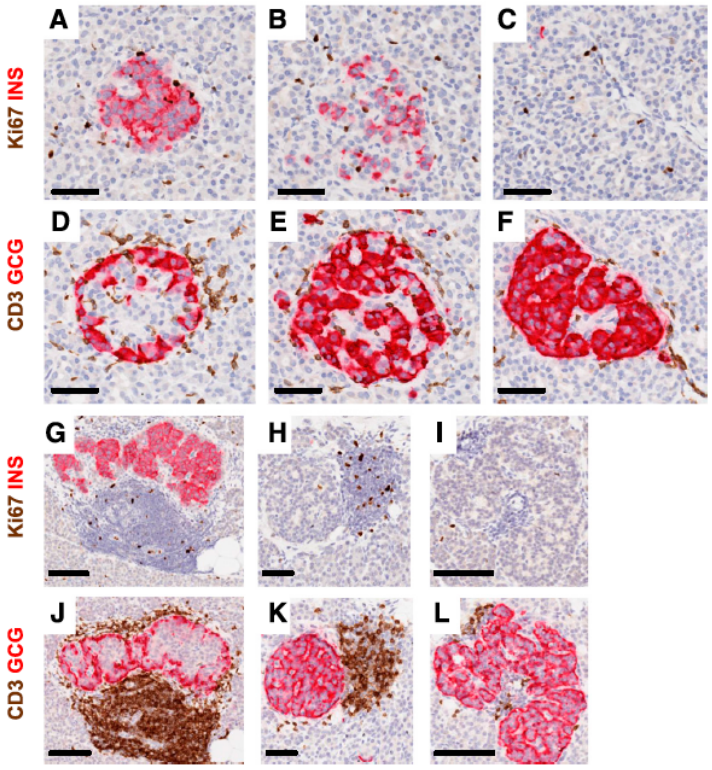

Figure 2—Heterogeneity of islet β-cells and the numbers of CD3$^+$ cells in insulitic islets in young donors at diabetes onset and after 5 years of disease duration. Representative islets were imaged for a 13-year-old at diabetes onset (nPOD 6228; *A–F*) and for another 13-year-old with T1D for 5 years (6243; *G–L*). Serial paraffin sections were stained using dual-IHC (Ki67 plus insulin [INS], CD3 plus glucagon [GCG]), as described in RESEARCH DESIGN AND METHODS. Decreasing proportions of β-cells are shown in different islets from both donors (*A–C* and *G–I*) as well as heterogeneity in numbers of CD3$^+$ cells per islet (*D–F* and *J–L*). Heterogeneity in CD3$^+$ counts between two insulin$^-$ islets of similar sizes is also shown for one donor (*K* and *L*). Scale bars: *A–F*, *H*, and *K*, 50 μm; *G*, *I*, *J*, and *L*, 100 μm.

Mean numbers of CD45$^+$ cells/islet in each donor were significantly correlated to mean numbers of both T cells (CD3$^+$) and B cells (CD20$^+$) per islet (Fig. 5). Absolute numbers of CD3$^+$ and CD20$^+$ cells varied between islets in a given donor, depending on the nature of the infiltrate (e.g., diffusely associated with islet, aggregate, and mixture). The numbers of both CD3$^+$ and CD20$^+$ cells appeared independent of islet insulin immunopositivity and diabetes duration (Supplementary Figs. 2 and 3). The numbers of CD3$^+$ cells were significantly correlated with the numbers of CD8$^+$ and CD4$^+$ cells/islet (Fig. 5*B*). Leukocyte numbers per islet were also plotted for both donor groups with insulitis, and high variability was observed, particularly for CD45$^+$, CD3$^+$ and CD20$^+$ (Fig. 5*C*). This variability was also seen in the AAb$^+$ donors without diabetes (Fig. 5*C*). Five donors had insulitis aggregates with >50 CD3$^+$ cells/islet; four were donors with T1D who were all <12 years of age but had widely varying diabetes durations (nPOD 6209, 6243, 6052, and 6070), and one was an AAb$^+$ donor without diabetes (nPOD 6267).

We also tested whether the numbers of leukocytes per islet were correlated with insulitis frequency, and found significant correlations with the numbers of CD45$^+$, CD3$^+$, CD20$^+$, CD8$^+$, and CD4$^+$ cells per islet, but not for the numbers of CD68$^+$ and CD11c$^+$ cells per islet (Supplementary Fig. 4 and Supplementary Table 4). Because of the variability between islets in the numbers of leukocytes per insulitic islet, we also tested whether a ratio of markers (CD3$^+$ cells/sum of CD3$^+$ and CD20$^+$ cells) would normalize interislet variability but found no significant correlation to insulitis frequency (Supplementary Fig. 4).

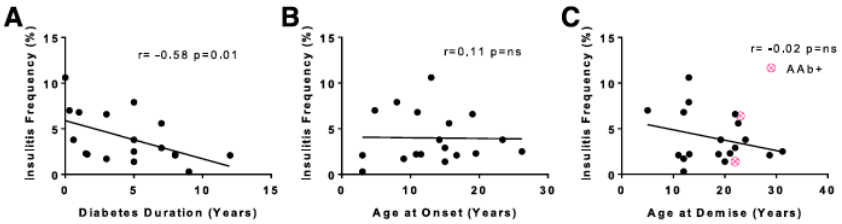

Figure 3—Insulitis frequency in relation to diabetes duration and donor ages at demise or disease onset. The insulitis frequency (%, total number of insulitic islets/total number of islets) is shown on the *y*-axis in comparison with years for diabetes duration (*A*), age at T1D onset (*B*), and age at demise (*C*). Data were displayed (pink) for the two AAb$^+$ donors with insulitis for age at demise but were excluded from statistical analyses because of the small sample size. Insulitis frequency (%) had a low but significant correlation to diabetes duration, but not to donor age at demise or disease onset. The linear regression line with Spearman *r* and *P* values are shown.

β-Cell and α-Cell Mass and Insulitis

Pancreata weights were variable in donors without diabetes: 71.1 ± 24.2 g (n = 49) in AAb$^-$ donors, 70.7 ± 28.0g (n = 15) in AAb$^+$ donors without insulitis, and 60.7 ± 17.9 in two AAb$^+$ donors with insulitis (Fig. 6*A*). As anticipated, both donor groups without diabetes (AAb$^-$ and AAb$^+$) showed increasing pancreas weight after age 4 years up to ~30 years (Supplementary Fig. 5*A* and *B*). Donors with T1D had a significantly lower mean pancreas weight (39.51 ± 17.9 g no insulitis, n = 60; 33.8 ± 12.9 g with insulitis, n = 16) compared with donors without diabetes (Fig. 6*A*). Pancreata weights were decreased at all ages in donors with T1D compared with donors without diabetes (Supplementary Fig. 5*C*).

β-Cell area was also variable in donors without diabetes: 1.18 ± 0.71% for AAb$^-$ donors (n = 57) and 0.67 ± 0.38% for AAb$^+$ donors without insulitis (n = 16) (Fig. 6*B*). The two AAb$^+$ donors with insulitis had a mean β-cell area of 2.26 ± 0.4%. As expected, significant reductions in β-cell area were observed for donors with T1D compared with those for donors without diabetes (Fig. 6*B*). β-Cell area was also significantly different between the two donor groups with T1D (0.02 ± 0.05% no insulitis, n = 54; 0.15 ± 0.17% with insulitis, n = 18) (Fig. 6*B*).

The mean β-cell mass was 797 ± 523 mg for AAb$^-$ donors (n = 45) and 448 ± 268 mg for AAb$^+$ donors without insulitis (n = 15) (Fig. 6*C*). β-Cell mass was 1,407 ± 649 mg in the two AAb$^+$ donors with insulitis. β-Cell mass was decreased in donors with T1D, regardless of insulitis status, compared with those without T1D; however, β-cell mass was significantly higher in T1D donors with insulitis (47.9 ± 53.4 mg, n = 17, $P < 0.05$) compared with those without insulitis (5.5 ± 10.1 mg, n = 53) (Fig. 6*C*). The mass of β-cells from five donors without diabetes (three AAb$^-$, two AAb$^+$) overlapped with the highest values for β-cell mass in donors with T1D and insulitis due to low pancreas weight (3) or low β-cell area (2). Estimated β-cell mass was further tested for correlation with insulitis frequency, but no association was found (Supplementary Fig. 6*A*, $P > 0.05$). Similarly, we compared β-cell mass to T1D duration and age at onset to determine whether insulin mass was greater in donors with shorter diabetes durations or younger donors; again, no significant associations were found (Supplementary Fig. 6*B* and *C*, $P > 0.05$).

In marked contrast to differences in β-cell area and mass, α-cell area and mass were not significantly different between groups (Fig. 6*D* and *E*). The mean α-cell area was 0.73 ± 0.45% in AAb$^-$ donors (n = 57), 0.62 ± 0.44% in AAb$^+$ donors without insulitis (n = 16), 1.37 ± 0.04% in AAb$^+$ donors with insulitis (n = 2), 1.04 ± 0.84% in donors with T1D and no insulitis (n = 57), and 1.37 ± 0.62% in donors with T1D and insulitis (n = 17). The mean α-cell mass was 495 ± 320 mg in AAb$^-$ donors, 402 ± 428 mg in AAb$^+$ donors without insulitis, 827 ± 224 mg in AAb$^+$ donors with insulitis, 396 ± 377 mg in donors with T1D and no insulitis, and 513 ± 375 mg in donors with T1D and insulitis.

Influence of HLA on Insulitis

Donors with T1D had a higher frequency of DRB1*03:01-DQB1*02:01 or DRB1*04-DQB1*03:02 genotypes (DR3/DR4) compared with AAb$^-$ or AAb$^+$ donors (35.9% vs. 0%, $P < 0.0001$ and $P = 0.0012$, respectively). HLA DR3/DR4 frequencies were not different when comparing the matched donors within the T1D group, with and without insulitis (Supplementary Table 3). The DRB1*1501-DQB1*0602 haplotype was very rare and considered protective for T1D. The overall frequency of this haplotype in AAb$^-$ donors (25%) was similar to the general U.S. Caucasian population (22%) (27). This haplotype was found in four AAb$^+$ donors with single autoantibodies and in none of those with multiple autoantibodies. In the T1D donors, only one had the DRB1*1501-DQB1*0602 haplotype, and, quite interestingly, this donor also had insulitis (nPOD 6195).

DISCUSSION

The concept of insulitis has been the subject of previous reports; however, this study is the first to report insulitis

Table 2—Insulitis prevalence in nPOD donors by age of onset and diabetes duration

	Diabetes duration ≤1 year			Diabetes duration >1 year			Totals*		
	Number donors insulitis+	Total number donors	Proportion with insulitis (%)	Number donors insulitis+	Total number donors	Proportion with insulitis (%)	Number donors insulitis+	Total number donors	Proportion with insulitis (%)
Age at onset (years)									
1.4–14.2	3	3	100	7	51	14	10	54	19
15–36	1	1	100	7	24	29	8	25	32
Total	4	4	100	14	75	19	18	79	23

Age-groups and diabetes durations adapted from In't Veld (6). *One donor with T1D had an unknown duration of disease (see RESEARCH DESIGN AND METHODS).

frequency, leukocyte subtypes, pancreatic weights, and β-cell and α-cell mass in organ donors encompassing the entire natural history of T1D (i.e., preclinical phase [no diabetes, islet autoantibody positive] as well as acute and chronic phases]. Beyond this, the current study is large, used standard operating procedure–driven protocols, and used only high-quality tissues. As a result, we believe the results noted herein provide much in the way of new information as well as validating, or refuting, past observations.

Insulitis was defined by the presence of six or more $CD3^+$ cells/islet and three insulitic islets/section (4). Overall, insulitis was identified in 18 of 80 donors with T1D (23%) and 2 of 18 AAb^+ donors without diabetes (11%). Notably, the numbers of donors with insulitis (insulitis prevalence) was higher for older donors and for longer disease durations in both young and older patients than reported using a meta-analysis of existing studies (6). Historically, insulitis prevalence has been reported in 51% of patients (41 of 81 patients) with disease duration of ≤1 year, compared with only 3% of patients (4 of 132 patients) with durations >1 year (6). As shown in Table 2, we observed an insulitis prevalence of 100% (4 of 4 donors) in donors with T1D durations of ≤1 year and 19% (14 of 75 of donors) in donors with durations of >1 year. We also observed a high proportion of donors with insulitis for diabetes durations up to 12 years (18 of 40 donors, 45%). At the same time, our overall insulitis prevalence of 23% (18 of 79 donors) in donors with T1D compares well with the overall insulitis prevalence rate of 21% (45 of 213 donors) from former studies (6). Heterogeneity in the distribution of insulitic islets could partly explain the lower detection rates from former studies using autopsy collections or biopsy specimens because fewer blocks and pancreas regions could be tested per patient (5,7).

Studies by Oram et al. (11) and several others (28–30) support the persistence of β-cells in patients with long-standing T1D. Our data also show the persistence of β-cells in donors with T1D, with and without insulitis, up to 12 years after disease onset. Although other investigators have also reported $insulin^+$ islets without insulitis in patients with T1D, few have emphasized that $insulin^-$ islets are also involved in the insulitic process (reviewed in Gepts [31]). The proportions of $insulin^+$ and $insulin^-$ insulitic islets in our study (33% $insulin^+$, 2% $insulin^-$) were similar to those reported by Foulis et al. (5) and others (32) (28–35% $insulin^+$, 1–5% $insulin^-$). Persistent insulitis after the loss of β-cells could imply the presence of degranulated and/or dedifferentiated β-cells, undetected by these methods, or, alternatively, the spread of autoimmunity or "innocent bystander" effects on other endocrine cells. Prospective studies demonstrated that the appearance of multiple islet autoantibodies is usually sequential rather than simultaneous and that their number (two or more) is a strong predictor of progression to T1D (1). Herein, insulitis was observed in two of five

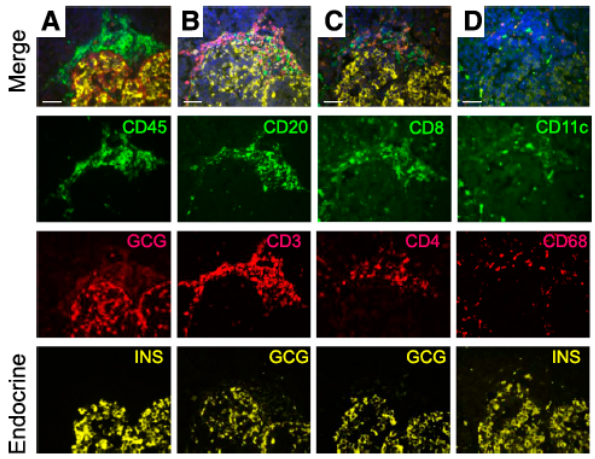

Figure 4—Insulitis leukocyte subtyping by multiple immunofluorescence. Serial sections of an islet with insulitic aggregate from a 5-year-old donor with T1D for 4 months (nPOD 6209) were imaged. The numbers of leukocytes/islet were counted for total leukocytes (CD45, *A*), B and T lymphocytes (CD20 and CD3, *B*), T-lymphocyte subsets (CD8 and CD4, *C*), and dendritic cells and macrophages (CD11c and CD68, *D*) as described in RESEARCH DESIGN AND METHODS. Islet endocrine cells were identified using glucagon (GCG) and insulin (INS) stains. The merged images (*A–D*) include DAPI-stained DNA, and the individual fluorescence channels are shown below the merged images. Scale bars: 50 μm.

donors (40%) with multiple autoantibodies, a finding that is similar to that in the study by In't Veld et al. (7), in which insulitis was detected in two of seven donors (29%) with multiple autoantibodies. Of note, the AAb^+ donors with insulitis in our study were younger (21 and 22 years of age compared with 46 and 52.5 years of age), and the entire pancreas was available for study compared with the former study of biopsy material. Interestingly, all four donors showed similar insulitis frequencies (1.4–6.4%, 3–9%) and normal β-cell mass, and all four donors had the autoantibody combination of $GADA^+$ and $IA\text{-}2A^+$.

Another novel aspect of our study is in showing similarities between the preclinical and clinical phases of T1D in the leukocyte subtypes in insulitic islets and independent of the presence of residual β-cells. Both phases were characterized by diffuse and/or focal aggregates and varying numbers of leukocytes/islet within and between donors. Several studies (7,30,32–38) have shown a predominance of $CD8^+$ T lymphocytes and macrophages in insulitic islets. The defining study was considered to be that of Willcox et al. (32), using 29 patients from the Foulis collection (5), and reexamination of 21 patients from the Foulis collection with two nPOD donors (6052 and 6113) was recently reported by Arif et al. (39). In the former study, insulitic islets were ranked by both insulin percentage and immune cell numbers (n = 279 insulitic islets), whereas only $insulin^+$ islets were evaluated in the latter study. All leukocytes were significantly reduced in $insulin^-$ islets (32). In marked contrast, our study found that $CD45^+$, $CD3^+$, $CD20^+$, and T-cell subsets increased linearly with the numbers of insulitic islets (insulitis frequency), irrespective of islet β-cells. Potential differences between studies may result from differences in age or disease duration or in technical differences in methods.

An intriguing hypothesis by Skog et al. (40) proposed that ascending infection through the pancreatic ducts could result in insulitis due to the greater susceptibility of β-cells compared with other endocrine cells. Though insulitic islets were frequently observed near pancreatic ducts in this study, no consistent pattern was observed for the presence of periductal infiltrates and insulitic islets. The heterogeneous leukocyte composition of insulitis might support this hypothesis if the offending agent (virus or bacterium) or its toxic by-products had minimal proclivity for ductular epithelial cells.

In the current study, donors with T1D had small pancreata regardless of age or disease duration. Pancreata weights were not different between AAb^- or AAb^+ donor groups without diabetes and are similar to the age-related changes reported in normal pancreas volume by Saisho et al. (41). Our current findings contrast with those of our previous report (42) using a subset (n = 8) of the 18 AAb^+ donors in this study. In our first study, all donors were >18 years of age and excluded if pancreatitis was noted in the medical chart or was discovered by histopathology. Age or pancreatitis exclusion criteria were not used in the current study. Because it is difficult to predict

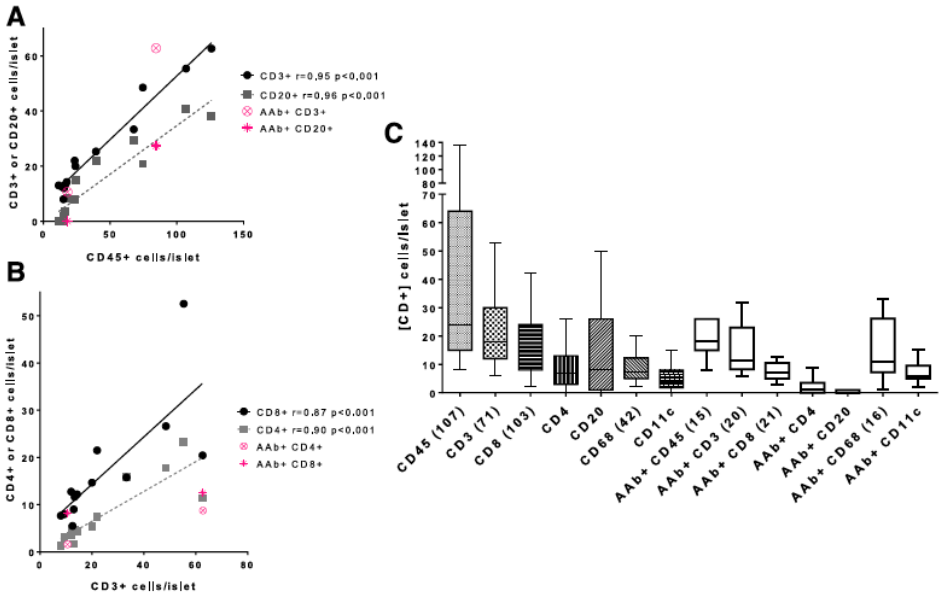

Figure 5—Leukocyte numbers increase in parallel with CD45$^+$ and CD3$^+$ numbers but are heterogeneous between islets. Numbers of CD45$^+$, CD3$^+$, CD20$^+$, CD8$^+$, and CD4$^+$ cells were counted for each insulitic islet and averaged per donor (n = 13 donors with T1D, 2–10 islets/donor). Data were also displayed (pink) for each leukocyte marker for the two AAb$^+$ donors with insulitis for comparison purposes but were excluded from statistical analyses because of the small sample size. Correlations between the numbers of leukocytes per islet were performed by Spearman analysis with linear regression lines shown. The numbers of CD45$^+$ cells per islet were significantly correlated with the numbers of CD3$^+$ and CD20$^+$cells per islet (*A*). The numbers of CD3$^+$ cells per islet were also significantly correlated with the numbers of CD8$^+$ and CD4$^+$ cells per islet (*B*). The numbers of leukocyte subtypes per islet were also analyzed to examine the variability in cell numbers among all islets for donors with T1D and two AAb$^+$ donors with insulitis, with means depicted by box-and-whisker plots with Tukey test error bars (nonparametric) (*C*). Numbers in parentheses following each marker indicate the total number of individual islets analyzed for each leukocyte marker (CD45) or set of markers. Variability in the overall numbers of leukocytes per islet was observed. The numbers of leukocytes per islet had similar trends in AAb$^+$ donors without diabetes.

when organ donors with multiple AAb$^+$ could progress to T1D, pancreas size loss in the natural history of T1D might be best studied in longitudinal clinical trials of AAb$^+$ subjects by noninvasive radiography (43,44). Beyond this, the notion of pancreatitis in individuals with T1D is of evolving interest, given the histopathological findings of multifocal CD3$^+$ infiltrates in acinar regions of donors with T1D. Indeed, Rodriguez-Calvo et al. (45) reported an increased number of exocrine CD8$^+$ T cells in nPOD donors with T1D and type 2 diabetes.

Reports indicate marked variability in β-cell area or mass in normal adults, with adult levels reached by 5 years of age (17,18,46,47). Indeed, some donors without diabetes (both AAb$^+$ and AAb$^-$) exhibit β-cell mass within the range observed for T1D donors. Reasons for this heterogeneity are unknown but could result from host genetic and environmental factors, including in utero and neonatal periods when β-cell mass is increasing. Rising rates of obesity could also account for some variability. Our β-cell area and mass data are in agreement with past literature that also reported high variability in both measures of islet β-cell content. For instance, our mean control β-cell area (1.2%) was similar to that reported in children and young adults by Meier et al. (18) (1.7% [age range 5.7–15 years of age], 1.3% [18.5–21.5 years of age]), as well as that reported by In't Veld et al. (7) in AAb$^+$ donors without diabetes (mean relative β-cell area of 1.2% [0.51–2.61 years of age]). In the report by In't Veld et al. (7), comparison of β-cell areas and mass in AAb$^+$ and AAb$^-$ donors did not show a significant difference. Two other groups (48,49) have also reported normal β-cell mass in patients with autoantibodies. Interestingly, β-cell mass in five donors without diabetes was in the upper range found for donors with T1D and insulitis and was related to young donor age, low pancreas weight, and/or low β-cell area.

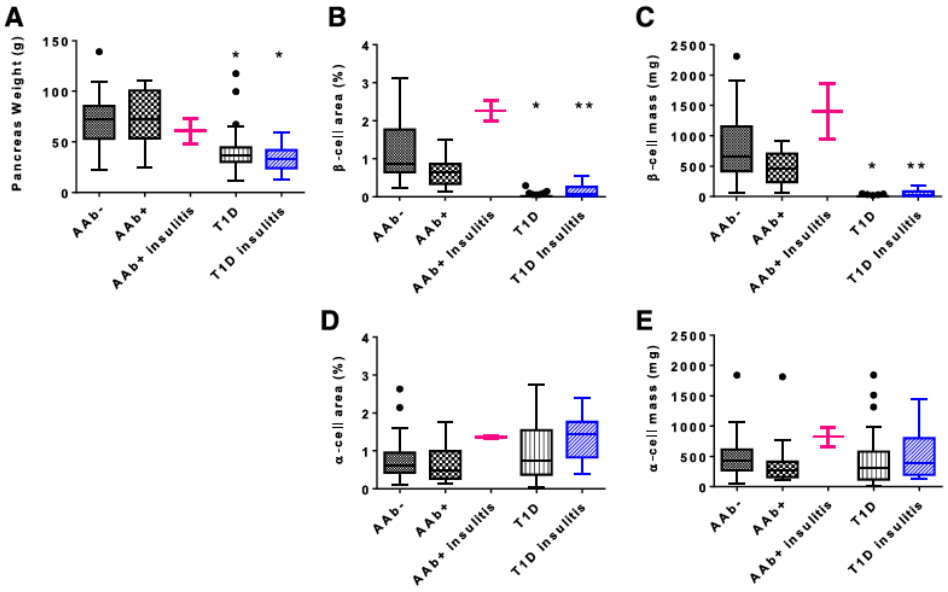

Figure 6—Pancreas weight and β-cell area and mass are decreased in donors with T1D. Group means were depicted by box-and-whisker plots with Tukey test error bars (nonparametric, outliers shown as black circles). Data were displayed (pink) for the two AAb$^+$ donors with insulitis and were excluded from statistical analyses because of the small sample size (n = 2). Pancreata weights were significantly lower in donors with T1D compared with AAb$^-$ and AAb$^+$ donors without diabetes, and no difference was detected between donors with T1D based on insulitis status (*A*). The β-cell area was significantly lower in both donor groups with T1D compared with AAb$^-$ and AAb$^+$ donors without diabetes (*B*). T1D donors with insulitis had a significantly higher β-cell area compared with T1D donors without insulitis (*B*). The β-cell mass was similar between AAb$^-$ and AAb$^+$ donors and was significantly different from that in groups of donors with T1D (*C*). T1D donors with insulitis had significantly higher insulin$^+$ β-cell mass compared with T1D donors without insulitis (*C*). The α-cell area (*D*) and mass (*E*) were not significantly different between groups. *Significantly different from AAb$^-$ and AAb$^+$ donors ($P < 0.001$). **Significantly different from AAb$^-$ and AAb$^+$ donors ($P < 0.001$) and T1D donors without insulitis ($P < 0.05$).

In terms of potential limitations, it must be emphasized that these data were obtained from pancreata of multiorgan donors with brain death who were subjected to intensive care procedures. Interpretations of data must be considered, as posed by In't Veld et al. (7), in light of the medications received during critical care and the duration of intensive care (reviewed in the study by Atkinson [50]). The majority of donors in this study had short hospitalization durations, yet further studies are needed to both validate and expand these findings. The computer-assisted image analysis algorithm used in this study has inherent limitations, with single minimum and maximum intensity thresholds applied to all sections. Alternatively, its unbiased approach using cross-sectional areas of the entire section could add greater consistency compared with manual point-counting methods. When studying organ donors, there is the possibility that the referring/initial diagnosis provided to nPOD may be of a questionable nature. To address this, full medical histories are carefully reviewed by board-certified endocrinologists and pathologists. Even when histories are limited, we carefully consider age at diagnosis, BMI, time to insulin dependence, history of insulin$^-$ dependence, treatment with oral agents, C-peptide level, HLA level, the presence of DKA, ketosis, and more.

In sum, the proportion of insulitic islets (insulitis frequency) in donors with T1D was inversely associated with disease duration, and not by age at onset, number of autoantibodies, or HLA genotype. The number of donors with insulitis (insulitis prevalence) was higher in older patients and for longer disease durations in both young and older patients than previously reported. In donors with T1D, the proportions of insulin$^+$ islets with insulitis was higher than in insulin$^-$ islets, yet insulin$^-$ islets were also affected by insulitis, particularly in donors with multiple AAb$^+$. Insulitic lesions were composed of a mixture of B and T lymphocytes, the numbers of which increased with insulitis frequency. The β-cell mass was significantly higher in donors with T1D and insulitis compared with donors without insulitis, whereas β-cell mass was similar

between AAb$^+$ donors and AAb$^-$ donors. These data underscore the chronic and heterogeneous nature of insulitis in individuals with T1D. The presence of insulitis as well as insulin$^+$ islets several years after diagnosis, and the limited correlation between insulitis frequency and disease duration suggest that the chronicity of islet autoimmunity extends well into the postdiagnosis period. Together with growing evidence for the chronicity of autoimmune responses and for the persistence of C-peptide secretion in patients, even decades after onset, our findings support the existence of a potentially long window of therapeutic opportunity (51,52).

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Author Contributions. M.C.-T. designed the experiments; performed pathology reviews, image analyses, and statistical calculations; and wrote the article. A.F. performed experiments and edited the article. J.S.K. and A.P. researched the data, performed the statistical calculations, and contributed to discussion and the editing of the article. C.W. designed the autoantibody studies, performed experiments, and edited the article. D.A.S. and M.A.A. contributed to the study design and discussion and edited the article. M.C.-T. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Prior Presentation. Preliminary immunotyping results of this study were presented at the Immunology of Diabetes Society 12th International Congress, Vancouver Island, BC, Canada, 15–19 June 2012, and 13th International Congress, Lorne, Victoria, Australia, 7–11 December 2013. Pancreata weights were reported in a subset of these donors (53). Of the 159 nPOD cases presented herein, 43 were previously examined by Coppieters et al. (30) (Supplementary Table 5).

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[CW3] Increased Complement Activation in Human Type 1 Diabetes Pancreata.

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Clive Wasserfall*	**Designed experiments** **Performed experiments** **Wrote manuscript**
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☐	7	JOURNAL OF PINEAL RESEARCH	7,278	10.391	0.00802
☐	8	FRONTIERS IN NEUROENDOCRINOLOGY	3,516	9.425	0.00659
☐	9	DIABETES	54,142	8.684	0.08485
☐	10	Obesity Reviews	9,124	7.883	0.02490
☐	11	Molecular Metabolism	1,626	6.799	0.00880
☐	12	DIABETES OBESITY & METABOLISM	8,333	6.715	0.02329
☐	13	ANTIOXIDANTS & REDOX SIGNALING	17,831	6.337	0.04018
☐	14	JOURNAL OF BONE AND MINERAL RESEARCH	25,813	6.284	0.04177
☐	15	DIABETOLOGIA	28,710	6.080	0.05111
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☐	20	JOURNAL OF CLINICAL ENDOCRINOLOGY & METABOLISM	76,719	5.455	0.11446
☐	21	ENDOCRINE-RELATED CANCER	6,547	5.267	0.01153

Increased Complement Activation in Human Type 1 Diabetes Pancreata

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OBJECTIVE—Evidence supporting an association between complement (C) and type 1 diabetes (T1D) includes the identification of C-fixing islet cell autoantibodies in T1D sera and genetic associations with the major histocompatibility complex III C4 region on chromosome 6. Therefore, we investigated whether C activation was present in pancreata from those with or at increased risk (positive for T1D associated autoantibodies) for T1D.

RESEARCH DESIGN AND METHODS—Immunohistochemical techniques were used to measure the C degradation product C4d in organ donor pancreata from patients with T1D and type 2 diabetes and autoantibody-positive and autoantibody-negative subjects.

RESULTS—Median C4d antigen density differed across the groups ($P < 0.0001$) and was highest in patients with T1D. C4d immunostaining localized to the blood vessel endothelium and extracellular matrix surrounding blood vessels and exocrine ducts. Receiver operating characteristic analysis resulted in 81.8% sensitivity and 94.4% specificity for C4d staining.

CONCLUSIONS—These data suggest that C activation is occurring within pancreata from patients with T1D and C4d may be a biomarker for T1D.

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A potential role for antibody-mediated complement (C) activation in the pathogenesis of type 1 diabetes (T1D) has been suggested from genetic (association with major histocompatibility complex III C4 region on chromosome 6) and immunological (presence of C-fixing islet autoantibodies in T1D sera) studies (1–4). Additional efforts support a role for C in the pathogenesis of complications (5,6). These data, along with the rarity of observed insulitis in humans, suggest the possibility of an alternative explanation of β-cell destruction; namely, that islet autoantibodies, present before disease onset, may fix C on the surface of β-cells and promote their lysis. Therefore, we quantified C4d, a marker of antibody-mediated C activation, in pancreata from individuals with T1D (including long-standing disease) and type 2 diabetes (T2D), as well as autoantibody-positive and autoantibody-negative nondiabetic subjects (7–9).

RESEARCH DESIGN AND METHODS

Human pancreata from cadaveric organ donors obtained from the Network for Pancreatic Organ Donors with Diabetes program were analyzed, including those with T1D ($n = 11$), those diabetes free with T1D-associated islet autoantibodies (AA^+) ($n = 16$), autoantibody-negative control subjects (AA^-) ($n = 11$), or those with T2D ($n = 7$) (10). The mean ± SE ages of the T1D, T2D, AA^+, and AA^- groups were 22.1 ± 2.2 years (range 10.7–37.0), 54.0 ± 4.6 years (39.3–74.0), 32.2 ± 4.2 years (2.2–69.2), and 32.1 ± 5.5 years (9.0–68.0), respectively. BMI was highest in T2D patients (35.5 ± 2.2 kg/m^2) compared with T1D (24.2 ± 1.3), AA^+ (24.4 ± 1.5), and AA^- (25.7 ± 1.7) subjects. Caucasians comprised the majority of patients who had T1D (73%), were AA^+ (69%), and were AA^- (90%). Of T2D patients, only 43% were Caucasian, with the remainder being black (29%), Hispanic (14%), and Asian (14%). There were no differences in age and BMI between T1D patients and either the AA^- or the AA^+ group. As expected, islet autoantibodies negatively correlated with duration of disease within the T1D group. ($r = -0.75$, $P < 0.05$).

One or more islet autoantibodies (GAD 65 [GADA], insulin [IAA], insulinoma associated protein 2 [IA2], zinc transporter 8 [ZnT8]) were present in 23 subjects (16 without and 7 of 9 with T1D). No sera were available in two T1D patients. Three T1D patients were positive for a single autoantibody ($n = 2$ mIAA; $n = 1$ GADA), three had two autoantibodies ($n = 2$ IA2 and mIAA; $n = 1$ IA2 and ZnT8), and one had three autoantibodies (GADA, IA2, and ZnT8). Among AA^+ subjects without diabetes, 12 were positive for a single autoantibody ($n = 11$ GADA; $n = 1$ ZnT8) and 4 were positive for two autoantibodies ($n = 1$ GADA and IA2, $n = 2$ GADA and mIAA, and $n = 1$ IA2 and ZnT8). All research procedures were approved by the University of Florida Institutional Review Board.

C4d analysis

Frozen pancreatic sections were analyzed using an anti-human C4d monoclonal antibody (clone 10–11; AbD Serotec, Raleigh, NC) according to manufacturer recommendations, with visualization using a DAB detection kit (Ventana Medical Systems, Tucson, AZ). Whole-section digital images were analyzed using ImageScope software (Aperio Technologies, Vista, CA). A positive pixel count (total positive pixels plus total negative pixels/total pixels) from sections incubated with negative control antisera was subtracted from the positive pixel count from sections incubated with anti-C4d antisera to provide a fraction (converted to %).

Statistical analysis

Potential group demographic differences were analyzed using one-way ANOVA with Bonferroni corrections. For nonparametric

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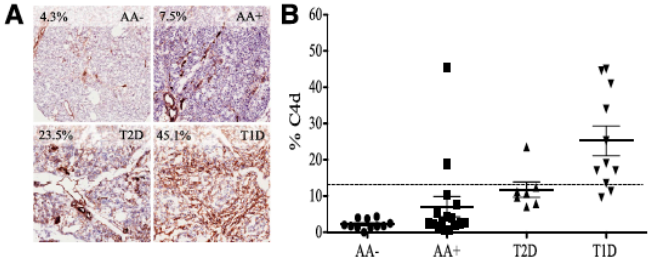

Figure 1—A: *C4d immunostaining in representative tissues from T1D, T2D, and AA$^+$ patients and AA$^-$ control subjects. Percentages in the* upper left corner *of each panel refer to the C4d antigen density.* B: *Comparison of C4d antigen density between groups. Clear differences between groups were present;* $P < 0.0001$, *ANOVA. T1D patients had higher C4d antigen density than both control subjects and nondiabetic AA$^+$ subjects* ($P < 0.0001$). *Dotted line, ROC C4d cutoff of 12.95% (area under the curve 0.94,* $P < 0.0001$). *This cutoff yielded a sensitivity of 81.8% and specificity of 94.4% in distinguishing T1D patients.*

analyses, the Spearman rank correlation test was used, whereas Pearson testing was used for correlation assessment. Receiver operating curve (ROC) analysis was used to establish sensitivity and specificity, whereas mean values were analyzed using a Mann-Whitney test (nonparametric).

RESULTS—In all groups, C4d immunostaining was largely localized to the blood vessel endothelium and extracellular matrix surrounding blood vessels and exocrine ducts depending on vessel size (Fig. 1*A* and ref. 11 [see ref. 11 for login information]). C4d staining was detected on the endothelium of very small blood vessels/capillaries. On larger blood vessels, C4d staining localized to both the endothelium and extracellular matrix/adventitia, as well as extracellular matrix surrounding ductal structures.

Mean C4d antigen density differed between the three groups ($P < 0.0001$). C4d antigen expression was more prevalent in pancreata from patients with T1D (mean ± SEM 25.2 ± 4.1%) than from subjects without diabetes (2.2 ± 0.4%, $P < 0.0001$) or no diabetes yet AA$^+$ (7.1 ± 2.8%, $P < 0.0001$) (Fig. 1*B*). C4d density did not differ between T2D, AA$^+$, and AA$^-$ individuals. When comparing control subjects and T2D patients with T1D subjects, ROC analysis of C4d density yielded a sensitivity of 81.8% and specificity of 94.4% at a cutoff of 12.95% (Fig. 1*B*) (area under the curve 0.94, $P < 0.0001$), indicating that pancreatic C4d immunostaining can distinguish autoimmune T1D from nonautoimmune diabetes. These data were confirmed in blinded fashion by an expert pathologist. When converting percent C4d pixels into a rank order, C4d deposition was significantly correlated with computer-aided image analysis ($\rho = 0.86$, $P < 0.001$).

The mean C4d deposition among T1D patients with histological evidence of insulitis (17.8 ± 4.3%, $n = 5$), defined as six or more CD3$^+$ cells adjacent to or within an islet, tended to be lower ($P = 0.08$) than in patients without insulitis (31.4 ± 5.7%, $n = 6$). In contrast to T1D patients with insulitis, among the AA$^+$ subjects without diabetes the only subject with histological evidence of insulitis (Network for Pancreatic Organ Donors with Diabetes identification no. 6197) had higher C4d density (45.5%). This subject was positive for GADA and IA2. Islet autoantibody positivity did not correlate with C4d antigen density (P = NS). Finally, no correlations were found between C4d density and either cause of death or cardiac downtime prior to or during terminal hospital stay (data not shown).

CONCLUSIONS—In this unique collection of pancreatic tissue from organ donors with long-standing T1D and AA$^+$ and AA$^-$ control subjects, we directly investigated C activation. We showed that C activation was present in pancreatic tissue from patients with T1D and thus may be a novel immunohistochemical biomarker for T1D. Furthermore, increased pancreatic C4d deposition appears to distinguish T1D from T2D patients and nondiabetic control subjects. Whether the increased C deposition is directly or indirectly involved in the pathogenic mechanisms leading to T1D cannot be ascertained from this study. The elevated C4d density in one AA$^+$ case that also presented with insulitis might suggest that C4d expression could be related to immune activation and beginning of β-cell destruction. Hyperglycemia and poor control may lead to enhanced C activation. Indeed, recent reports have suggested that serum peptides corresponding to C activation are increased in both humans with diabetes and animal models (12,13) and may be involved in both vascular (5,6) and renal complications (14) associated with diabetes. Complement activation products could not be measured in our study because serum was not collected in a manner that would avoid artifactual C activation. In addition, HbA_{1c} measurements were not obtained in sufficient numbers of patients to test this hypothesis. Future studies will investigate C4d localization (i.e., vascular, exocrine, endocrine) and will determine whether C4d colocalizes with downstream, more final, effectors of the C cascade such as components of the membrane attack complex.

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No potential conflicts of interest relevant to this article were reported.

P.R. and C.W. designed and performed the experiments and wrote the manuscript. B.C. and M.C.-T. performed experiments and provided critical manuscript review. A.P., M.A., and D.S. provided experimental design and discussion input, as well as critical manuscript review. D.S. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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[CW4] Persistence of Pancreatic Insulin mRNA Expression and Proinsulin Protein in Type 1 Diabetes Pancreata.

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Cell Metabolism
Short Article

Persistence of Pancreatic Insulin mRNA Expression and Proinsulin Protein in Type 1 Diabetes Pancreata

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SUMMARY

The canonical notion that type 1 diabetes (T1D) results following a complete destruction of β cells has recently been questioned as small amounts of C-peptide are detectable in patients with long-standing disease. We analyzed protein and gene expression levels for proinsulin, insulin, C-peptide, and islet amyloid polypeptide within pancreatic tissues from T1D, autoantibody positive (Ab+), and control organs. Insulin and C-peptide levels were low to undetectable in extracts from the T1D cohort; however, proinsulin and INS mRNA were detected in the majority of T1D pancreata. Interestingly, heterogeneous nuclear RNA (hnRNA) for insulin and INS-IGF2, both originating from the *INS* promoter, were essentially undetectable in T1D pancreata, arguing for a silent *INS* promoter. Expression of PCSK1, a convertase responsible for proinsulin processing, was reduced in T1D pancreata, supportive of persistent proinsulin. These data implicate the existence of β cells enriched for inefficient insulin/C-peptide production in T1D patients, potentially less susceptible to autoimmune destruction.

INTRODUCTION

Type 1 diabetes (T1D) develops as a result of endogenous insulin insufficiency due to autoimmune destruction of the insulin-producing pancreatic β cells (Atkinson et al., 2014). Two long-standing dogmas in T1D maintain that symptomatic onset occurs when 90%–95% of β cells are destroyed and that, within months to a few years after recent onset, all β cells are lost (Atkinson et al., 2014; Eisenbarth, 1986). However, recent data collected from pancreata with disease of varying durations suggest that β cell mass at diagnosis is more variable than once appreciated. In addition, β cells, while rare in number, can exist for decades after disease onset (Campbell-Thompson et al., 2016; Keenan et al., 2010; Meier et al., 2005). Not surprisingly, this emerging concept has further fueled an interest in identifying factors that either promote the formation of new β cells or promote enhanced survival and function of existing β cells, in combination with an immunotherapy capable of blocking the destruction of such cells (Ludvigsson, 2014).

Contemporaneous with this pathology-based literature, emerging metabolic data from living T1D patients support reconsideration of the notion that complete β cell loss occurs in T1D (both quantitatively and functionally). Indeed, a citation classic involving analysis of NIH Diabetes Control and Complications Trial (DCCT) study participants using the conventional assay noted that after 5–15 years of disease, only 22% had detectable C-peptide (The DCCT Research Group, 1987). However, a vast majority of subjects with established T1D, upward of 80%, have been posited as being so called "microsecretors" of insulin, made possible by newly developed ultrasensitive C-peptide assays (Oram et al., 2014; Wang et al., 2012).

With this, we sought to better characterize the molecular mechanisms of endocrine hormone production and processing in the pancreas throughout the natural history of T1D. These studies have only recently become possible due to the availability of high-quality tissues through organ donation programs, such as the JDRF Network for Pancreatic Organ donors with Diabetes (nPOD) (Campbell-Thompson, 2015). We hypothesized that in T1D, a population of endocrine cells selected for resistance to autoimmune destruction may exist, possibly representing a depot for unprocessed proinsulin given extremely low levels of insulin and C-peptide in T1D. To address this concept, we determined the expression (both protein and gene) of a series of hormones in human pancreatic tissues obtained from relevant study groups, including those with long-standing T1D. These analyses included IHC, acid-ethanol extractions of pancreatic tissue blocks followed by quantification of the various peptide hormones by ELISA, gene expression studies of tissue blocks by real-time qPCR, and *in situ* hybridization (ISH) of tissue sections for mRNA.

Table 1. Donor Demographics

Donor Type	N	Gender (N) M	F	Age Median (Range)	Disease Duration Median (Range)	Age at Disease Onset Median (Range)
No Diabetes	50	32	18	26.9 (0.3–75)	N/A	N/A
Autoantibody Positive	16	9	7	33.2 (2.2–69)	N/A	N/A
Type 1 Diabetes	40	24	16	27.5 (10.7–61)	13.0 (1–52)	12.4 (2–33.8)

Network for Pancreatic Organ Donors with Diabetes (nPOD) tissue donors are categorized by disease state (left column), and for each donor type, the number of donors (N), the number of male (M) and female donors (F), age (years), disease duration (years), and age at disease onset (years) are listed. N/A indicates not applicable.

RESULTS AND DISCUSSION

Pancreatic Hormone Production by Immunofluorescence Staining

All pancreatic tissue blocks were obtained from 106 study organs (Table 1), with a summary of all assays per donor summarized in Table S1. The study groups include either those that had no history of diabetes and were T1D autoantibody (Ab) negative (controls), those with no diabetes but were Ab positive (Ab+, Table S2), and those diagnosed with T1D.

As a histological illustration of our patient groups, we performed immunolocalization for proinsulin (green), insulin (red), and glucagon (yellow) on an islet from a representative control (no history of diabetes, Figures 1A, 1F, 1K, and 1P), along with random proinsulin and insulin staining of individual cells in the exocrine pancreas (Figure 1A, inset). Representative images from a patient with short-duration T1D (Figures 1B, 1C, 1G, 1H, 1L, 1M, 1Q, and 1R) illustrate the heterogeneity and progression of disease with islets displaying significant proinsulin and insulin staining as well as islets where only glucagon is detected (Figure 1C, inset). Here, we note a patient with longer-duration T1D, demonstrating the continued expression of both proinsulin and insulin despite 7 years of disease (Figures 1D, 1I, 1N, and 1S). Analogous to the cells staining positive for insulin in the exocrine tissue of the control donor, a representative patient with 2 year duration of T1D similarly illustrates the presence of cells in the exocrine pancreas that harbor proinsulin, insulin, and glucagon (Figures 1E, 1J, 1O, and 1T, inset).

Persistence of Proinsulin in T1D Pancreata Identified by Protein Extraction

Following acid-ethanol extraction of protein from randomly localized pancreatic blocks, levels of proinsulin, insulin, C-peptide, islet amyloid polypeptide, and glucagon were determined and normalized against total protein. Extracted insulin ($p = 0.60$), proinsulin ($p = 0.95$), and C-peptide ($p = 0.71$) levels were comparable for single Ab+ subjects ($n = 12$) versus the four subjects with multiple Ab; hence, all Ab+ donors were analyzed together as one group. Insulin protein was detected in amounts similar to those determined from control pancreas tissues in only five of the T1D pancreata, while the majority of samples (17/24) displayed insulin levels at the lower end of the assay detection limits with two additional samples that were completely undectable (Figure 2A; Figure S1A, $p < 0.0001$). However, proinsulin protein levels in T1D pancreata were surprisingly similar to the Ab+ cohort, albeit slightly lower than controls (Figure 2B, $p < 0.01$). C-peptide levels were very low to undetectable in T1D pancreata relative to the control and Ab+ tissues (Figure 2C, $p < 0.0001$, not detected [ND] = 8/24). Consequently, proinsulin/insulin and proinsulin/C-peptide ratios were significantly elevated in T1D pancreatic extracts versus control and Ab+ pancreata (Figures S1B and S1C, $p < 0.0001$). In line with the C-peptide data, levels of islet amyloid polypeptide, a β cell co-secretory molecule, were essentially undetectable in the majority of T1D pancreata relative to controls (Figure 2D, ND = 13/18). Due to limited sample availability, glucagon levels were only determined in a subset of controls and T1D donors, yet no significant differences in the quantity of this analyte were observed between these groups (Figure 2E).

When analyzing the relationship between proinsulin and insulin levels within the pancreatic extracts, there was a significant correlation between these analytes in control (Figure 2F, $p < 0.0001$) but not in the T1D or Ab+ pancreata (Figures 2G and 2H). We then performed a correlation analysis comparing the percent of insulin-positive area from IHC-stained tissue sections against the extracted insulin for each respective patient. For both T1D and control pancreata, the r values were above 0.4, with the correlation for T1D reaching significance (Figures S1D and S1E).

Gene Expression Analysis Identifies Persistant INS mRNA Expression in T1D Pancreata

To further investigate the persistence of proinsulin protein in T1D pancreata (Figure 2B), we next assayed insulin (INS) mRNA, INS heterogeneous nuclear RNA (hnRNA), and the mRNAs encoding INS-IGF2, islet amyloid polypeptide (IAPP), glucagon (GCG), and somatostatin (SST) expression by real-time qPCR. It should be noted that we provide Cq values for all genes since we felt it invalid to normalize when, in so many instances, we observed undetectable expression. Contrary to our expectations, but consistent with the detection of proinsulin protein (Figure 2B), we observed low, but reproducible, levels of INS mRNA in T1D pancreata (Figure 2I). Although the T1D INS mRNA was much lower than control, we observed consistent expression across pancreata, even in those with long duration T1D. We next studied *de novo* transcription from the *INS* gene promoter, using primers that would only detect unspliced hnRNA for INS (Table S3). Surprisingly, compared with control pancreata, we detected very little, if any, *de novo* synthesis of INS hnRNA, indicating that the *INS* promoter was essentially silent in T1D pancreata (Figure 2J, ND = 17/30 in T1D samples versus 0/34 controls). Levels of INS-IGF2 read through mRNA, which also originates from the human *INS* promoter, supports an inactive *INS* promoter since 28/31 T1D pancreata demonstrated no detectable mRNA (Figure 2K).

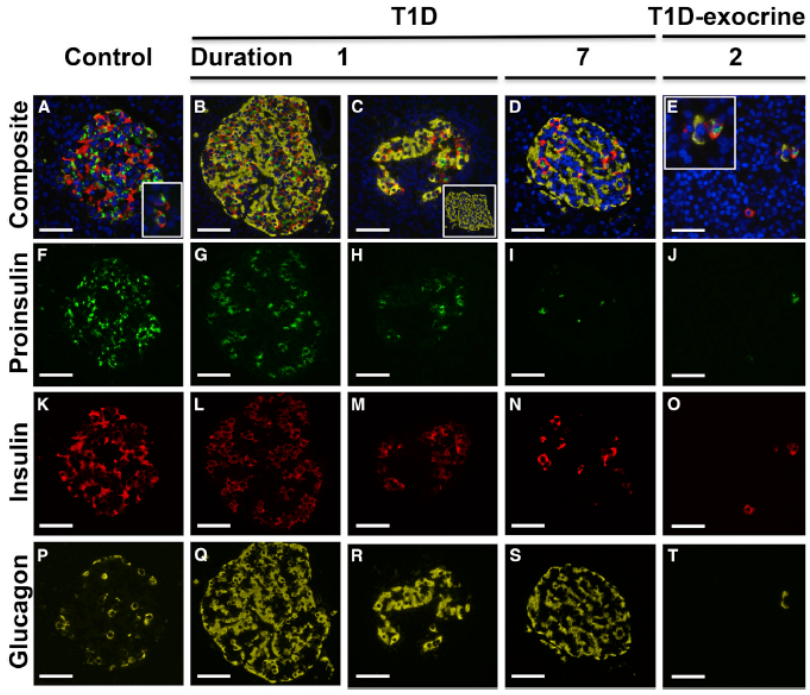

Figure 1. Proinsulin, Insulin, and Glucagon Expression in Type 1 Diabetes

(A–E) Composite images of pancreas sections stained for proinsulin (green), insulin (red), glucagon (yellow), and DAPI (nuclei, blue) are shown for sections containing islets from a control 24-year-old male Caucasian (nPOD 6131) (A), a 12-year-old male African American with T1D for 1 year (nPOD 6052) (B and C), a 23-year-old female Caucasian with T1D for 7 years (nPOD 6070) (D), and a section containing pancreas exocrine tissue from a 12.5-year-old female Caucasian with T1D for 2 years (nPOD 6371) (E).

(F–T) The individual channels for proinsulin (green; F–J), insulin (red; K–O), and glucagon (yellow; P–T) are also shown.

Insets display: proinsulin- and insulin-positive cells in the exocrine pancreas (A), an insulin-negative, proinsulin-negative, glucagon-positive islet (C), and proinsulin-, insulin-, and glucagon-positive cells (E) in the exocrine pancreas.

Scale bars represent 50 μm. See also Figure S1.

To assess the notion of whether these INS hnRNA results were potentially influenced by sample degradation afforded by the action of digestive enzymes or variability in pancreatic recovery procedures influenced by these results (e.g., warm and cold ischemia time), we measured hnRNA levels for calmodulin 1 (*CALM1*), serine protease inhibitor Kazal-type 1 (*SPINK1*), cyclophilin A (*PPIA*), and chymotrypsin C (*CTRC*) by real-time qPCR. We observed that hnRNA levels for these pancreatic genes were comparable across T1D and control samples (Figure S2). Collectively, these data support an interpretation that the *INS* promoter displays significantly reduced activity in the vast majority of T1D subjects.

In agreement with the the islet amyloid polypeptide protein data (Figure 2D), IAPP mRNA levels were substantially lower in T1D, with 17/31 undetectable, while control pancreata contained detectable message in all cases (Figure 2L). In contrast, we observed no significant differences between T1D and control pancreata for GCG and SST (Figures 2M and 2N). These data suggest that T1D pancreata have cells, either in islets or scattered in the exocrine regions of the pancreas, that contain INS mRNA and proinsulin protein but with limited *INS* promoter activity and little to no production of the co-secretory molecule IAPP.

Distribution of Insulin or Glucagon Single Positive Cells in the Exocrine Tissue

To further explore the intrapancreatic location of these cells, we performed IHC using insulin (red) and glucagon (blue) antibodies (Figures 3A–3E), as well as ISH for INS mRNA (Figures 3F–3J), in a representative control and longer-duration (7 and 35 years) T1D pancreata. These efforts demonstrated the presence of both insulin-positive and -negative islets in the 7-year-duration

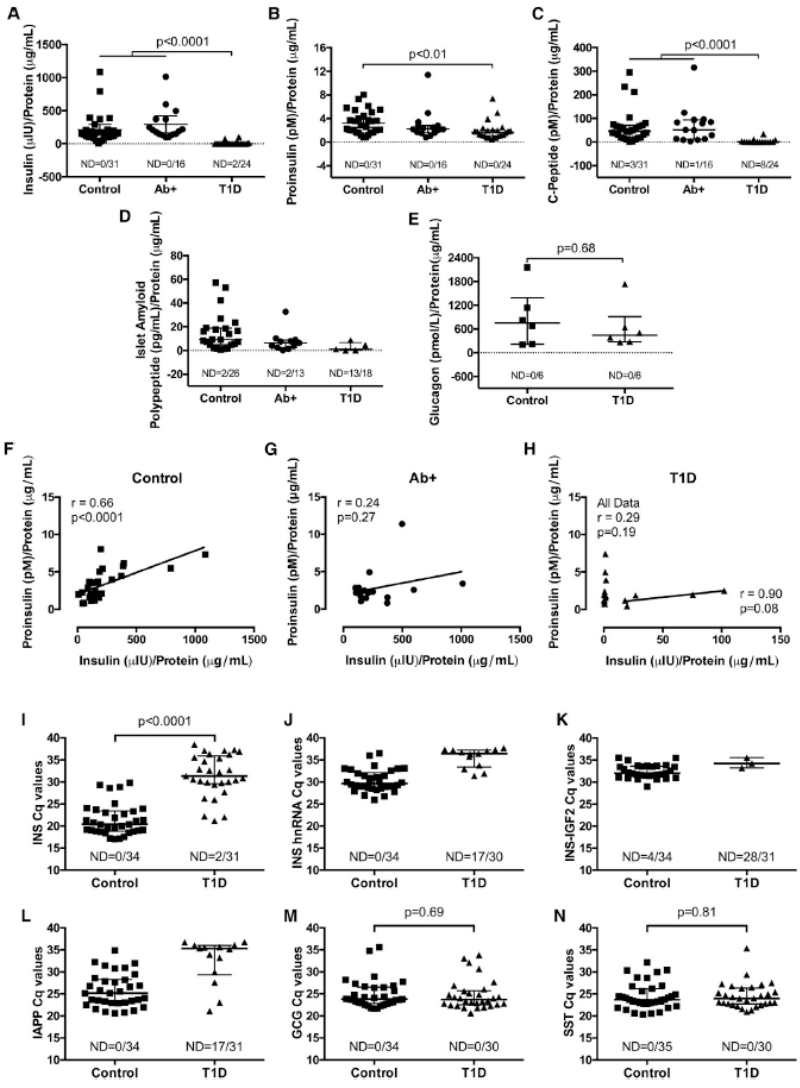

Figure 2. Total Protein Extracts and Gene Expression from Human Pancreas Sections

(A–E) ELISAs of acid-ethanol extracts from human pancreas tissue sections for insulin (μInternational Units, μIU) (A), proinsulin (B), C-peptide (C), islet amyloid polypeptide (D), and glucagon (E) (values were normalized against total protein). ND, not detected/examined are noted on each graph. Data are presented as median with interquartile range.

(F–H) Correlation between extracted proinsulin and extracted insulin in controls (F), autoantibody-positive (Ab+) (G), and T1D (H) is shown. For T1D subjects, correlation analysis is reported for all data, as well as a separate analysis of the five subjects with insulin detected in the normal range (Detectable INS Only), with the latter represented by the trend line.

(legend continued on next page)

patient (Figure 3B), whereas no insulin-positive islets were detected in the long-duration pancreas (Figures 3D and 3E, insets). Interestingly, we observed small clusters or single insulin-positive cells scattered through the exocrine pancreas in both the control (Figure 3A) and the 7-year-duration T1D pancreas (Figure 3C). These scattered insulin-postive cells were also present in the long-duration T1D pancreas (35 years) and were either detected in the exocrine pancreas (Figure 3D) or localized adjacent to ducts (Figure 3E).

We performed a semiquantitative assessment of insulin-positive cells within islets, noting both small clusters (2–5 insulin-positive cells) and single cells, in a subset of control (n = 5), short-duration (0–7 years; n = 5), and longer-duration (8–35 years; n = 6) T1D pancreata, involving co-staining for insulin and glucagon (Figures 3K–3O). Whereas control pancreata contained significant numbers of insulin-positive cells in islets, reduced numbers of such cells were identified in the short-duration T1D cases, while in longer-duration pancreata, they were essentially absent (Figure 3K). Additionally, pseudo-atrophic (glucagon+insulin−) islets were only detected in the T1D groups (Figure 3L). Consistent with the number of insulin-positive cells per islet (Figure 3K), the number of glucagon+insulin+ islets showed a similar distribution (Figure 3M, $p < 0.01$). Interestingly, control pancreata contained a significant population of both insulin-positive single cells (Figure 3N, $p < 0.01$) and small clusters (Figure 3O, $p < 0.01$), while short- and long-duration T1D pancreata harbor both populations, albeit in reduced numbers relative to controls. Similar to these single insulin-positive cells in longer-duration T1D pancreata, we also observed glucagon-positive cells in both the exocrine pancreas and peri-ductal regions (Figure S3).

Consistent with these IHC findings (Figures 3A–3E and 3K–3O), studies involving acid-ethanol extraction (Figures 2A and 2B; Figure S1), RNA expression of proinsulin/insulin (Figure 2I), and ISH for INS mRNA clearly demonstrate that T1D pancreata harbor INS mRNA in islets (Figure 3G) as well as in many cells scattered through the exocrine pancreas (Figure 3H). Here, we would highlight, in particular, those in the exocrine region of longer-duration T1D pancreata (Figures 3I and 3J). Our observations not only corroborate previous findings of residual β cells in long-duration T1D pancreas (Keenan et al., 2010), but extend them by providing semiquatitative data on insulin-positive cell numbers. Both efforts support the need to better understand the production of endocrine hormones, at both the protein and RNA levels, throughout the natural history of T1D.

Proconvertase PCSK1 Is Diminished in T1D Pancreata

Proinsulin is processed to insulin and C-peptide by the prohormone convertases (PCSK1 and PCSK2) and carboxypetidase E (CPE) (Goodge and Hutton, 2000). Glucagon is similarly processed by PCSK2 and CPE in α cells, which do not express PCSK1 (Friis-Hansen et al., 2001; Orskov et al., 1987) and which are spared destruction in T1D. To address a mechanistic explanation for the presence of INS mRNA and proinsulin protein in contrast to low levels of mature insulin and essentially undetectable C-peptide in T1D pancreata (Figures 2A–2C and 2I), we addressed whether inflammation might contribute to defective proinsulin processing in T1D. This action was taken given previous *in vitro* reports that proinflammatory cytokines inhibit the protein levels of PCSK1 and PCSK2 (Hostens et al., 1999). Specifically, we measured IFN-γ, IL-1β, and TNFα expression in the pancreas by real-time qPCR (Figure S4). IFN-γ was undetectable in both groups, whereas IL-1β levels were similar (Figure S4A). mRNA levels for TNFα were undetectable in approximately 30% of T1D and 50% of control samples (Figure S4B), precluding comprehensive statistical analysis for this cytokine. Hence, while reducing the potential that proinflammatory cytokines underscore our mRNA-based observations, we believe additional studies addressing this issue are warranted moving forward.

We also evaluated the expression levels of three prohormone convertases/proteases in control and T1D pancreata. PCSK1 mRNA expression was reduced among T1D pancreata compared to controls (Figure 3P, $p < 0.01$). In contrast, PCSK2 and CPE were expressed at similar levels in controls and T1D tissues (Figures 3Q and 3R). Indeed, the lower levels of PCSK1 in T1D pancreata are supportive of the reduction/absence of insulin and C-peptide. Furthermore, the detection of low levels of insulin in some pancreata may reflect the gradual, but slow, maturation from any residual PCSK1 activity in T1D pancreata.

Conclusions

Herein, in aggregate, we report the persistence of proinsulin protein and INS mRNA within the pancreata of individuals with varying durations of T1D, including those with long-duration disease. Evidence for this includes direct staining of proinsulin by immunolocalization and IHC (Figures 1 and 3A–3E), measurable extracts of proinsulin by ELISA (Figure 2B), ISH staining (Figures 3F–3J), and qPCR analysis of INS mRNA (Figure 2I) in β cells within islets as well as in scattered single cells within the exocrine regions of T1D pancreata. Furthermore, cell counts demonstrate the remarkable heterogeneity and persisitence of single insulin-positive cells and small clusters in T1D pancreata. While insulin levels were significantly lower and C-peptide, as well as islet amyloid polypeptide, essentially undetectable in T1D pancreata compared with no diabetes controls, proinsulin and INS mRNA were detectable in virtually every T1D subject. The novel finding regarding complete absence of INS hnRNA and the INS-IGF2 mRNA in many T1D pancreata, given that both messages originate from the *INS* promoter, demonstrates that the *INS* gene is most likely silent in T1D pancreata, while the presence of INS mRNA is suggestive of the persistence of long-lived message (Evans-Molina et al., 2007; Lee and Gorospe, 2010; Welsh et al., 1985).

The significant correlation between proinsulin and insulin concentrations in extracts from the control pancreata (Figure 2F) further implies that these assays are truly measuring the cognate analytes. While proinsulin is synthesized, it is likely not

(I–N) Real-time qPCR Cq values for control and T1D pancreata for INS (I), unspliced INS heterogeneous nuclear RNA (hnRNA) (J), INS-IGF2 readthrough mRNA (K), IAPP (L), GCG (M), and SST (N) expression were compared. For INS hnRNA, INS-IGF2, and IAPP, statistical analyses were not performed due to the number of T1D samples with undetectable RNA (ND, not detected/examined). Data are presented as median with interquartile range.
p values are indicated on the figure.

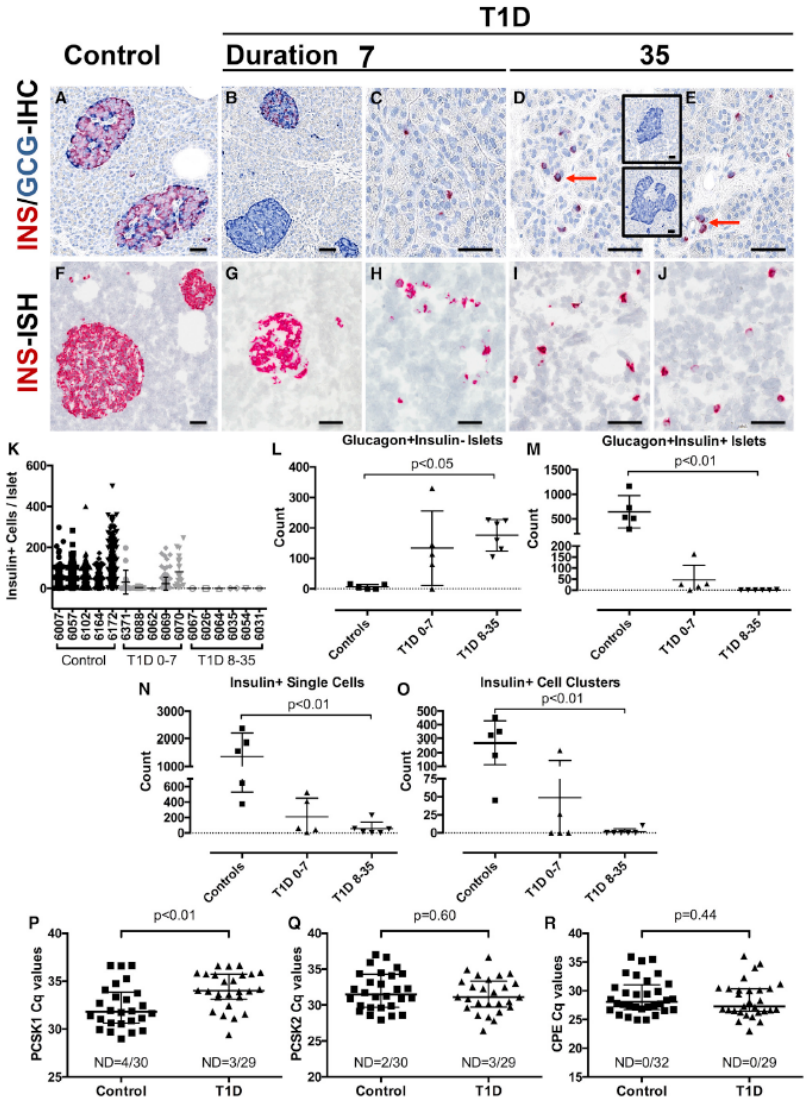

Figure 3. Evaluation of Insulin/Glucagon IHC, INS ISH, Prohormone Convertase, and Protease Gene Expression from Human Pancreas

(A–J) Insulin (red) and glucagon (blue) protein was detected by IHC (A–E), and insulin mRNA (pink) was detected by ISH (F–J) in pancreas sections from donors without diabetes (A and F; control; nPOD 6172) and T1D pancreata with 7 (B, C, G, and H; nPOD 6070) and 35 year duration (D, E, I, and J; nPOD 6031). Insets in (D) and (E) illustrate glucagon-only-positive islets. Red arrows indicate single insulin-positive cells located in the exocrine tissue and peri-ductal. Scale bars represent 50 μm.

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undergoing appropriate processing into mature insulin and C-peptide in the majority of T1D pancreata (Figure 2H). The presence of proinsulin (Figure 2B), coupled with the low levels of insulin along with the near absence of C-peptide and islet amyloid polypeptide (Figures 2A, 2C, and 2D), further supports the notion that the molecular machinery for processing the full-length proinsulin protein may be disrupted. In addition, our data demonstrating a significant reduction in the levels of PCSK1 mRNA in T1D pancreata also provide a strong argument for the observed persistence of proinsulin. Alternatively, our findings could also be interpreted as supporting the notion that proinsulin (along with islet amyloid polypeptide) is so rapidly secreted that there is insufficient time for it to be retained in residual β cells for full processing to insulin. This concept has been previously associated with type 2 diabetes (Alarcon et al., 2016).

In sum, these data indicate that proinsulin is present in the pancreas in individuals with long-standing T1D; whether this is occurring in dedifferentiated β cells (Talchai et al., 2012) or in cells attempting to become β cells cannot be concluded from this data. The absence of insulin hnRNA and INS-IGF2 mRNA is suggestive of the *INS* gene not being actively transcribed (Evans-Molina et al., 2007; Lee and Gorospe, 2010; Welsh et al., 1985), potentially as a means to avoid autoimmune killing (Rui et al., 2017). Therefore, there is a clear need for further studies of the mechanisms underlying long-lasting INS mRNA and proinsulin protein expression despite low to no *INS* promoter activity. Future analysis of the lineage of the cells expressing proinsulin/insulin in long-standing T1D pancreata and the molecular machinery involved in processing the full-length protein into insulin and C-peptide is warranted. Indeed, it is our belief that these observations are important for expanding our understanding of the pancreas and β cell biology in the context of T1D.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2017.08.013.

AUTHOR CONTRIBUTIONS

C.W. conceived of the study, researched the data, and wrote the manuscript; H.S.N. conceived of the study, researched the data, and reviewed/edited the manuscript; M.C.-T. researched the data and reviewed/edited the manuscript; D.B. and M.B. researched the data and reviewed/edited the manuscript; L.H., I.K., A.P., C.R., E.B., and P.A. contributed to discussion and reviewed/edited the manuscript; M.A. conceived of the study and reviewed/edited the manuscript.

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(K–O) For control (n = 5) and T1D subjects (n = 11), IHC analysis of glucagon and insulin within pancreas sections from control donors, as well as short (0–7 years; n = 5) or long (8-35 years; n = 6) duration T1D, were analyzed for insulin-positive cells per islet (K), cell counts within the islets (L and M), and exocrine tissue (N and O). Data are presented as mean ± SD.
(P–R) qPCR for control versus T1D pancreata PCSK1 (P), PCSK2 (Q), and CPE (R). Data are presented as median with interquartile range.
p values indicated on the figure.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Proinsulin	Developmental Studies Hybridoma Bank	GS-9A8
Insulin	Dako	Cat# A056401; RRID: AB_2617169
Glucagon	Dako	Cat# A056501
Glucagon	Abcam	Cat# ab10988, K79bB10; RRID: AB_297642
Biological Samples		
Human Pancreas	JDRF nPOD	Table S1; https://www.jdrfnpod.org
Critical Commercial Assays		
Proinsulin	Alpco	Cat# 80-PINHU-E01.1
Insulin	Alpco	Cat# 80-INSHU-E01.1
C-Peptide	Alpco	Cat# 80-CPTHU-E01.1
Glucagon	Mercodia	Cat# 10-1271-01
IAPP	Millipore	Cat# HMHEMAG-34K
Bradford Total Protein Assay	Thermo Fisher Scientific	Cat# 23236
OPAL 4 color amplification kit	PerkinElmer	Cat# NEL94001KT
EnVision G/2 Doublestain System	Dako	Cat# K5355
RNeasy Plus Mini Kit	QIAGEN	Cat# 74134
RNAscope 2.0 High Definition Kit	Advanced Cell Diagnostics	Cat# 310036
Insulin Probe	Advanced Cell Diagnostics	Cat# 313571; NCBI: NM_000207.2
Oligonucleotides		
Primers	This paper, Table S3	https://www.ncbi.nlm.nih.gov/tools/primer-blast/
Software and Algorithms		
GraphPad Prizm (v.6.02)	GraphPad software	http://www.graphpad.com
Aperio eSLIDE (v.12)	Leica Biosystems	http://www.leicabiosystems.com/digital-pathology
ImageScope (v.12.1.0.5029)	Leica Biosystems	http://www.leicabiosystems.com/digital-pathology/digital-pathology-management/imagescope/
Image Analysis Software (HALO)	Indica Labs	http://www.indicalab.com

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for reagents may be directed to, and will be fulfilled by the corresponding author Mark A. Atkinson (atkinson@ufl.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Donors and Sample Processing

The JDRF nPOD program (https://www.jdrfnpod.org) recovered transplant-quality pancreata from organ donors with T1D as previously described (Campbell-Thompson et al., 2012b). All procedures were approved by the University of Florida Institutional Review Board (201400486) and the United Network for Organ Sharing (UNOS) according to federal guidelines, with informed consent obtained from each donor's legal representative. For each donor, a medical chart review was performed in addition to assays for T1D associated Ab (Wasserfall et al., 2016) (Table S2) and C-peptide (Campbell-Thompson et al., 2012a), with T1D diagnosed according to the guidelines established by the ADA. Demographic data, hospitalization duration, and organ transport time were

obtained from hospital records or UNOS. Pancreata were recovered, placed in transport media on ice, and shipped via organ courier to the University of Florida where tissues were processed by a licensed Pathology Assistant as previously described (Campbell-Thompson et al., 2012b).

METHOD DETAILS

Immunofluorescence

Sections (4 μm) from formalin fixed paraffin embedded samples were evaluated for immunolocalization (Table S1). Immunolocalization was performed after deparaffinization and rehydration, blocking, and incubation with primary antibodies to proinsulin (GS-9A8, Developmental Studies Hybridoma Bank, Iowa City, IA), insulin (A056401, Dako, Carpinteria CA), and glucagon (A056501, Dako). Detection of primary antibody binding was performed by multiplex staining using polymer horseradish peroxidase (HRP, ARH1001EA, PerkinElmer, Waltham MA) followed by tyramine amplification with fluorophores 520, 570, and 670 with DAPI counterstain (OPAL 4-color, NEL94001KT, PerkinElmer). Slides were mounted and sections visualized using a Zeiss AxioskopPlus microscope.

Acid-Ethanol Extraction of Pancreatic Proteins

This method was adapted from a method developed for extraction of whole rodent pancreas (Andersson and Sandler, 2001). It is not feasible to perform such a procedure on whole human pancreas, but from initial experimentation with control tissue, we determined 150 μm cryosections of pancreas (3x50 μm) collected into cryotubes to be suitable. 1mL of acid-ethanol (1.5% HCl in 70% ethanol) was added to each cryotube, and this was incubated overnight at −20°C. The following day the tissue was homogenized using a dounce and again incubated overnight at −20°C. The following day the tubes were centrifuged at 800 x g and the resulting supernatant neutralized 1:1 with 1M Tris pH 7.5 for use in subsequent Bradford, ELISA, and Luminex assays (described below).

Proinsulin, Insulin, C-peptide, Islet Amyloid Polypeptide, and Glucagon by ELISA and Luminex

Commercially available kits from ALPCO (Salem, NH) were utilized to measure total proinsulin, insulin and C-peptide from pancreas protein extracts as indicated in Table S1. The proinsulin, insulin and C-peptide assays are specific and do not cross react with each other. Glucagon was measured with a commercial ELISA assay provided by Mercodia (Winston Salem, NC). Islet amyloid polypeptide was measured by an assay from Millipore (Billerica, MA) using magnetic bead technology (Luminex). A total protein Bradford assay from Thermo-Fisher (Waltham, MA) was performed on each supernatant and used to normalize mass and extraction efficiency.

Immunohistochemistry

Sections (4 μm) from formalin fixed paraffin embedded pancreas tissues were deparaffinized and rehydrated with serial passage through changes of xylene and graded ethanol. All slides were subjected to heat induced antigen retrieval in Target Retrieval Solution (Dako). The tissue sections were stained for insulin as a part of routine collection protocol for nPOD tissues (previously described; Campbell-Thompson et al., 2012b, 2016) or double stained for insulin (polyclonal guinea pig anti-insulin,1:1000 dilution, Dako (Santa Clara, CA)) and glucagon (monoclonal mouse anti-glucagon, 1:5000 dilution, Abcam, Cambridge, MA) by immunohistochemistry (IHC), scanned using an Aperio CS Scanscope (Leica/Aperio, Vista, CA), and stored in the nPOD online digital pathology database (eSLIDE version 12, Leica/Aperio). For donors indicated in Table S1, scanned images of insulin-stained slides available from the block(s) nearest to the tissues used for total protein extraction (described above) were evaluated for fractional insulin area using Indica Labs, Inc image analysis software (Corrales, NM). For insulin and glucagon double stained slides, antigen-antibody binding was visualized using the EnVision G/2 Doublestain System (Dako). For control (n = 5) and T1D subjects (n = 11), glucagon + insulin positive islets, glucagon positive islets, insulin positive single cells, and clusters of two to five insulin positive cells were annotated by hand using the Aperio viewing platform and analyzed using Image Scope, Leica Biosystems analysis software (Version 12.1.0.5029, Buffalo Grove, IL).

RNA Extraction

Tissue from pancreas was flash frozen in RNAlater (QIAGEN, Valencia, CA) an average of 16h from cross clamp. Total RNA was isolated following homogenization in QIAGEN RNeasy Plus Mini Kit isolation buffer as per the manufacturer's instructions including treatment with DNase 1. RNA concentrations were determined using a Nanodrop 2000C (Thermo Scientific, Waltham, MA) and when necessary, integrity was verified by visualization of ribosomal RNA by gel electrophoresis and ethidium bromide staining.

Primer Design

We utilized the public Primer-Blast software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/; Ye et al., 2012) which incorporates the Primer3 program (Rozen and Skaletsky, 2000) for primer design, genome-wide BLAST analysis along with the Needleman-Wunsch (NW) global alignment algorithm (Needleman and Wunsch, 1970) to identify internal homology between primers and any unwanted targets in the human genome and to satisfy the requirements for primer specificity compared to both the human transcriptome and genome. Primers were designed as exonic primers spanning an intron when possible, with an optimal T_m of 60°C. For hnRNA studies, RNA (free of genomic DNA) was isolated from frozen human pancreatic tissue and evaluated by real time qPCR using an exon derived primer and a primer in the adjacent intron to evaluate hnRNA levels, thus evaluating promoter

activity. Human gene symbols, based on HUGO were used throughout this study with primer sequences and the NCBI accession numbers provided in Table S3.

RT-qPCR

All samples were confirmed to be free of DNA contamination in no reverse transcriptase controls with an exon/intron primer pair. cDNA was produced with SuperScript II (Invitrogen, Carlsbad, CA) using oligo dT priming and subsequently utilized for real time quantitative PCR (RT-qPCR) using Thermo Luminaris Color HiGreen Fluorescein qPCR Master Mix (Thermo Scientific). 0.5-1 μg of total RNA was used for each 20 μl cDNA reaction which was then diluted to 200 μl and 2 μl of diluted cDNA employed for each 25 μl RT-qPCR reaction containing 600 nM of each primer pair. Individual RT-qPCR reactions were carried out in duplicate in a Biorad MyiQ. Un-normalized Cq values were displayed in all data since many of the genes displayed no detectable amplification (ND) from the T1D pancreata, thus precluding the use of normalization factors and ultimately statistical analysis. We have however extensive data on multiple reference genes from these same patient samples verifying total RNA integrity and lack of genomic DNA contamination. Regarding each gene primer set, we obtain proper amplification from the no diabetes controls with total reproducibility. For each donor, the specific genes examined by RT-qPCR are indicated in Table S1.

In Situ Hybridization

Sections (4 μm) from fresh frozen pancreas samples were evaluated for *in situ* hybridization (ISH) as indicated in Table S1. ISH was performed using the RNAscope 2.0 High Definition kit (Advanced Cell Diagnostics (ACD), Hayward, California, USA) according to manufacturer's instructions. A human insulin probe (ACD catalog number 313571, NCBI reference sequence NM_000207.2) was hybridized to sections followed by alkaline phosphatase detection with Fast Red chromogen. Slides were mounted and scanned using an Aperio CS Scanscope (Leica/Aperio, Vista, CA) with image files stored in an online pathology database (eSLIDE version 12, Leica/Aperio).

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical parameters including exact n, the definition of center, dispersion and precision measures (mean and 95% confidence intervals or median and interquartile range) are reported in the Figures and Figure Legends. Data were judged to be statistically significant when $p < 0.05$ by either Kruskal-Wallis with Dunn's post-test for for multiple comparisons or unpaired, two-tailed Mann-Whitney test for comparison of two groups, and the Spearman correlation with linear regression as indicated in Figures and Figure Legends. Data were analyzed and graphed using GraphPad Prism software version 6.02.

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Clive Henry Wasserfall

Anlage_1

Anlage 1

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