Differential insulitic profiles determine the extent of beta cell destruction and the age at onset of type 1 diabetes

Pia Leete[1], Abby Willcox[1], Lars Krogvold[2,3], Knut Dahl-Jørgensen[2,3], Alan K. Foulis[4], Sarah J Richardson*[1] and Noel G Morgan*[1]

[1]Institute of Biomedical & Clinical Science, University of Exeter Medical School, Exeter, UK EX2 5DW

[2]Paediatric Department, Oslo University Hospital, Oslo, Norway

[3]Faculty of Medicine, University of Oslo, Norway

[4]GG&C Pathology Department, Southern General Hospital, Glasgow

Running title: Insulitis and age at onset of type 1 diabetes

Word count: 3982

***Corresponding authors:**

Sarah J Richardson

Institute of Biomedical & Clinical Science

University of Exeter Medical School

RILD Building, Barrack Road,

Exeter, EX2 5DW, UK

s.richardson@exeter.ac.uk

+44-1392-408225

Noel G Morgan

Institute of Biomedical & Clinical Science

University of Exeter Medical School

RILD Building, Barrack Road,

Exeter, EX2 5DW, UK

n.g.morgan@exeter.ac.uk

+44-1392-408300

Diabetes Publish Ahead of Print, published online February 8, 2016

Type 1 diabetes (T1D) results from a T-cell mediated destruction of pancreatic beta cells following the infiltration of leukocytes (including CD8+, CD4+ and CD20+ cells) into and around pancreatic islets ("insulitis"). Recently, we reported that two distinct patterns of insulitis occur in patients with recent-onset T1D from the UK and that these differ principally in the proportion of infiltrating CD20+ B-cells (designated "CD20Hi" and "CD20Lo" respectively). We have now extended this analysis to include patients from the nPOD (USA) and DiViD (Norway) cohorts and confirm that the two profiles of insulitis occur more widely. Moreover, we show that patients can be directly stratified according to their insulitic profile and that those receiving a diagnosis before the age of 7 years always display the CD20Hi profile. By contrast, individuals diagnosed beyond the age of 13 years are uniformly defined as CD20Lo. This implies that the two forms of insulitis are differentially aggressive and that patients with a CD20Hi profile lose their beta cells at a more rapid rate. In support of this, we also find that the proportion of residual insulin-containing islets (ICIs) increases in parallel with age at onset of T1D. Importantly, those diagnosed in, or beyond, their teenage years retain ~40% ICIs at diagnosis, implying that a functional deficit rather than absolute beta cell loss may be causal for disease onset in these patients. We conclude that appropriate patient stratification will be critical for correct interpretation of the outcomes of intervention therapies targeted to islet-infiltrating immune cells in T1D.

Type 1 diabetes (T1D) is widely perceived as an autoimmune condition arising from the selective destruction of pancreatic β-cells by influent immune cells targeted to the islets of Langerhans (1-4). This concept has been examined extensively in animal models but many fewer studies have been undertaken in humans. Indeed, In't Veld has noted that as few as 150 T1D cases with insulitis have been scrutinized worldwide since the beginning of the last century (5; 6). This is unfortunate because the extent of insulitis observed in animal models is more fulminant than in most human patients, implying that additional studies are required to improve our understanding of the process in humans (5). Accordingly, we have exploited a large collection of autopsy pancreas samples recovered from patients who died soon after the diagnosis of T1D, to investigate the immune infiltrate in inflamed islets (2; 7; 8). This has revealed aspects of the temporal changes which accompany β-cell destruction and has shown that the principal cells involved in the destructive process are likely to be CD8+ cytotoxic T-cells (2; 7; 8). However, the studies also yielded a more unexpected outcome in that they revealed a potential role for CD20+ B-cells in some patients based on the finding that these cells infiltrate islets in large numbers, in parallel with the influx of CD8+ T-cells (7).

Building on this background, we have also highlighted an unexpected dichotomy in the composition of the immune cell infiltrates present in the inflamed islets of patients with recent-onset T1D (8). Thus, it was found that while significant numbers of CD20+ B-cells infiltrate the islets in many individuals; this is not universally true. Rather, other patients could be found in whose inflamed islets very few CD20+ cells were present. To denote this difference, the terms "CD20Hi" and "CD20Lo" were coined to differentiate the two profiles of insulitis and it was shown that each was present among a group of recent-onset patients diagnosed with T1D before the age of 20 years (8). Importantly, it was also noted that the variation in B-cell numbers occurs independently of the presence of CD8+ T-cells. Despite this, it was also clear that the total population of infiltrating immune cells is least abundant in cases where CD20+ cell numbers are depleted. Accordingly, the two states might also be considered as relatively "hyper-immune" (CD20Hi) or "pauci-immune" (CD20Lo), respectively.

In order to develop a greater understanding of the functional significance of these two insulitic profiles in human T1D, we now report a more complete characterization of the phenomenon in three different pancreas collections: those harvested at post-mortem (UK) (9), biopsies from living donors (DiViD; Norway (10)) and samples from organ donors (nPOD; USA (11; 12)). The studies confirm the existence of two distinct profiles of insulitis and

imply that these patterns exert a profound influence on the rate of disease progression and the extent of β-cell destruction in humans. As such, these new results suggest very strongly that correct patient stratification will be essential when testing the efficacy of targeted immunotherapeutic agents in human populations, and imply that caution should be exercised when interpreting the outcomes of earlier trials where this was not achieved.

Experimental

Pancreas samples

Insulitis was examined in 21 human pancreas sections recovered at the time of post-mortem from patients among a UK cohort of patients with T1D who had died within 3 months of diagnosis (7; 9). These comprised 14 female and 7 males with ages ranging from 1–19 years (mean 10.1 ± 1.3y). For studies of the extent of residual insulin positivity, sections from all 151 patients within this collection were evaluated, irrespective of disease duration or the presence of insulitis (63 males; 88 females).

In addition, insulitis was also studied in pancreas sections recovered by laparoscopic minimal tail resection from 6 living adult donors who had participated in the DiViD study (10), as well as in 2 organ donors with recent-onset T1D from the nPOD cohort (Table S1). Participants in the DiViD study were aged between 25-35 years and their biopsies were taken between 3-9 weeks after initial diagnosis. The 2 nPOD donors were 4.75y and 11y old at time of death and their disease duration was 120 days and 0 days respectively. Collection of pancreatic tissue in the DiViD study was approved by The Norwegian Governments Regional Ethics Committee and all samples were studied with ethical approval.

Immunohistochemistry

Serial sections (4 μm) were cut from each pancreas and mounted on glass slides. Samples from the UK cohort were processed and labelled using a standard immunoperoxidase technique for paraffin sections as described previously (7; 8). All antigens, except insulin and glucagon, were unmasked by heat-induced epitope retrieval. In brief, sections to be labelled with anti-CD20+ anti-serum (M0755; Dako) were heated in 10mM citrate buffer, pH 6 for 20 min in a microwave oven at full power (800W). Those to be labelled with anti-$CD4^+$ (ab846; Abcam) or anti-CD8+ (ab4055; Abcam) were heated in 10mM Tris, 1mM

ethylenediamine-tetraacetic acid, pH 9. Primary antibodies were applied for 1h at room temperature, with the exception of anti-CD4, which was incubated at 4°C overnight (Table S2). Antigen visualization was achieved via the Dako REAL™ Envision™ Detection System. Some slides were processed in the absence of primary antibody or with isotype control antisera to confirm the specificity of labelling.

Pancreas sections from the DiViD and nPOD collections were examined by a combination of immunofluorescence and immunohistochemistry using horseradish peroxidase and alkaline phosphatase staining, as appropriate. Where possible, and to minimize sample usage, at least two antigens per section were examined in parallel. Antigens were unmasked by heat-induced epitope retrieval (20 min at full power in a microwave oven; 800W) in 10 mmol/L citrate buffer (pH 6). Combined staining was achieved by using guinea pig anti-insulin (A0564; Dako) in combination with mouse anti-glucagon (ab10988; Abcam) and visualized using a Vector Red Substrate kit for detection of alkaline phosphatase activity (AK-5100 and SK-5100; Vector Laboratories, U.K.). Binding of rabbit anti-CD8 and mouse anti-CD20 sera was visualized using secondary antibodies tagged with appropriate fluorescent probes (Alexafluor anti-mouse 488 (A11029; Invitrogen, Paisley, U.K.) or anti-rabbit 568 (A1136; Invitrogen)). Cell nuclei were stained with DAPI.

Image acquisition and analysis

Brightfield image acquisition and analysis were performed on a Nikon 50i Microscope fitted with a DS-Fi camera and DSL2 camera control unit. Images were captured and analyzed using the ImageJ platform. Immunofluorescence image collection and processing was achieved using a Leica DM4000 B LED upright fluorescence microscope and Leica Image analysis software (LAS AF).

Statistical analysis

10 islets were selected randomly from within each stained section among the UK cohort and the number of CD8+, CD4+ and CD20+ cells within the islet area and in the endocrine-exocrine interface in close contact with the islet boundary were recorded. The mean number ± SEM of each immune cell subtype was calculated for each patient as an average of the 10 individual values from each islet. The insulin positive area of each islet was assessed and expressed as percentage of the total area as described previously and scored as (+++), (++) or (+) respectively. In the DiViD and nPOD cohorts, immune cells were counted from ~30

islets per patient section and expressed as above. Statistical significance was calculated using Kruskal-Wallis with Dunn's multiple comparison test, Student's t-test or by Chi-squared analysis, as appropriate.

Results

Profiles of insulitis in patients with type 1 diabetes

Detailed analysis was undertaken of the numbers of CD8+, CD4+ and CD20+ cells within, or in close proximity to, 10 randomly selected inflamed islets across multiple cases from among the UK collection of recent-onset T1D patients. This confirmed the existence of the two independent profiles ("CD20Hi" and "CD20Lo"; Fig.1A & B). The results also revealed that these profiles are patient-specific in that all inflamed islets within any given individual display the same phenotype.

A more dynamic evaluation of the profile of immune cell infiltration in relation to β-cell loss was also undertaken in patients from each group (Fig.1 C-F). This analysis confirmed that CD8+ T-cells always comprise the majority population within inflamed islets, irrespective of the number of CD20+ cells present. This was evident both when the absolute number of each cell type was examined (Fig.1C & D) and when the relative proportions of each were calculated (Fig.1E & F). It was also clear that the number of CD8+ T-cells varied according to the progression of beta-cell loss (Fig.1C & D). CD4+ T-cell numbers also tended to rise as the extent of β-cell loss neared totality but their absolute number (and relative proportion) was always much lower than the number (or proportion) of CD8+ cells. Importantly, the most marked difference between the two phenotypes was in the proportion of CD20+ B-cells found within, or near to, inflamed islets. Among one group (CD20Hi) these were abundant and they increased in parallel with the CD8+ cells as β-cell death progressed (Fig.1C, E). By contrast, in the second group (CD20Lo individuals) they were the least abundant cell type at all stages of the disease process (Fig.1D, F).

In addition to the variation in the absolute number of CD20+ cells, the ratio of CD20+ to CD4+ cells also varied consistently between the two phenotypes (Fig.1). Thus, in CD20Hi cases the ratio of CD20+:CD4+ cells in inflamed islets was greater than 1.0 at all stages of β-cell loss, whereas this ratio was consistently less than 1.0 in CD20Lo patients. On this basis, it seemed that estimation of the CD20+:CD4+ cell ratio might offer greater utility as a means

to sub-classify patients. Accordingly, we focused on this ratio to define the CD20Hi (CD20+:CD4+ >1.0) and CD20Lo (CD20+:CD4+ <1.0) sub-groups in subsequent studies.

Since the two profiles of insulitis may represent differentially aggressive phenotypes with variable rates of disease progression (8), the ratio of CD20+:CD4+ cells was examined in patients from the UK at increasing age at diagnosis. This revealed a striking relationship despite the limitation that all patients studied had been diagnosed below the age of 20 years (Fig.2). Notably, it was clear that CD20Hi patients tended to be diagnosed at younger ages than those defined as CD20Lo. Indeed, when patients within the UK cohort were stratified into those diagnosed with T1D at between 1 and 6 years of age vs those diagnosed at 13 years or beyond, the separation between the two phenotypes was absolute (Fig.2). All individuals within the younger group were classified as CD20Hi while those diagnosed at the older age were CD20Lo. Patients who developed T1D between the ages of 7 and 13 years fell into either group.

To verify these conclusions, we next examined the profile of insulitis in pancreas samples from among the nPOD collection and from the DiViD cohort of living organ donors. In practice, only two patients who met our criteria for recent-onset T1D (within 120 days of diagnosis) were available from nPOD (6209 and 6228) while all six of the DiViD patients conformed to this criterion. Strikingly, the insulitic profile in each of the inflamed islets of the six DiViD patients (all of whom were diagnosed beyond the age of 20 years) was CD20Lo (Fig.3). A similar profile was found in one of the two nPOD patients (6228; diagnosed originally at 13 years of age). By contrast, the second nPOD case (6209) was diagnosed much earlier in life (<5 years) and was defined as CD20Hi.

The influence of age at diagnosis on the proportion of residual insulin-containing islets in the pancreases of patients with type 1 diabetes

In view of the finding that age at diagnosis is predictive of the profile of insulitis in patients with recent-onset T1D, and that individuals diagnosed at younger ages appear to display a more aggressive phenotype, we considered the possibility that the proportion of residual insulin-containing islets (ICIs) might also differ between these groups. Accordingly, we calculated the proportion of such islets in sections of pancreas from each patient. This was achieved by using glucagon immunoreactivity to define the total islet number, with simultaneous co-staining for insulin to monitor the proportion of residual ICIs. Initially, this analysis was performed for the patients within the UK cohort and these were stratified

according to age: group 1) from 1-6 years at diagnosis; group 2) 7-12 years; group 3) 13 years or greater. The results revealed a strongly positive relationship in which individuals who had been diagnosed at the youngest ages retained a much lower proportion of ICIs than those diagnosed in the older age group ($p<0.001$; Fig.4).

Extending this analysis to include pancreas samples from the two additional nPOD cases as well as those from the six DiViD patients, reinforced these conclusions. Thus, the DiViD patients retained a mean of 25% ICIs in the pancreas sections studied, at diagnosis (Fig.4) and this was also the situation in nPOD 6228 (CD20Lo; Fig.4). By contrast, nPOD case 6209, whose insulitic profile was consistent with a more aggressive inflammatory infiltrate, retained only 10% ICIs at diagnosis.

Having examined patients soon after diagnosis, the analysis was extended to include UK cases having a disease duration of between 1-10 years (Supplementary Fig. 1). Consistent with the data from recent–onset patients, individuals diagnosed at the younger ages retained fewest ICIs.

In view of this striking outcome, we extended the dataset further to examine the entire collection of T1D pancreas samples available within the UK cohort (a total of 151 patients) irrespective of their disease duration or the presence of insulitis (Fig. 5). The proportion of residual ICIs present in at least one pancreatic section (selected randomly from those available for study) from each block was calculated. When stratified into three groups according to the original age at diagnosis (1-6 years; 7-12 years; >13 years) the proportion of residual ICIs increased from a mean of 14±3% in those diagnosed early in life, to 39±4% in those diagnosed from their teens or beyond ($p<0.001$). Indeed, among the latter group, 56 of 69 patients still retained residual ICIs in the pancreas section studied. This was significantly more than the equivalent proportion among those in the youngest age group (19 of 43; $p<0.004$ by Chi squared analysis). Similar findings were made in the nPOD cohort when the proportion of cases with residual ICIs was examined (Supplementary Fig. 2 & Supplementary Table 2).

Discussion

It is often assumed that insulitis and β-cell loss proceed in a broadly consistent manner in the majority of patients developing T1D and that the final descent into clinical disease occurs

when a defined proportion of the initial β-cell mass has been lost. Estimates vary as to the precise extent but it is frequently argued that >80% loss may be required to precipitate the disease (13-16). However, in accord with Klinke (17), we find that this may be an over-simplification. Importantly, we note that β-cell numbers do not necessarily dwindle to very low levels before diabetes is manifest but that many patients become diabetic while still retaining significant numbers of these cells. We also find that the process and extent of β-cell loss varies with age such that individuals who develop T1D early in life (before the age of 7 years) lose β-cells to a much greater extent than those who are diagnosed with the illness in their teenage years or beyond. Similar conclusions that patients with an early disease onset may have a more fulminant form of the illness have also been reached previously (18; 19) and we now attribute this variation to the existence of two distinct profiles of insulitis which yield very different rates of β-cell loss (8).

Careful examination of multiple islets across a range of patients reveals that all of the inflamed islets within a given patient display the same insulitic profile but that this profile differs significantly between individuals. Importantly, this variation does not occur in a purely stochastic manner but, rather, two identifiable forms of insulitis can be differentiated. Both are characterized by a preponderance of CD8+ T-cells suggesting that these are likely to be the key cytotoxic mediators in each subtype of the disease. By contrast, there is much greater variation in the proportion of CD20+ B-cells; these are abundant in the inflamed islets of some individuals but are almost absent from the islets of others. As a result, the terms "CD20Hi" or "CD20Lo" were coined to denote these two profiles (8). This designation is not intended to imply that CD20+ cells are necessarily (or selectively) cytotoxic in one form of the disease but, rather, to emphasize that these cells may play an important accessory role in the destructive process, and that this is exerted more effectively in one form than in the other. Comparison of the number (or proportion) of CD4+ cells within inflamed islets revealed no significant differences among this cell population but suggested that they could provide a useful means to distinguish the two profiles, based on measurement of the ratio of CD20:CD4 cells. Thus, among CD20Hi patients this ratio was greater than 1.0 while it was below 1.0 in those defined as CD20Lo. Accordingly, we propose that monitoring of the ratio of CD20:CD4 cells may provide a useful and robust means to distinguish the two subtypes of insulitis.

The justification for our conclusion that the two profiles of insulitis are functionally different lies in the striking finding that children who develop diabetes below the age of 7 years

uniformly display a CD20Hi profile, while the islets of those patients diagnosed in or beyond their teenage years are always CD20Lo. This extraordinary level of differentiation was unexpected and it was important to establish that it did not reflect a hidden variable arising from the study of a individuals whose pancreas was harvested in a particular way or from one specific geographical region (the majority of our observations having been made using samples collected at autopsy in the UK from individuals who had died in ketoacidosis (9)). In noting this, it must also be emphasized that extending the analysis is not a trivial task because the number of recent onset T1D cases available for study worldwide is very small. Nevertheless, we were able to confirm the findings by analysis of two organ donors with recent onset T1D from the nPOD collection (6209 and 6228 (11; 12)) and six newly diagnosed adults who had consented to pancreatic biopsy in the Norwegian DiViD study (10). These followed identical patterns to those seen in the larger group from the UK and allowed the compilation of a more extensive dataset to verify the conclusions. This revealed statistically significant differences in several parameters assessed at the islet level between those diagnosed at younger ages (under 7 years) and those who were older at disease onset (13 years or over). Most notably, all patients diagnosed at >13 years displayed a CD20Lo islet profile while young children had a CD20Hi profile. Not surprisingly, individuals who were diagnosed with T1D between 7-12 years of age could fall into either category, as might be expected if two overlapping distribution profiles exist.

Analysis of the age-related variation in disease pathology revealed a second important feature; namely that the proportion of ICIs remaining at diagnosis of T1D varies significantly between groups. Consistent with the results of a previous meta-analysis (17), we found that those diagnosed in their early years had a much lower proportion of ICIs than patients with an older age at onset. This difference was so pronounced that age at diagnosis could be used as a strong surrogate to predict the proportion of ICIs present at diagnosis and the profile of insulitis that would be found (whether CD20Hi or CD20Lo). Moreover, when this analysis was extended to include all 151 samples from within the UK cohort and 116 from nPOD (irrespective of disease duration) stratified according to age at diagnosis, these relationships still held. Younger patients (aged under 7 years) had a significantly lower proportion of residual ICIs than those who presented with T1D in their teenage years or beyond (even when many years had passed between the initial diagnosis and the availability of the pancreas for analysis).

In drawing this conclusion it is important to recognize that the present analysis was undertaken on only a small number of sections from any given pancreas. Indeed, in many cases the proportion of ICIs was estimated on a single section. This then raises an important question about the extent to which an individual set of data derived from one section can be considered representative of the entire pancreas. This is especially true given that the processes of insulitis and β-cell loss occur in a lobular manner across the gland (20-23). Such considerations inevitably mean that it would be dangerous to draw firm conclusions from a single pancreatic section taken from one individual patient studied in isolation. However, a key strength of the present study is that we were able to examine sections selected at random from multiple patients and thereby to counter the likelihood of systematic bias. On this basis, we consider that the cumulative results provide a representative view of the pathological mechanisms causing T1D and that wider generalization of the conclusions is valid. If this were not the case, then factors such as the proportion of ICIs would be expected to distribute more randomly in the analysis rather than segregating in parallel with age at disease onset, as we find.

The discovery that many patients develop T1D at a time when their β-cell mass remains relatively high suggests that, in these individuals, β-cell dysfunction rather than cell death may be a significant contributory factor leading to glucose intolerance. Indeed, this is supported directly by study of the insulin secretory capacity of the islets isolated from pancreas biopsies recovered from the DiViD cohort (24). These patients all displayed a CD20Lo profile of insulitis and, in accord with this, they were diagnosed beyond their teenage years and they retained a large proportion of ICIs at disease onset. Importantly, their islets were largely unresponsive to a glucose stimulus upon isolation but gradually regained responsiveness during tissue culture under *ex vivo* conditions (24). This implies that the ICIs had become dysfunctional *in vivo* rather than experiencing an irreversible decline arising from complete β-cell demise. These results therefore offer the prospect that, by appropriate therapeutic intervention, the residual β-cells of patients with a CD20Lo insulitic profile might also be reactivated *in vivo* and that, in combination with an appropriate immunotherapeutic regime, this could lead to a reduced requirement for exogenous insulin.

One important conclusion arising from the present data is that due care should be exercised when designing immunotherapeutic interventions in patients with T1D. In particular, it will be important to ensure that patients are stratified according to their insulitic profile (for which age at onset appears a strong surrogate) and that the regimen is tailored appropriately. One

obvious example to illustrate this principle arises from the outcome of trials with the anti-CD20 monoclonal antibody, rituximab (25; 26). Since we find that infiltrating CD20+ cells are present preferentially in the islets of patients diagnosed below the age of 7 years it is, perhaps, likely that such individuals may be most sensitive to the therapy whereas older patients will be more refractory.

In conclusion, we confirm that insulitis occurs in two distinct profiles in patients with T1D. These differ principally in the proportion of CD20+ B-cells (expressed relative to CD4+ cells) present within the infiltrate and this appears to lead to a more or less aggressive phenotype respectively. Not only do these phenotypes lead to differential rates of β-cells loss (as judged by marked differences in the age at onset between the groups) but they also culminate in loss of glucose tolerance when dramatically different numbers of β-cells remain in the pancreas. In the youngest patients, β-cell loss is rapid and extensive. By contrast, those diagnosed in their teenage years or beyond, have a less aggressive disease and they experience clinical onset when a significant β-cell reserve is still measurable. These results emphasize that, in the future, it will be critical to stratify patients appropriately according to their insulitic profile and age at onset of disease in order to design and trial effective therapies for T1D.

Acknowledgements. The authors thank specialist nurse Trine Roald, Oslo University Hospital, Norway, whose invaluable efforts were essential to the success of the DiViD study.

Author Contributions. P.L. performed data collection and analysis and edited the manuscript. A.W. performed data collection and analysis and reviewed the manuscript. L.K, K.D-J and A.K.F collected patient material and revised the manuscript. S.J.R and N.G.M. designed the study, performed data analysis and wrote the manuscript. N.G.M and S.J.R. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Duality of interest. The authors report no potential conflicts of interest in relation to this article.

Funding. We are pleased to acknowledge financial support from the European Union's Seventh Framework Programme PEVNET [FP7/2007-2013] under grant agreement number

261441. The participants of the PEVNET consortium are described at http://www.uta.fi/med/pevnet/publications.html. Additional support was from a JDRF Career Development Award (5-CDA-2014-221-A-N) to S.J.R. KDJ has received grants from the South-Eastern Norway Regional Health Authority and the Novo Nordisk Foundation. The research was also performed with the support of the Network for Pancreatic Organ Donors with Diabetes (nPOD), a collaborative type 1 diabetes research project sponsored by the Juvenile Diabetes Research Foundation International (JDRF, JDRF 25-2013-268) and with a JDRF research grants awarded to the nPOD-V consortium, JDRF 25-2012-516 and JDRF 25-2012-770. Organ Procurement Organizations (OPO) partnering with nPOD to provide research resources are listed at www.jdrfnpod.org/our-partners.php.

Figure 1. Complement of immune cells in inflamed islets of patients with type 1 diabetes. The numbers of CD8+ (circles), CD4+ (squares) and CD20+ (triangles) cells were quantified in at least 10 inflamed islets in each of 20 pancreases available within the UK cohort of recent-onset T1D patients. These are displayed as the cumulative mean values per islet (±SEM) of each cell type, stratified according to the average number of CD20+ cells present. Left hand panels (A,C,E) are from patients with a mean of >3 CD20+ cells per inflamed islet (10 cases); right hand panels (B,D,F) are from those with <3 CD20+ cells per inflamed islet (10 cases). Panels A & B show the average number of immune cells per islet independent of islet insulin content. Panels C-F show the data analyzed in relation to the insulin content of each islet; displayed either as the absolute mean number of immune cells (C & D) or as the relative proportion of each immune cell subtype (E & F). Islet insulin content was calculated relative to total islet area and is expressed as (+++) (++) (+) and (-) to denote decreasing insulin immunopositivity (>65%; 33-64%; <33% and zero, respectively).

Figure 2. Ratio of CD20+:CD4+ cells in the inflamed islets of patients with type 1 diabetes from the UK cohort. Patients with recent-onset T1D from within the UK cohort were stratified according to their age at diagnosis of diabetes and the ratio of CD20+:CD4+ cells calculated in 10 inflamed islets from each patient. Each point represents the mean ratio in an individual patient and the differentially hatched panels define the increasing age ranges at diagnosis (1-6 years (left; stippled pattern) 7-13 years (centre; diagonal hatches) and 14-20 years (right; horizontal hatches)).

Figure 3. Ratio of CD20+:CD4+ cells in the inflamed islets of patients with type 1 diabetes from multiple cohorts. Islets were examined in pancreas samples from patients with recent-onset T1D among the UK cohort (circles) the nPOD collection (squares) and the DiViD cohort (triangles) and stratified according to their age at diagnosis of diabetes. The ratio of CD20+:CD4+ cells was calculated in up to 30 inflamed islets from each patient. Each point represents the mean ratio in an individual patient displayed in relation to their age at diagnosis.

*$p<0.001$ relative to those diagnosed at 1-6 years.

Figure 4. Proportion of residual insulin-containing islets found in pancreas sections from patients with recent-onset type 1 diabetes. The proportion of insulin-containing islets (expressed as %) in pancreatic sections from patients with recent-onset T1D among the UK cohort was determined and displayed in relation to the age at diagnosis. Arrows indicate the equivalent values in the 6 patients from the DiViD cohort (all of whom were diagnosed beyond the age of 20 years) and the 2 nPOD cases (6228; diagnosed at 13y; 6209 diagnosed at <5y).

*P<0.001 relative to those diagnosed at 1-6 years.

Figure 5. Proportion of residual insulin-containing islets found in pancreas sections from 151 patients with type 1 diabetes among the UK cohort, irrespective of disease duration. The proportion of residual insulin-containing islets was calculated (%) in at least one pancreatic section from all 151 patients with type 1 diabetes among the UK cohort. These were then stratified into 3 groups according to their age at initial diagnosis of T1D.

*p<0.001 relative to those aged 1-6 years.

References

1. Roep BO, Tree TI: Immune modulation in humans: implications for type 1 diabetes mellitus. Nature reviews Endocrinology 2014;10:229-242
2. Morgan NG, Leete P, Foulis AK, Richardson SJ: Islet inflammation in human type 1 diabetes mellitus. IUBMB life 2014;66:723-734
3. Atkinson MA, Eisenbarth GS, Michels AW: Type 1 diabetes. The Lancet 2014;383:69-82
4. Campbell-Thompson ML, Atkinson MA, Butler AE, Chapman NM, Frisk G, Gianani R, Giepmans BN, von Herrath MG, Hyoty H, Kay TW, Korsgren O, Morgan NG, Powers AC, Pugliese A, Richardson SJ, Rowe PA, Tracy S, In't Veld PA: The diagnosis of insulitis in human type 1 diabetes. Diabetologia 2013;56:2541-2543
5. in't Veld P: Rodent versus human insulitis: why the huge disconnect? Current opinion in endocrinology, diabetes, and obesity 2015;22:86-90
6. In't Veld P: Insulitis in human type 1 diabetes: The quest for an elusive lesion. Islets 2011;3:131-138
7. Willcox A, Richardson SJ, Bone AJ, Foulis AK, Morgan NG: Analysis of islet inflammation in human type 1 diabetes. Clinical and experimental immunology 2009;155:173-181
8. Arif S, Leete P, Nguyen V, Marks K, Nor NM, Estorninho M, Kronenberg-Versteeg D, Bingley PJ, Todd JA, Guy C, Dunger DB, Powrie J, Willcox A, Foulis AK, Richardson SJ, de Rinaldis E, Morgan NG, Lorenc A, Peakman M: Blood and islet phenotypes indicate immunological heterogeneity in type 1 diabetes. Diabetes 2014;63:3835-3845
9. Foulis AK, Liddle CN, Farquharson MA, Richmond JA, Weir RS: The histopathology of the pancreas in type 1 (insulin-dependent) diabetes mellitus: a 25-year review of deaths in patients under 20 years of age in the United Kingdom. Diabetologia 1986;29:267-274
10. Krogvold L, Edwin B, Buanes T, Ludvigsson J, Korsgren O, Hyoty H, Frisk G, Hanssen KF, Dahl-Jorgensen K: Pancreatic biopsy by minimal tail resection in live adult patients at the onset of type 1 diabetes: experiences from the DiViD study. Diabetologia 2014;
11. Campbell-Thompson M: What can organ donor specimens tell us about type 1 diabetes? Pediatric diabetes 2015;
12. Pugliese A, Vendrame F, Reijonen H, Atkinson MA, Campbell-Thompson M, Burke GW: New Insight on Human Type 1 Diabetes Biology: nPOD and nPOD-Transplantation. Current diabetes reports 2014;14:530
13. Atkinson MA: ADA Outstanding Scientific Achievement Lecture 2004. Thirty years of investigating the autoimmune basis for type 1 diabetes: why can't we prevent or reverse this disease? Diabetes 2005;54:1253-1263
14. Gale EA: Can we change the course of beta-cell destruction in type 1 diabetes? The New England journal of medicine 2002;346:1740-1742
15. Battaglia M, Atkinson MA: The streetlight effect in type 1 diabetes. Diabetes 2015;64:1081-1090
16. Kloppel G, Lohr M, Habich K, Oberholzer M, Heitz PU: Islet pathology and the pathogenesis of type 1 and type 2 diabetes mellitus revisited. Surv Synth Pathol Res 1985;4:110-125
17. Klinke DJ, 2nd: Extent of beta cell destruction is important but insufficient to predict the onset of type 1 diabetes mellitus. PloS one 2008;3:e1374
18. Pipeleers D, Ling Z: Pancreatic beta cells in insulin-dependent diabetes. Diabetes Metab Rev 1992;8:209-227
19. Lernmark A, Kloppel G, Stenger D, Vathanaprida C, Falt K, Landin-Olsson M, Baskin DG, Palmer JP, Gown AM, Petersen JS, et al.: Heterogeneity of islet pathology in two infants with recent onset diabetes mellitus. Virchows Arch 1995;425:631-640
20. Atkinson MA, von Herrath M, Powers AC, Clare-Salzler M: Current concepts on the pathogenesis of type 1 diabetes-considerations for attempts to prevent and reverse the disease. Diabetes care 2015;38:979-988

21. Rodriguez-Calvo T, Suwandi JS, Amirian N, Zapardiel-Gonzalo J, Anquetil F, Sabouri S, von Herrath MG: Heterogeneity and Lobularity of Pancreatic Pathology in Type 1 Diabetes during the Prediabetic Phase. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society 2015;63:626-636
22. Richardson SJ, Morgan NG, Foulis AK: Pancreatic pathology in type 1 diabetes mellitus. Endocrine pathology 2014;25:80-92
23. Richardson SJ, Willcox A, Bone AJ, Morgan NG, Foulis AK: Immunopathology of the human pancreas in type-I diabetes. Seminars in immunopathology 2011;33:9-21
24. Krogvold L, Skog O, Sundstrom G, Edwin B, Buanes T, Hanssen KF, Ludvigsson J, Grabherr M, Korsgren O, Dahl-Jorgensen K: Function of isolated pancreatic islets from patients at onset of type 1 diabetes; Insulin secretion can be restored after some days in a non-diabetogenic environment in vitro. Results from the DiViD study. Diabetes 2015;
25. Pescovitz MD, Greenbaum CJ, Bundy B, Becker DJ, Gitelman SE, Goland R, Gottlieb PA, Marks JB, Moran A, Raskin P, Rodriguez H, Schatz DA, Wherrett DK, Wilson DM, Krischer JP, Skyler JS, Type 1 Diabetes TrialNet Anti CDSG: B-lymphocyte depletion with rituximab and beta-cell function: two-year results. Diabetes care 2014;37:453-459
26. Pescovitz MD, Greenbaum CJ, Krause-Steinrauf H, Becker DJ, Gitelman SE, Goland R, Gottlieb PA, Marks JB, McGee PF, Moran AM, Raskin P, Rodriguez H, Schatz DA, Wherrett D, Wilson DM, Lachin JM, Skyler JS, Type 1 Diabetes TrialNet Anti CDSG: Rituximab, B-lymphocyte depletion, and preservation of beta-cell function. The New England journal of medicine 2009;361:2143-2152

Fig. 1

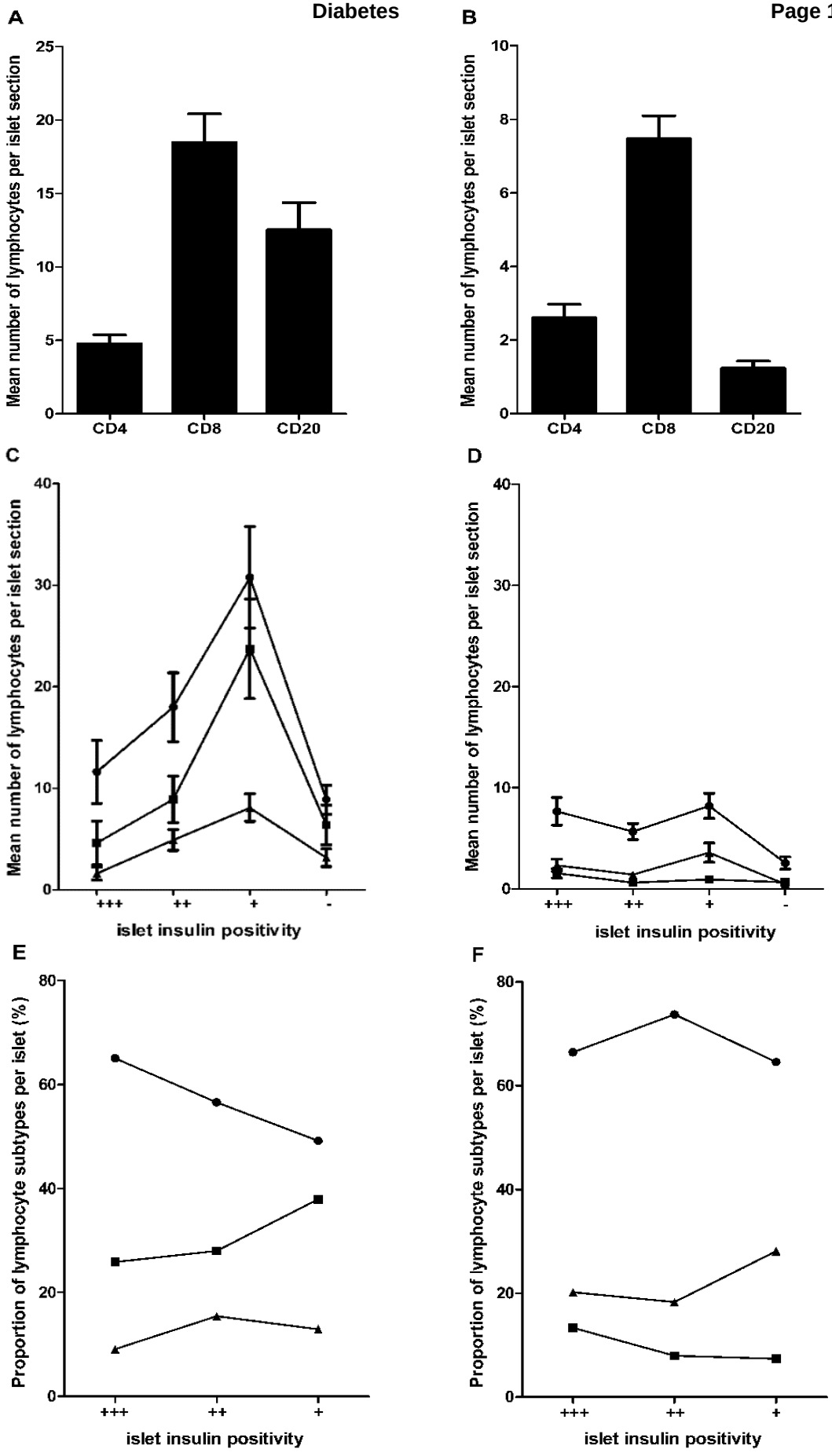

A
Mean number of lymphocytes per islet section
25
20
15
10
5
0
CD4
CD8
CD20
B
Mean number of lymphocytes per islet section
10
8
6
4
2
0
CD4
CD8
CD20
C
Mean number of lymphocytes per islet section
40
30
20
10
0
+++
++
+
-
islet insulin positivity
D
Mean number of lymphocytes per islet section
40
30
20
10
0
+++
++
+
-
islet insulin positivity
E
Proportion of lymphocyte subtypes per islet (%)
80
60
40
20
0
+++
++
+
islet insulin positivity
F
Proportion of lymphocyte subtypes per islet (%)
80
60
40
20
0
+++
++
+
islet insulin positivity

Fig. 2

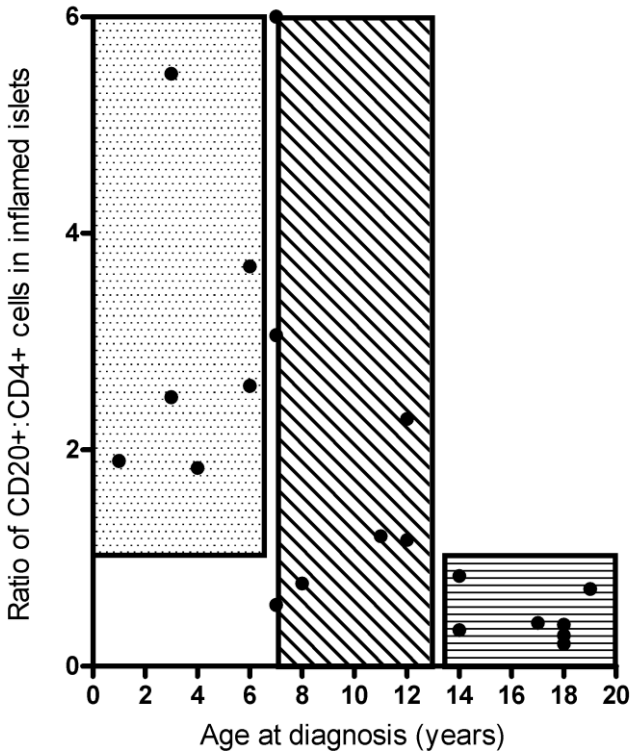

Ratio of CD20+:CD4+ cells in inflamed islets
0
2
4
6
0 2 4 6 8 10 12 14 16 18 20
Age at diagnosis (years)

Fig. 3

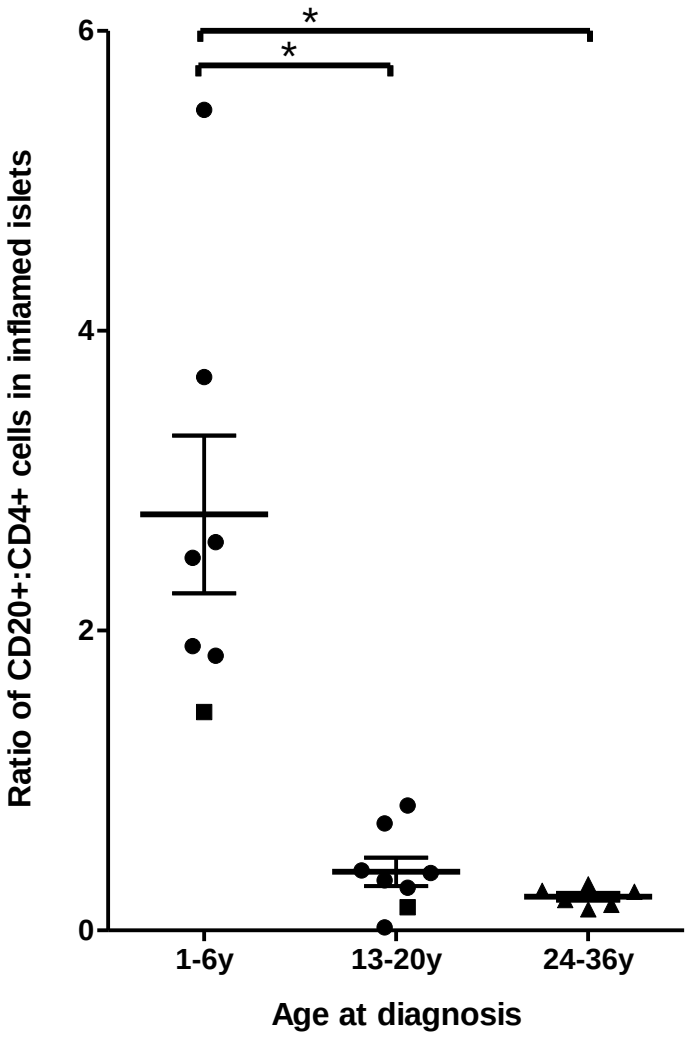

*
*
6
4
2
0
Ratio of CD20+:CD4+ cells in inflamed islets
1-6y
13-20y
24-36y
Age at diagnosis

Fig. 4

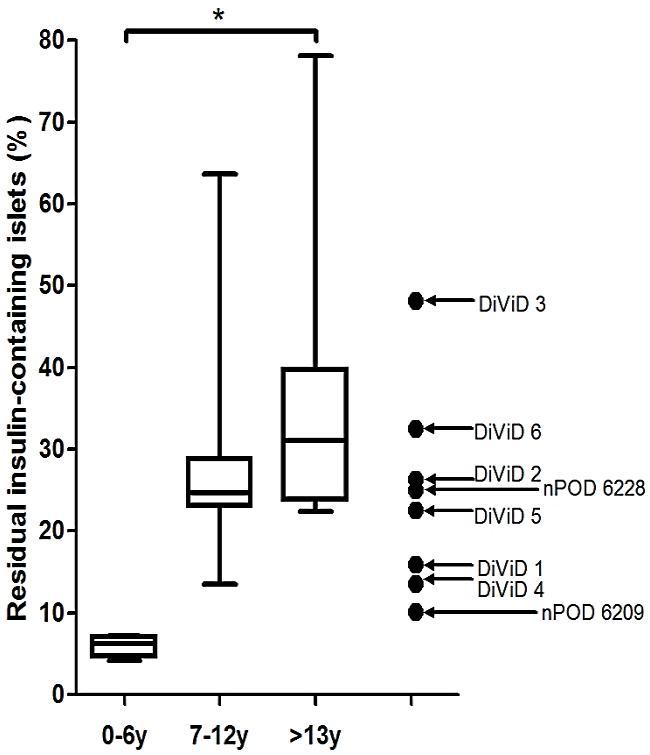
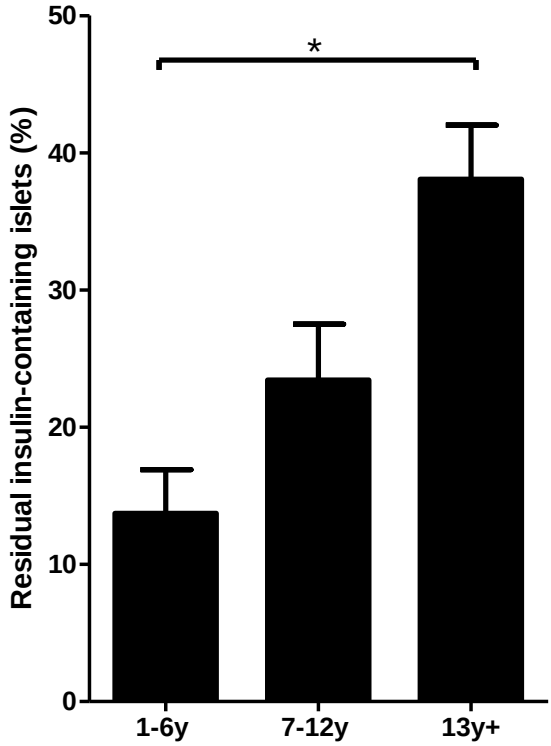

*
Residual insulin-containing islets (%)
80
70
60
50
40
30
20
10
0
0-6y
7-12y
>13y
DiViD 3
DiViD 6
DiViD 2
nPOD 6228
DiViD 5
DiViD 1
DiViD 4
nPOD 6209

Fig. 5


Residual insulin-containing islets (%)
50
40
30
20
10
0
*
1-6y
7-12y
13y+

Supplementary table 1. Characteristics of the patients studied from among the nPOD and DiViD cohorts.

Patient No	Age at diagnosis	Gender	Duration (weeks)
DiViD 1	25	F	4
DiViD 2	24	M	3
DiViD 3	34	F	9
DiViD 4	31	M	5
DiViD 5	24	F	5
DiViD 6	35	M	5
nPOD 6209-01PB	4.75	F	<13
nPOD 6228-04PB	13	M	0

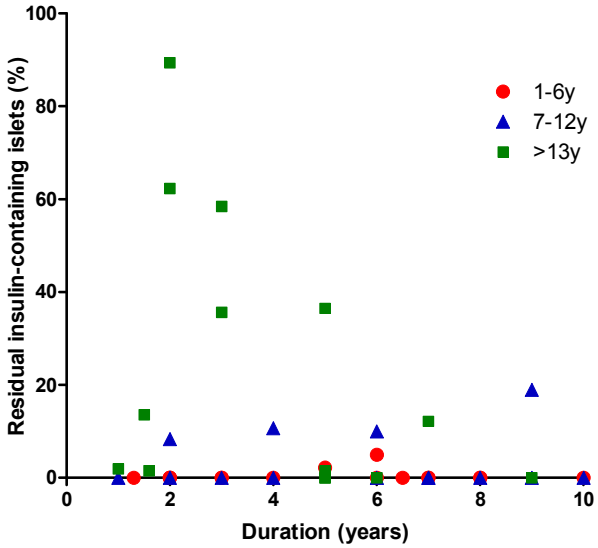
Supplementary Table 2: Retention of insulin-containing islets in nPOD cases stratified according to age at onset and duration of disease (1-10y and 11-25y).

The proportion of cases with residual ICIs declines with disease duration but this effect is least marked in those diagnosed in their teenage years and beyond (when almost 40% of patients still retain ICIs at up to 25 years of disease duration).

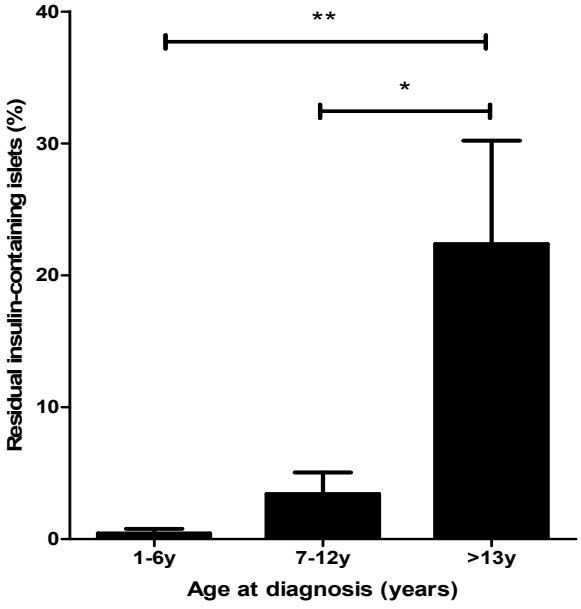
	All cases regardless of duration		**Duration 1-10y**		**Duration 11-25y**	
Age at diagnosis (years)	Total No. cases (N)	Cases with residual ICIs/ N (%)	Total No. cases (N)	Cases with residual ICIs/ N (%)	Total No. cases (N)	Cases with residual ICIs/ N (%)
1-6y	33	6/33 (18.2)	8	3/8 (37.5)	8	0/8 (0.0)
7-12y	32	9/32 (25.0)	9	6/9 (66.7)	13	2/13 (15.4)
>13y	51	25/51 (49.0)	22	14/22 (63.6)	22	8/22 (36.4)

Supplementary Figure 1:

(A) Proportion of residual insulin-containing islets found in pancreas sections from 44 patients with type 1 diabetes among the UK cohort (disease duration of 1-10 years). These were stratified into 3 groups according to their age at initial diagnosis of T1D; 1-6y (n=16; red circles), 7-12y (n=14; blue triangles) and >13y (n=14; green squares). The data show that individuals diagnosed at younger ages have fewest residual ICIs. This difference is statistically significant between the 1-6y vs >13y age groups ($p<0.006$).



(**B**) Proportion of residual ICIs in patients who died 1-10y post-diagnosis of T1D stratified according to age at onset of disease. The residual ICIs (mean ± SEM %) differed significantly at increasing ages at onset; ** $p<0.01$; * $p<0.05$.



Supplementary Figure 2: Proportion of cases with residual insulin-containing islets found in pancreas sections with type 1 diabetes among the nPOD cohort. Data were extracted from the histopathology reports available at: https://npoddatashare.coh.org/labkey/login/home/login.view.

Among 116 patients studied, a total of 40 had residual insulin-containing islets at the time of death. When cases were stratified based on the age at diagnosis, it was revealed that more of those diagnosed at >13 years retained ICIs than diagnosed earlier irrespective of duration of disease (Chi squared; P=0.0067).