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Type 1 diabetes in the pancreas: A histological perspective

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Abstract

Aims: This review aims to introduce research in the pancreas to a broader audience. The pancreas is a heterocrine gland residing deep within our abdominal cavity. It is the home to our islets, which play a pivotal role in regulating metabolic homeostasis. Due to its structure and location, it is an impossible organ to study, in molecular detail, in living humans, and yet, understanding the pancreas is critical if we aim to characterise the immunopathology of type 1 diabetes (T1D) and one day prevent the triggering of the autoimmune attack associated with ß-cell demise.

Methods: Over a 100 years ago, we began studying pancreatic histology using cadaveric samples and clever adaptations to microscopes. As histologists, some may say nothing much has changed. Nevertheless, our microscopes can now interrogate multiple proteins at molecular resolution. Images of pancreas sections are no longer constrained to a single field of view and can capture a thousands and thousands of cells. AI-image-analysis packages can analyse these massive data sets offering breakthrough findings.

Conclusion: This narrative review will provide an overview of pancreatic anatomy, and the importance of research focused on the pancreas in T1D. It will range from histological breakthroughs to briefly discussing the challenges associated with characterising the organ. I shall briefly introduce a selection of the available global biobanks and touch on the distinct pancreatic endotypes that differ immunologically and in ß-cell behaviour. Finally, I will introduce the idea of developing a collaborative tool aimed at developing a cohesive framework for characterising heterogeneity and stratifying endotypes in T1D more readily.

KEYWORDS

biobanks, endotypes, islets, pancreas, stratification, T1D, Type 1 diabetes

1 | INTRODUCTION

1.1 | Context

Type 1 diabetes (T1D) is a lifelong condition mediated by genetic, autoimmune and environmental components. A consistent feature of T1D is the autoimmune destruction of insulin-containing ß-cells in the pancreas. However, it is increasingly accepted that there is considerable heterogeneity in the aetiology and long-term health outcomes between individuals.

1.2 | Incidence

The global incidence of T1D is projected to double by 2040, and the most rapid growth is expected in low- and middle-income countries (LMICS).[1]

Incidence in many European countries continues to rise and remains highest in children under 5 years of age, the age group most deeply affected by the condition in the longer term.[2] Traditionally a diagnosis of youth, research now suggests that nearly half of incident T1D cases occur in the over 30s.[3]

Finding a preventative therapy is an urgent, unmet need.

1.3 | Impact of T1D

All people living with T1D (PLWT1D) depend on the (self) administration of exogenous insulin to avoid lethal hyperglycaemia. They must also balance their nutritional needs and energy expenditure with near-constant monitoring of their blood glucose and lifestyle choices. For some individuals, T1D can be unpredictable, even with diligent management, and this unpredictability can lead to suboptimal glucose control, increased rates of diabetes-associated complications and poor mental health.

Alarmingly, even in countries with modern and universal health care, T1D is often only diagnosed when individuals are hospitalised because of diabetic ketoacidosis,[4] which, at best, is deeply traumatic and, at worst, fatal. Astonishingly, in the UK, a diagnosis before age 10 years still results in between 14 and 18 life-years lost.[5] In LMICs, the same child would be unlikely to live past 25.[1]

Globally, it is estimated that approximately 167,000 people still lose their lives every year due to complications associated with T1D.[1]

1.4 | There is currently no cure, nor preventative treatment, for T1D

Recent technological advances in strategies to manage diabetes have significantly improved the lives of PLWT1D in the UK; however, research to identify preventative interventions or a functional cure for T1D is ongoing.

In the last decade it has become increasingly clear, including from studies in the pancreas, that heterogeneity exists in the pathognomic features of T1D.[6–8] Molecular differences in disease aetiology suggest at least two distinct phenotypes of disease.[9] These differences may underlie the limited success of previous immunotherapy trials[10]: For example, in 2009, a clinical trial administering rituximab (a potent anti-CD20 (B lymphocyte) monoclonal antibody) to 57 recently diagnosed individuals, reported a preservation of ß-cell function for over a year,[11] but stated that, in post hoc analysis, it was the youngest participants of the trial who benefitted the most. Soon after, it was reported that in the Protégé study (which examined the anti-CD3 (T-cell targeting) monoclonal antibody, Teplizumab), recently-diagnosed children aged between 8 and 11 years also retained more C-peptide than their older counterparts after 1 year.[12] Conversely, when recently-diagnosed individuals were treated with an anti-thymocyte immunoglobulin (ATG), those individuals who were described as being diagnosed 'older' were more likely to be responders (i.e. have beneficial outcomes).[13] Whilst *post hoc* analysis carries the risk that assumptions will be made based on underpowered populations, it is still tempting to hypothesise that with more targeted study populations, overall results would likely have been even more striking in such trials.

In the last year, the first-ever disease-modifying drug proven to delay the onset of T1D has been licenced in the United States. Teplizumab has now been shown to delay diagnosis by an average of 2 years in a cohort of trial participants who were identified as having a high risk of developing T1D and aged between 8 and 49 years.[14] The drugs' recent licencing is providing significant hope to the diabetes community that more therapies will arrive and that pharmaceutical companies will continue

Key Points

- Type 1 diabetes is first and foremost an autoimmune-mediated disease of the pancreas and imparts a huge burden on people living with the condition. However, T1D does not affect everyone equally.
- For those individuals not familiar with research focussing on the pancreas, this review will briefly touch on early pancreatic studies, more recent explorations of the organ, and outline some of the challenges faced by researchers aiming to study the initiation and progression of type 1 diabetes in its target organ.
- This essay will summarise one side of a debate about the existence of endotypes and offer a simple framework to explore the effect of age versus the potential of different modalities of pathology in the pancreatic environment.
- Finally, this piece will touch on new ways in which research is being expanded to characterise the molecular landscape of the pancreas in type 1 diabetes.

to invest in T1D. Tantalisingly, whilst there were some non-responders, a proportionally higher number of individuals remain diabetes-free in the treatment arm of the trial than in the placebo group. However, Teplizumab also carries significant risks of adverse effects, and because it is also prohibitively expensive,[15] it is therefore not widely available. Thus, it will be important to discover and target those who will most benefit from the drug.

Critically, if we are to implement immunotherapies (or, indeed, vaccines against confirmed triggers of autoimmunity) that aim to prevent clinical onset of T1D—it will be important to establish population-wide methods for early detection of individuals at high risk of T1D. This exciting endeavour is currently being explored in research in the UK[16] and elsewhere.[17]

However, what remains clear is that, despite these advances, we still do not fully understand the aetiopathology(s) of T1D in its target organ, the pancreas.

1.5 | Aims

This review will give a brief overview of historical and current histological work in the pancreas, aiming to understand the drivers of the disease and the impact of age in the context of the pancreatic environment and T1D development. The piece will outline some challenges faced in obtaining pancreatic samples to study the active pathology. It will also touch on how expanding our understanding of what is 'normal' in the human pancreas is essential for work aimed at finding the initial triggering factor or factors leading to T1D.

I will then close by celebrating the emergence of increased data sharing and propose a simple tool that may assist in the basic stratification of disease profiles. By aligning patient and phenotype data, the tool aims to separate features of T1D that purely align with age and development from those that specifically align with other T1D associated phenotypic traits, which may support more stratifiable classification of clinically relevant endotypes to assist trialists in identifying the right people for the right trial, and differentiate where age at diagnosis is an adjunct of a more or less aggressive endotype of the disease.

2 | DISCUSSION

2.1 | Type 1 diabetes, at its core, is a disease of the pancreas

Diabetes is diagnosed when people can no longer control their blood glucose. In the case of T1D, this is caused by insulin insufficiency due to catastrophic loss of (functional and actual) pancreatic ß-cells, associated with an autoimmune process which targets the islets of Langerhans and is primarily mediated by T lymphocytes. This immune process is called insulitis.[18]

Historically, it was believed that T1D only manifests when 90% of ß-cells are destroyed. However, we now know that some people retain significant ß-cell mass at diagnosis, despite requiring exogenous insulin provision.[19,20] In either case, ß-cell loss predates clinical manifestation, but we do not yet know when the destruction begins nor what initially triggers the process.

What is clear is that the pancreas plays a central role in the early pathogenesis of T1D, and studying the human organ at a molecular level is key to deciphering the etiopathology of T1D.[21] However, both the location and nature of the organ render the organ challenging to access for research (see Figure 1).[22–24]

2.2 | Global collaborations: Combining research outputs from pancreatic biobanks

Crucially, to build a coherent and robust understanding of the pancreas in T1D, we must study the disease across ages and disease stages, but given that access to the living, in situ, pancreas, in humans, is not possible, and biopsying the organ carries significant inherent risks (as described in Figure 1c) most research relies on cadaveric pancreas samples.

Biobanks containing such material exist, but samples are rare.[25] Of the roughly 800 examples of T1D-associated pancreases available globally, only around a quarter were collected close to diagnosis (which is important for studying the active pathology), and even fewer represent recent-onset T1D in children (See Figure 2).

The majority of pancreatic tissue from young people who sadly died before or not long after receiving a diagnosis of T1D is housed within the Exeter Archival Diabetes Biobank (EADB).[25] Professor Alan Foulis collated this (primarily autopsy) biobank from different hospitals across the United Kingdom over four decades ago. For many of the EADB donors, there was only one formalin-fixed paraffin-embedded block of pancreatic tissue derived at autopsy.

Thankfully, since then, diagnosis and diabetes treatment methods have been much improved, meaning that it is much rarer for individuals to pass away at onset. However, this also means that the EADB cohort remains a key resource for studying early-onset and recently diagnosed diabetes in the pancreas.

There are newer collections of pancreas, such as the JDRF network of Pancreatic Organ Donors with

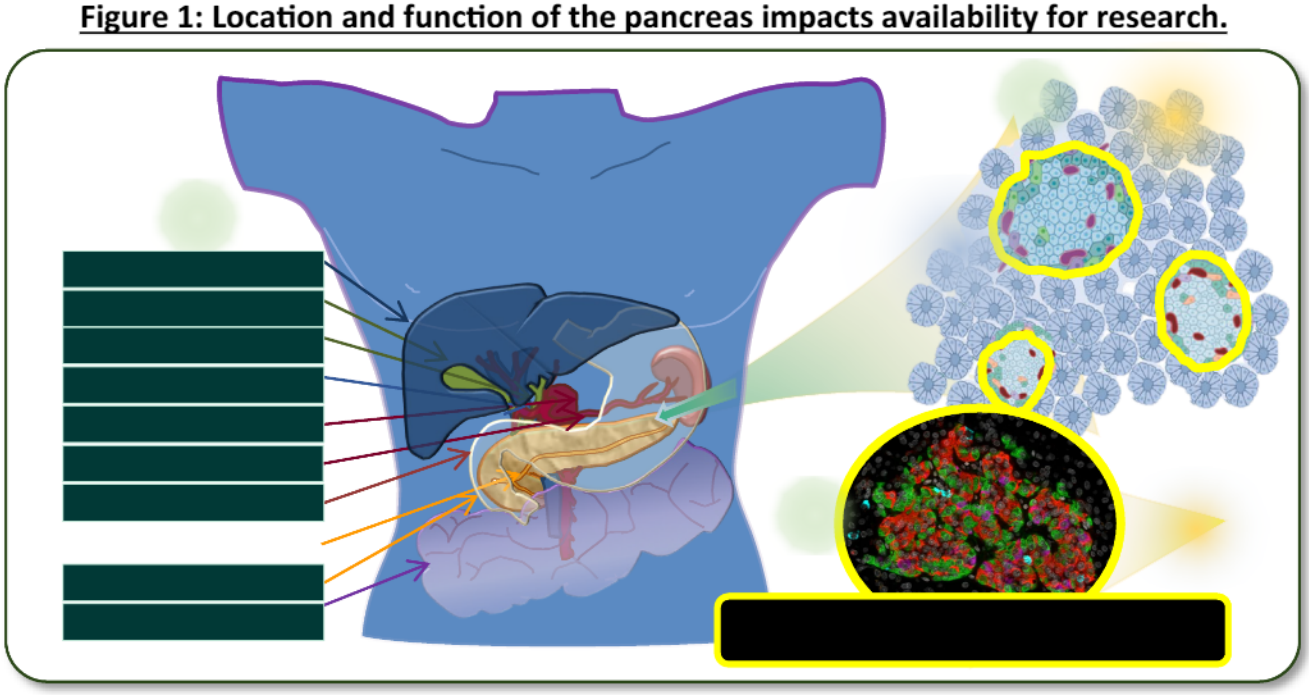


FIGURE 1 Location and function of the pancreas impacts availability for research. (a): The human pancreas accounts for approximately 1% of body weight in adults and is situated In the upper quadrants of the abdominal cavity, adjacent to the stomach, spine, spleen and duodenum, the latter encircles the pancreatic head, which is also in contact with the bile duct and rests on the inferior vena cava and renal vein. The body of the pancreas is contiguous with the aorta, supra-mesenteric artery, left kidney and renal blood vessels. (b): The pancreas is a heterocrine gland with both an endocrine (secreting hormones into the blood circulation) and exocrine function (secreting digestive substances into the pancreatic ductal system which drains directly into the duodenum). Ninety-eight per cent of the pancreas is acinar tissue which drains ~230 mL per day of digestive juice comprised of enzymes, including lipases, proteases and amylase into the upper duodenum. Frequent complications of pancreatic surgery include anastomotic leaks, postoperative fistulas, sepsis and intra-abdominal abscess formation, with postsurgical complication rates reaching 60%, and half of these are considered extremely serious. (c): The islets of Langerhans can contain five types of endocrine cells, and in healthy individuals, insulin-producing ß-cells dominate the islet area in most regions of the pancreas. Islet architecture is important for glucose-stimulated insulin secretion, and whilst the islet architecture within islets appears to be less organised in man vs rodents, it is apparent that alpha (glucagon positive) cells, and delta (somatostatin (SST)-positive) cells are more commonly situated in closer proximity to the islet borders. Islets also contain gamma (pancreatic polypeptide (PP)-positive) cells and epsilon (ghrelin-positive) cells, although PP-cells are more often located in the pancreatic head—which is embryologically distinct from the mid- and tail regions of the pancreas. Each hormone is thought to be involved in metabolic homeostasis in some way. Image: (a) Torso—copyright Pia Leete; (b) Expanded Acinar and islet diagram created with BioRender; (c) IF image: courtesy of Conor McMullen.

Diabetes (nPOD) and the Human Pancreas Analysis Program (HPAP), in the United States. Unlike EADB, they tend to contain a range of optimally preserved, disease-relevant material (including pancreas, serum and various other organs), and are collected from mostly brain-dead donors. The collection of these samples is facilitated by the American 'opt-out' organ donation programme, where the donors within the research biobanks were incompatible with transplantation programmes, and thus, instead, offer rich repositories of valuable research opportunities focused on T1D, T2D and other associated autoimmune pathologies.[26] The benefit of studies conducted within these collections is that the samples have been optimally collected and preserved and also have some lifestyle, familial, ethnic, genetic and epigenetic data which can be accessed and linked to any findings in the pancreas.

Another small adult cohort of T1D-affected pancreas biopsies has also been collected from living trial participants: A Nordic collection called the Detection of Virus in Diabetes Study (DiViD) strove to provide research-specific, optimally preserved tissue from the pancreatic tail of living individuals, both without and with recent-onset diabetes.[27] The original aim of the trialists was to study the potential of viral triggers for T1D. However, due to complications associated with surgery, the trial was curtailed after only six consenting adults were included. Despite the necessity of the early closure of this trial, the value of these six T1D samples cannot be overstated, generating 39 research articles to date; they continue to offer opportunities for a deeper understanding of tissue untraumatised by diabetic ketoacidosis or intensive care interventions.

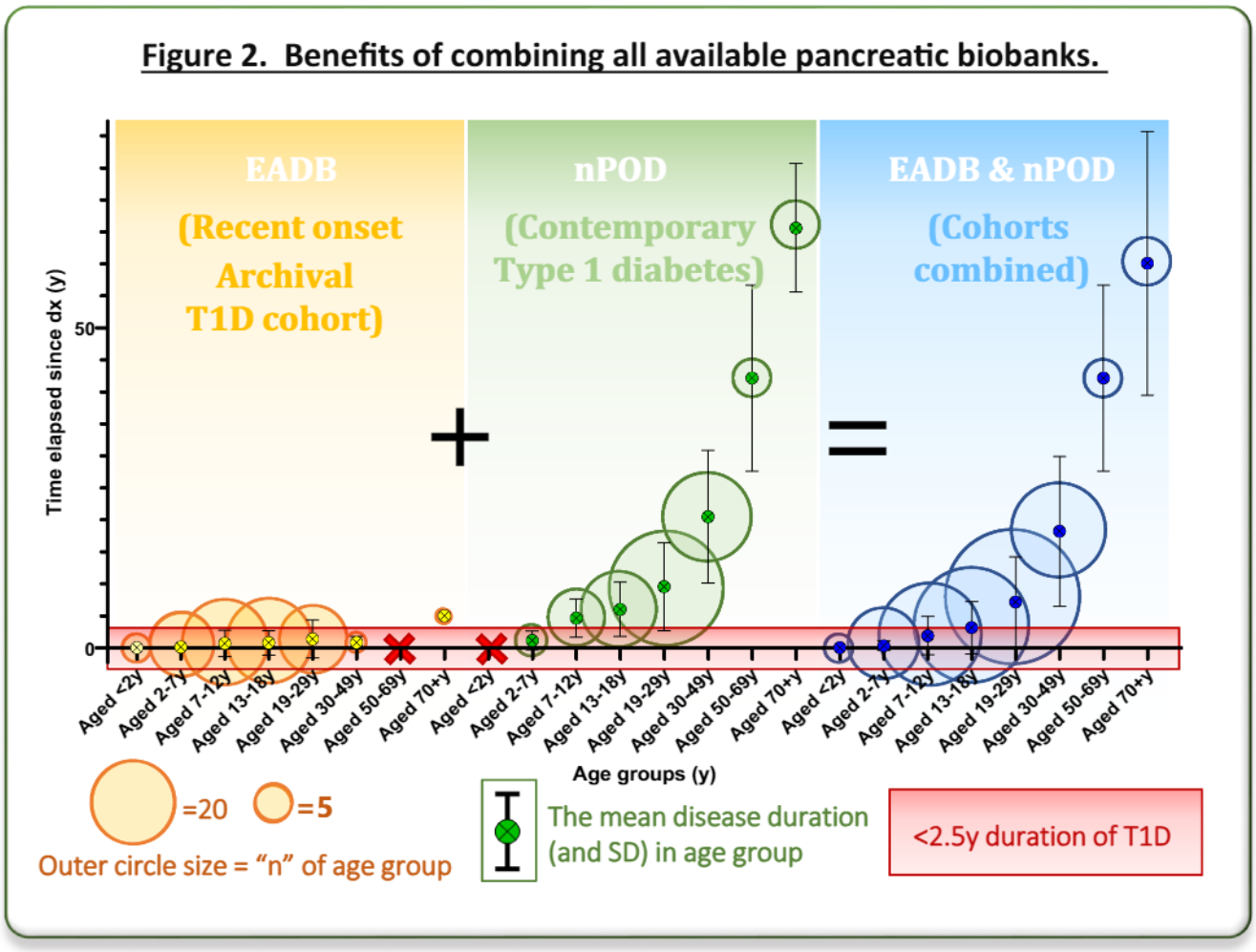


FIGURE 2 Benefits of combining all available pancreatic biobanks e.g. EADB and nPOD. Each global pancreatic biobank has strengths and shortcomings: For example, the EADB cohort contains over 70 pancreatic samples from individuals who had been diagnosed within 2 years, and nearly all of whom were <21 years of age, and of these, 35 were aged ≤10 years. However, with half of incident T1D occurring in individuals over 30 years, it is important to include a full spectrum of individuals in trials and investigations attempting to understand heterogeneity in the pathognomic features and aetiological processes of disease onset and progression. Conversely, the JDRF network of Pancreatic Organ Donors with Diabetes (nPOD) collection contains <20 equivalent samples in (US), of which only four cases which can teach us about early-onset (<10 years) disease. However, the nPOD (and other more recent biobanks) contain adult cases, most of which have longer-duration disease. As we now know, individuals who are diagnosed older are also more likely to retain ß-cells for longer (despite their need for insulin therapy), and therefore including these 'ß-cell positive' individuals in studies also allows pancreatic researchers to explore why these particular ß-cells survive, and why they are not secreting more insulin.

Individually, each cohort has strengths and weaknesses (reviewed here[25]). However, when combined, the joint biobanks offer opportunities to study T1D in the pancreas, in all age groups, and across a wide range of disease durations (e.g. Figure 2).

However, due in part to its inaccessibility and the tissue's rarity, the pancreas remains an 'enigmatic organ'. Although early studies explored the healthy landscape of the organ in man (see section 2.2 and Figure 3), much is still unknown about the molecular detail of the pancreas's developmental architecture and tissue maturation in healthy individuals. This 'framework of knowledge' is critical if we are ever to fully understand the point at which the resilience of normal function is overcome by pathology. Thus, as an important addition to the global pancreatic biobanks, the UK Quality in Organ Donation (QUOD) initiative is working with researchers to characterise the pancreas comprehensively. It now holds over 100 pancreas samples for research.[25]

2.3 | Early islet studies in the healthy pancreas

In 1869, Paul Langerhans famously identified "small cell" clusters with their own vasculature in a rabbit pancreas.

These micro-organs were named 'Islets of Langerhans' by Laguesse in 1893, who (among others) also linked them to carbohydrate metabolism. In 1901, Opie, a Scottish pathologist, first differentiated distinct pathologies in the cadaveric human pancreas, describing a 'pancreatitis of the exocrine compartment' (in which the islets remained untouched) versus an inflammation that was specific to diabetes, which conversely focussed on the islets and did not damage the exocrine.[28]

In 1933, another Scottish pathologist, named Ogilvy, examined 100 cadaveric human pancreases, including babies aged from 3 weeks premature, up to an adult of 62 years of age[29] (methods and data more fully summarised in Figure 3). This comprehensive study attempted to cover a whole lifespan since, in 1933 life expectancy did not often exceed 65 years.[30] Ogilvy compared body weight to pancreatic weight (Figure 3a,b), where the former is starkly different to our modern cohorts (Figure 3c,d). He also developed a method to characterise the relative weight, size and abundance of islets within a whole pancreas (Figure 3e–g). Ogilvy believed that members of his cohort were unlikely to have diabetes as he clearly stated that none were 'undernourished' (despite having a mean adult body weight of ~49 kg). This work may ultimately serve as a backdrop to our current endeavours to understand the continuing increases in T1D incidence.

This detailed early work revealed that during childhood, adolescence and throughout adulthood, changes in pancreatic mass and islet size roughly correlate with body weight, peaking at approximately 20 years of age (Figure 3), whilst islet numbers expand much more rapidly, reaching their maxima in early childhood. Ogilvy's work also accurately revealed that islets are smaller and more numerous in the earliest phases of life.

Notably, the 1933 cohort was from the post-World War 1 era, and the individuals included in the study were significantly lighter than most adults today (mean adult weight in the 1933 cohort vs the nPOD cohort is approximately 49 kg vs. 76 kg respectively) (Figure 3a,b). However, whilst the current obesity epidemic in Western societies potentially contributes to the increased mean cohort weight, people are also, on average, 10 cm taller now than a century ago, likely due to improved nutrition. It is interesting to note that despite a significant increase in overall pancreas size ($p < 0.005$) when taken as a proportion of mean body weight, the pancreas today may not be keeping pace with the increase in body mass in modern society (Figure 3c,d).

Ogilvy's work also reveals massive developmental changes to the pancreatic landscape during the earliest years of life (0-6 years), which we know is a time to be important in the development of T1D *(see below)*. Understanding how these changes might relate to the developing immune system, the onset of diabetes, and the initiation of autoimmunity may offer insights into why an early onset of diabetes carries such profound consequences[31] and whether, if onset can be delayed beyond a certain point, it might become possible to divert the disease trajectory towards a less aggressive ß-cell loss.

2.4 | The Islets of Langerhans in focus

The human pancreas contains between one and 3 million islets which are stochastically distributed throughout the acinar tissue and can now be studied histologically using a variety of techniques from single-plex and multiplex standard brightfield to multiplex fluorescence confocal microscopy (Figure 4a,b), to barcoded Hiplex RNA and DNA scope technologies which can visualise over 1000 targets per section and each can be analysed in the spatial context (see section 5).

Islets are complex micro-organs comprised of five endocrine cell types, which differ in proportions depending on age and the location of the islet within the gland,[32] with the most obvious difference being the abundance of pancreatic polypeptide cells in the head of the organ.[33] A higher proportion of delta cells are also present in the islet of infants (Figure 4b,c).[32] In the adult pancreas, the majority of islet mass is made up of islets that are approximately 100-200 µm in diameter (meaning roughly 10 might fit on the tip of a pin-head), which is thought to be an optimal size for islets to function most effectively (A comprehensive review of islet research can be found here: Ref. 34).

A protective basement membrane encases healthy islets, shielding them from immunological (and presumably chemical) assault. Islets are highly innervated and vascularised and seemingly respond independently of each other in a staggered response to fluctuating blood glucose levels.

This phased response concept is supported by research in a rodent model that revealed a niche of so-called 'sleeping' islets maintained in a hypoxic, quiescent state under normoglycemic conditions.[35] A reduction in functioning islets was then created by surgically removing half of the experimental animal's pancreas, thereby reducing total islet numbers, and the remaining active islets could no longer maintain glucose within a tolerable range. At this time, the 'sleeping islets' awoke, returning to an active state and becoming glucose-responsive. To understand whether the process was reversible, the rodent received a second pancreas via transplantation, giving it a surplus of islets. This additional capacity led

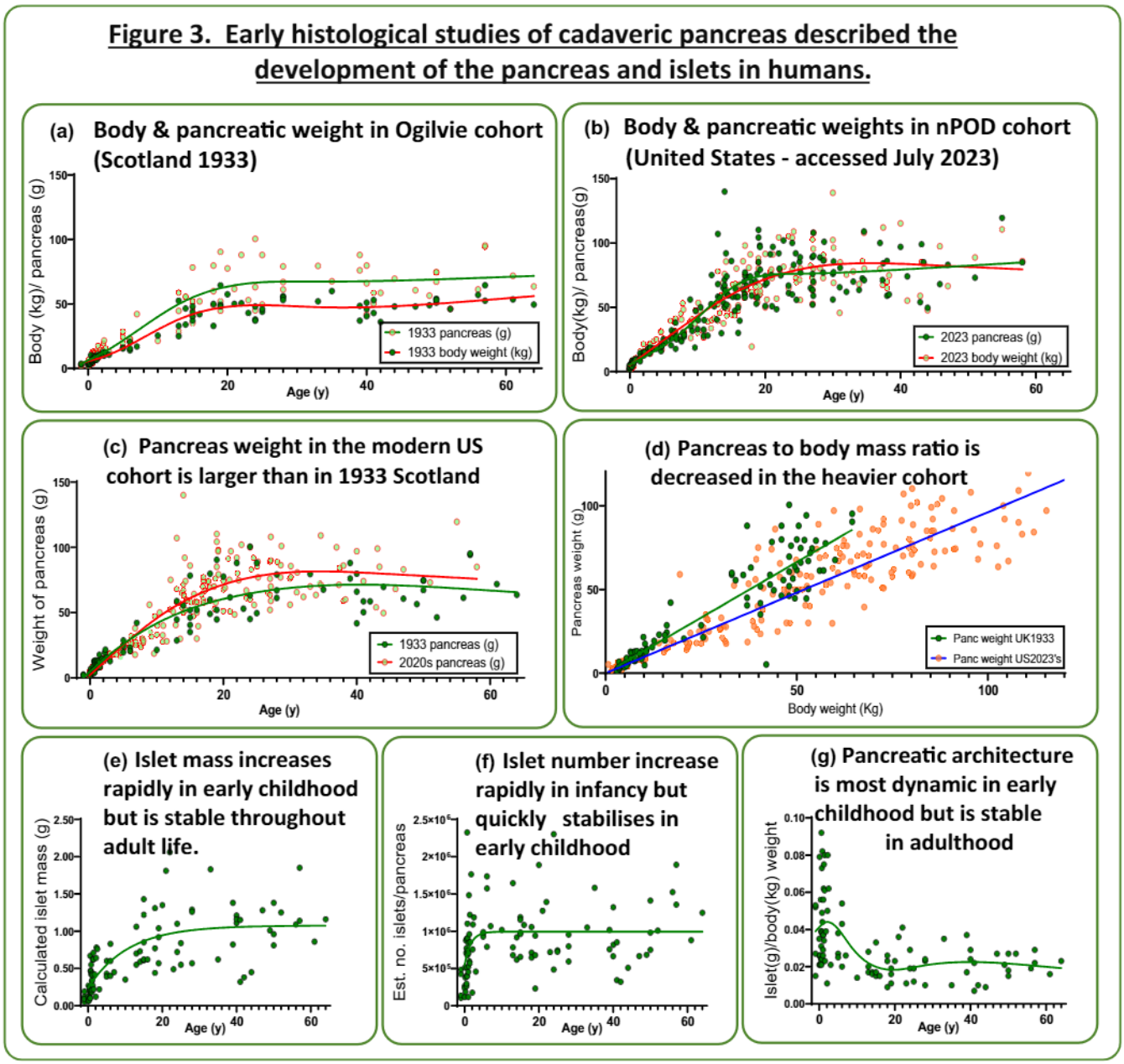


FIGURE 3 Early histological studies of human cadaveric pancreas accurately describe morphology and basic features of development and the mature organ. (a–g) In 1933, Ogilvy captured body weight, pancreatic weight, and age and sex of 100 deceased individuals at various ages between -3 weeks and 62 years. Using an azan stain he examined sections of the formalin-fixed paraffin-embedded pancreas taken from the head body and tail of the organ. This dye stains only the exocrine compartment, leaving the islet transparent, allowing Opie to turn his light microscope into a projector. The apparatus threw shadows of the exocrine onto cartridge paper. Cutting out the shapes of islets created by his projections, Ogilvy then quantified 45 fields of view taken from each organ's head, middle and tail, calculating islet mass, average islet diameter, weight, total islet numbers and islet/exocrine ratios. These studies revealed that absolute pancreatic and body weight develop in parallel, and pancreatic growth plateaus and stabilises at approximately 20 years (a in a 1933 Scottish cohort; & b confirmed in nPOD samples). Although the nPOD cohort is heavier, and (c) pancreas size is significantly larger in nPOD cohort cases (Kolmogorov–Smirnov test $p < 0.005$), proportionally, there is less pancreas per kg body weight (d). The cause and impact of these changes are yet to be determined but are likely to involve better available nutrition and healthcare vs. >100 years ago. Ogilvy also demonstrated that whilst the absolute mass of the pancreas grew in tandem with the mass of the growing child, islet mass develops fastest in childhood (e); that there is a massive expansion of islet numbers in infancy, which is complete by approximately 2 years of age (f), and the proportion of islet mass to body weight is 4-fold higher in infancy (g). The implications of this rapid islet expansion warrant further study using advanced techniques to examine molecular, RNA and DNA signatures to characterise what (if any) impacts T1D susceptibility.

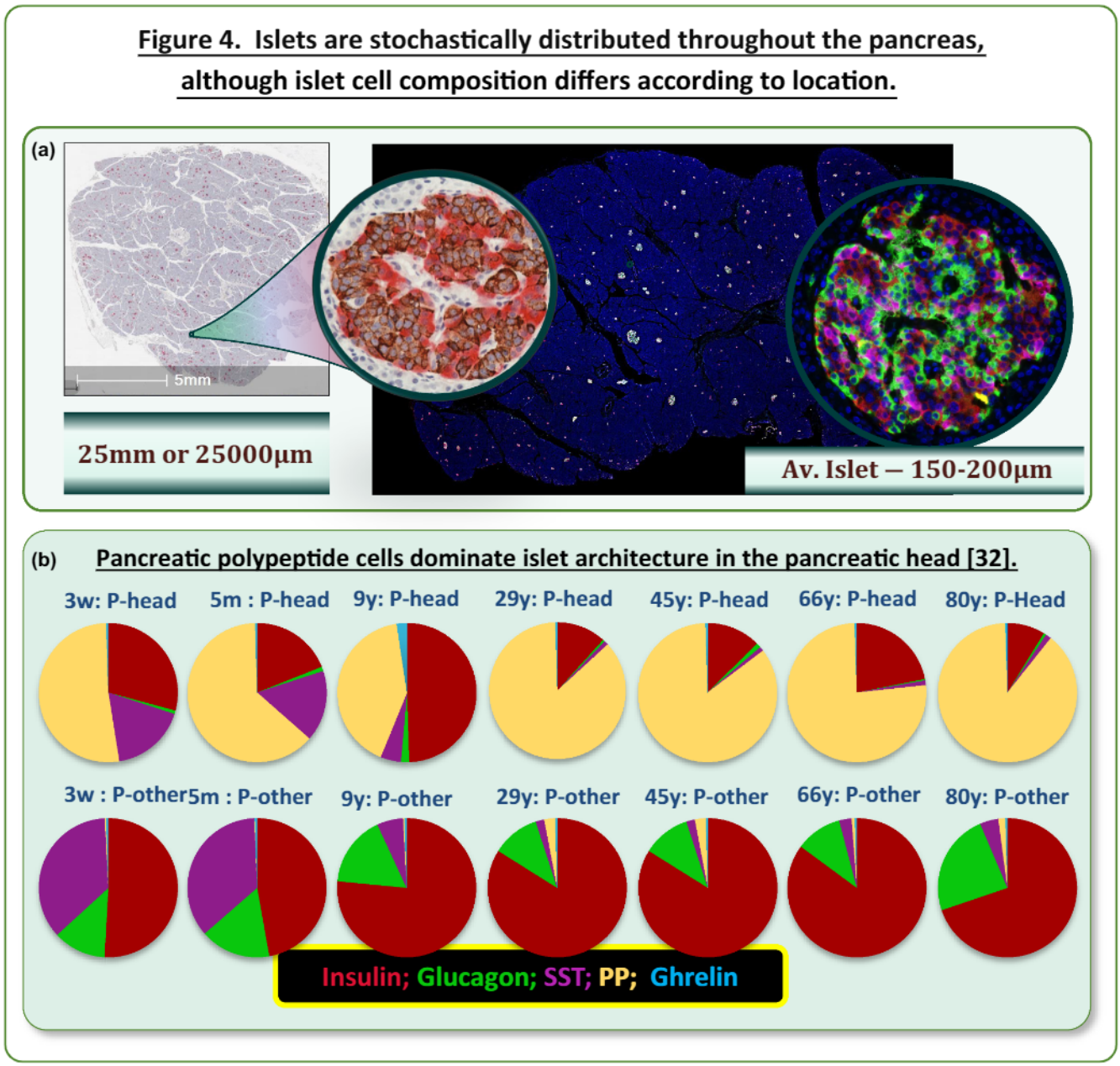


FIGURE 4 Islets are stochastically distributed throughout the pancreas, although islet cell composition differs between the head (or uncinate region) and the remainder of the organ (a). Pancreatic polypeptide cells dominate islet architecture in the pancreatic head (b).[32] Islet architecture is important for function: In the body and tail of the pancreas, the insulin-producing ß-cells represent the largest cell fraction at around ~60% of islet mass in man (80% in rodents). Alpha-cells, producing insulins' counterregulatory hormone, glucagon, account for ~30% of islet mass, but are rarer in the pancreatic head. Delta cells act to inhibit both alpha and ß-cells, ameliorating any extreme glucose-stimulated responses, and make up a further 10% of islet mass. Both alpha and delta cells are increasingly understood to be important for understanding pancreatic pathology in T1D. The most striking difference in cell type variability across the adult pancreas is the pancreatic polypeptide-positive gamma cells, which predominate in the pancreatic head but are exceptionally rare in the body and tail. Ghrelin-positive epsilon cells are the rarest of the cell types overall.

some of the islets (in the native and transplanted pancreases) to sink back into a dormant state. Further work to understand what differentiates these subgroups of islets is ongoing.

The massive number of islets and the idea that some may be 'spare', coupled with how extensive the loss of ß-cells can be when some people are first diagnosed with T1D, suggests a certain degree of redundancy within the system. It is tantalising to imagine that such dormant islets may be the residual islets which appear to be maintained in some individuals with T1D, potentially hidden from the worst ravages of the immune attack. If we can find such 'sleeping' islets in humans, it might offer insight as to why some individuals retain

ß-cell mass and yet require exogenous insulin provision. They may even offer an opportunity for a functional cure for T1D in some people.

3 | INSULITIS—THE ISLET LESION IN T1D

Although Opie first described an islet-associated inflammatory state in 1901, and Oglivy built on from his first study in 1933 to explore diabetes in the pancreas, studying a further selection of 60 samples in 1955, it was Willy Gepts, in 1965, who first attempted to characterise 'insulitis', the specific inflammatory lesion associated with diabetes.[36] Gepts noted that insulitis was a rare lesion that was easier to find in pancreatic samples from children and thus explicitly sought out those samples to study. He did not propose, however, that this observation might suggest distinct etiopathology between age groups. More extensive studies followed, also employing the most inflamed (youngest) cases, and these more clearly defined the insulitic lesion as having a preponderance of lymphocytes vs macrophages (e.g.[37]).

3.1 | Immunotypes in T1D

In 2011, by staining the tissue for insulin and undertaking morphometric analysis of the relative insulin-to-islet area, Willcox et al. devised a method which utilised the percentage of insulin-positive area per islet as a proxy for the stage of ß-cell loss from each islet (See graphical representation in Figure 5).[38] By staging the islets in order of insulin positivity and plotting the prevalence of a series of immune cell subsets in relation to these 'gated' islets, a model was created which described the dynamic sequence of events associated with ß-cell demise in the islet for the first time. This model suggested an ongoing infiltration of CD8+ T cells and CD20+B lymphocyte numbers throughout the phase of ß-cell destruction, numbers peaking just before total ß-cell loss from the islet. Although present, macrophages (CD68+) and CD4+T cells remained largely stable in numbers throughout the stages of ß-cell loss. In keeping with the notion that autoimmune cells target ß-cells, the model also revealed that the immune cells migrated elsewhere upon complete ß-cell loss from the islet. Willcox's study also showed the unequivocal involvement of B lymphocytes in the islet milieu in humans. This seminal work has since been corroborated using imaging mass spectrometry in the nPOD cohort.[39]

In 2016, to characterise insulitis further and expand in the more modern and geographically diverse cohorts, the insulitis in pancreas samples from nPOD and DiViD cohorts were then studied.[20] Using these samples, and re-analysing the older datasets, insulitis, ß-cell loss, age at diagnosis and disease duration in each individual were examined separately, unexpectedly revealing a second profile of inflammation.

Whilst several individuals, as expected, showed the same profile as had previously been described in the Willcox study (Figure 6; panels a vs. b), the second profile, possibly masked by the high numbers of immune cells in the other profile, showed only sporadic islet infiltration, and in those few islets that were inflamed, the inflammation was considerably less florid. No single islet contained more than 3 B-lymphocytes per lesion, leading to this immune phenotype being classified as CD20Lo (vs CD20Hi for the more B lymphocyte enriched profile described by Willcox et al.) (Figure 5; panel c).

Importantly, when the CD20Lo cohort was further examined, and even though these individuals had an absolute requirement for exogenous insulin provision, they retained surprisingly high numbers of insulin-containing islets at diagnosis (Figure 6e,f (i)) and tended to have been diagnosed beyond their teens (Figure 5; panel d). Conversely, all individuals diagnosed <7 years exhibited a CD20Hi profile, most of whom retained less than 10% of their expected ß-cell mass.[20]

Critically, all those in the tween-aged group (diagnosed between 7 and 12 years) exhibited one or other of the two immunotypes (Figure 6d, blue box: red = CD20Hi, green CD20Lo). It has since been shown that individuals with CD20Lo profile are more likely to retain insulin-positive islets at diagnosis (in higher numbers and for several years longer) than their CD20Hi age-group peers (Figure 5; panel f (i)&(ii)).[40] Long-enduring ß-cells in those diagnosed older have also been described in the Joslin Medallist study—where some individuals (diagnosed older), who lived for 50 years (or more) with T1D,[41] still retained insulin-positive ß-cells alive in their islets.

The higher-than-expected ß-cell mass in the CD20Lo-immunotype and yet the requirement for exogenous insulin provision may suggest some ß-cell dysfunction or dysregulation occurs in these individuals.

3.2 | Immunotype-associated patterns of proinsulin processing in T1D

Further studies of the EADB and nPOD cohorts revealed additional ß-cell-specific differences between individuals, specifically in the intracellular distribution of proinsulin (PI) and mature insulin (I).[8] In preliminary studies, a substantial number of islets appeared to exhibit high rates of aberrant PI:I colocalisation, hinting that proinsulin had

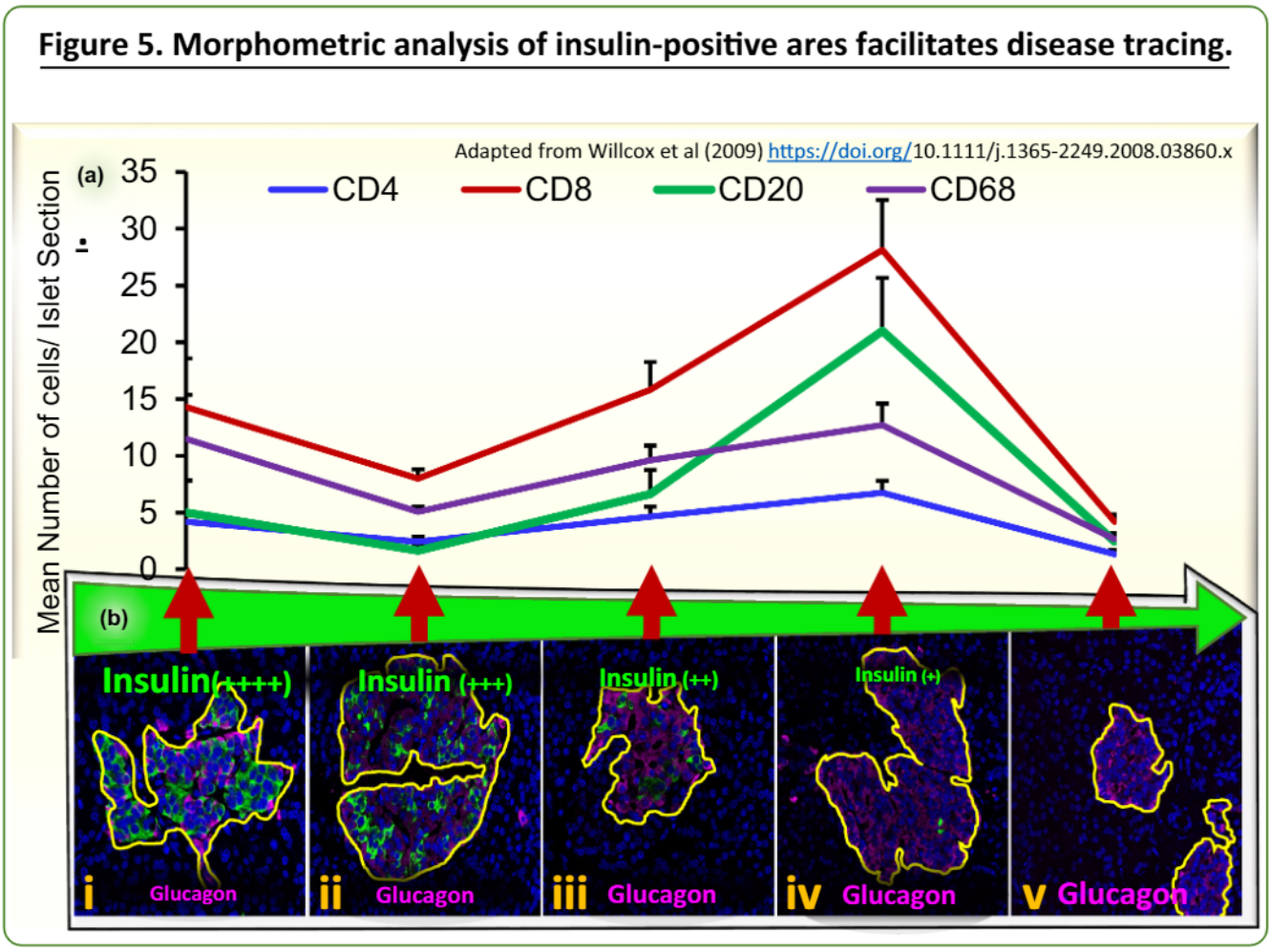


FIGURE 5 Morphometric analysis of insulin-positive areas facilitates disease progression tracing. (a): Four-micrometre serial sections of pancreatic tissue, derived from 29 recently diagnosed individuals, were originally stained using standard HRP protocols. Validated antisera were used to visualise cells positive for insulin (to detect ß-cells), CD4 (T-helper cells), CD8 (Cytotoxic T-cells), CD20 (B-lymphocytes) and CD68 (macrophages), and immune cells were considered part of the insulitic lesion if they were contiguous with the islet membrane, either directly or indirectly via other immune cells present within the lesion). Islets were then scored according to the relative area of insulin and immune cell numbers calculated. (b): Insulitis studies have also now progressed to fluorescent imaging allowing for multiple antigens per section. Disease progression, however, is still estimated in a similar manner and islets are binned according to insulin positivity and the mean number of each immune cell subtype (or protein expression profile) in each bin (y-axis) is calculated and plotted along the x-axis (red arrows). In this model, it is assumed that progressively more ß-cells are lost over time as the disease progresses, (i) An islet replete with insulin and few immune cells (ii) insulin: glucagon ratio is diminished (iii) few ß-cells remain, and alpha-cells dominate the islets (iv) even with evidence of a single insulin-positive ß-cell, the islet remains under attack. However, when ß-cell destruction is complete (v) immune populations dissipate. (NB: It is important to note that 'pseudotime' cannot describe units of time (such as minutes and hours) but instead serves as a tool to map sequential occurrences and disease trajectory).

not been correctly processed prior to being trafficked to the ß-cells membrane, possibly aligning with reports of aberrantly secreted proinsulin in T1D.[42]

To further examine this observation, 32 pancreas samples from individuals of various ages and disease statuses were examined (Figure 8), and PI:I colocalisation rates were quantitatively assessed using the Manders Overlap Coefficient (MOC). (MOC is described in Figure 7).[8]

In healthy islets, insulin and proinsulin usually inhabit separate but adjacent areas of the ß-cell (Figure 7a), rarely appearing to colocalise (Figure 7b), where less than 1% of non-diabetes affected islets show high levels of MOC (Figure 7c) (unpublished).

However, significant variability in MOC values occurred within and between individuals affected by T1D (Figure 8a).

After dissecting the data, it was clear that in every individual with a CD20Hi-immunotype (whether diagnosed at <7 years or between 7 and 12 years), the ß-cells within islets appeared to show high rates of PI:I colocalisation (Figure 8b); whilst islets in every CD20Lo-affected individual, only a minority subset of islets exhibited a > 0.5

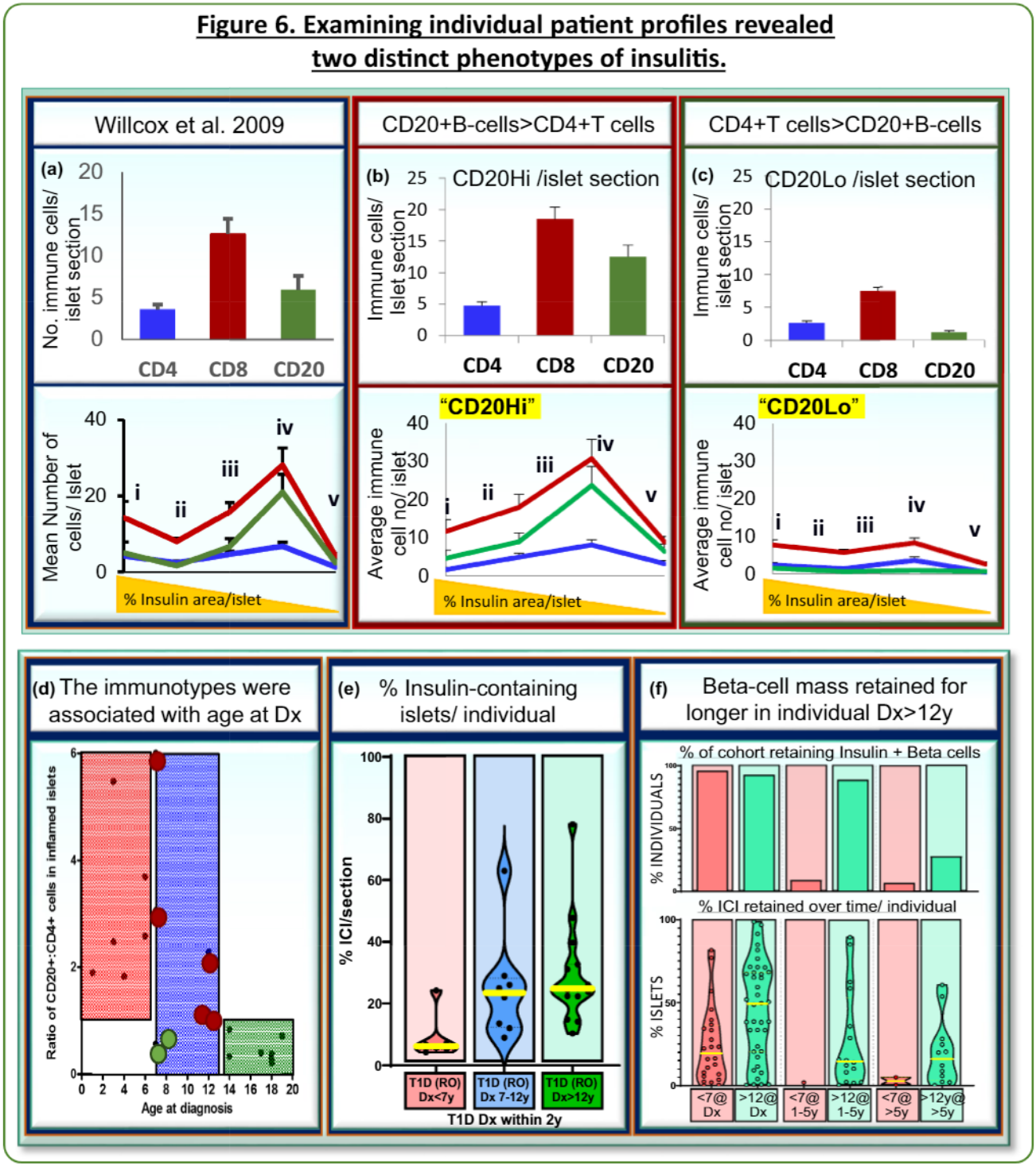


FIGURE 6 Examining individual patient profiles revealed two distinct phenotypes of insulitis. Examining the data from each individual in EADB insulitis cohort (a) revealed that some individuals (b) exhibited such high levels of inflammation, that they masked others who (despite islet-ß-cell loss) exhibited a much lower level of inflammation, with almost no B lymphocyte involvement (c). To examine this further, comparable studies in the nPOD and DiViD cohorts were undertaken and combined with EADB data, revealing that the profiles were consistently associated with age at diagnosis (d–f: red colour box's and dots represent individuals with high levels of inflammation and high number of B-lymphocytes, vs the green box and dots which represent the low CD20 profile, with reduced levels of inflammation) and degree of ß-cell destruction at diagnosis (in e). All individuals <7y at diagnosis (<7y Dx) had high numbers of CD20+ cells and a more hyper-inflamed profile (CD20Hi) (d), and although the majority still retained some insulin positivity at diagnosis (fi), levels were significantly lower (fii) than in those Dx >12y. NB. The more 'hyper-immune' CD20Hi-insulitis is observed consistently in those diagnosed under the age of 7 years, and whilst many of the CD20Hi-population in these studies were derived from EADB, the pancreas of two similar children in nPOD were concordant with EADB-CD20Hi hyper-immune profile and massive loss of ß-cells.

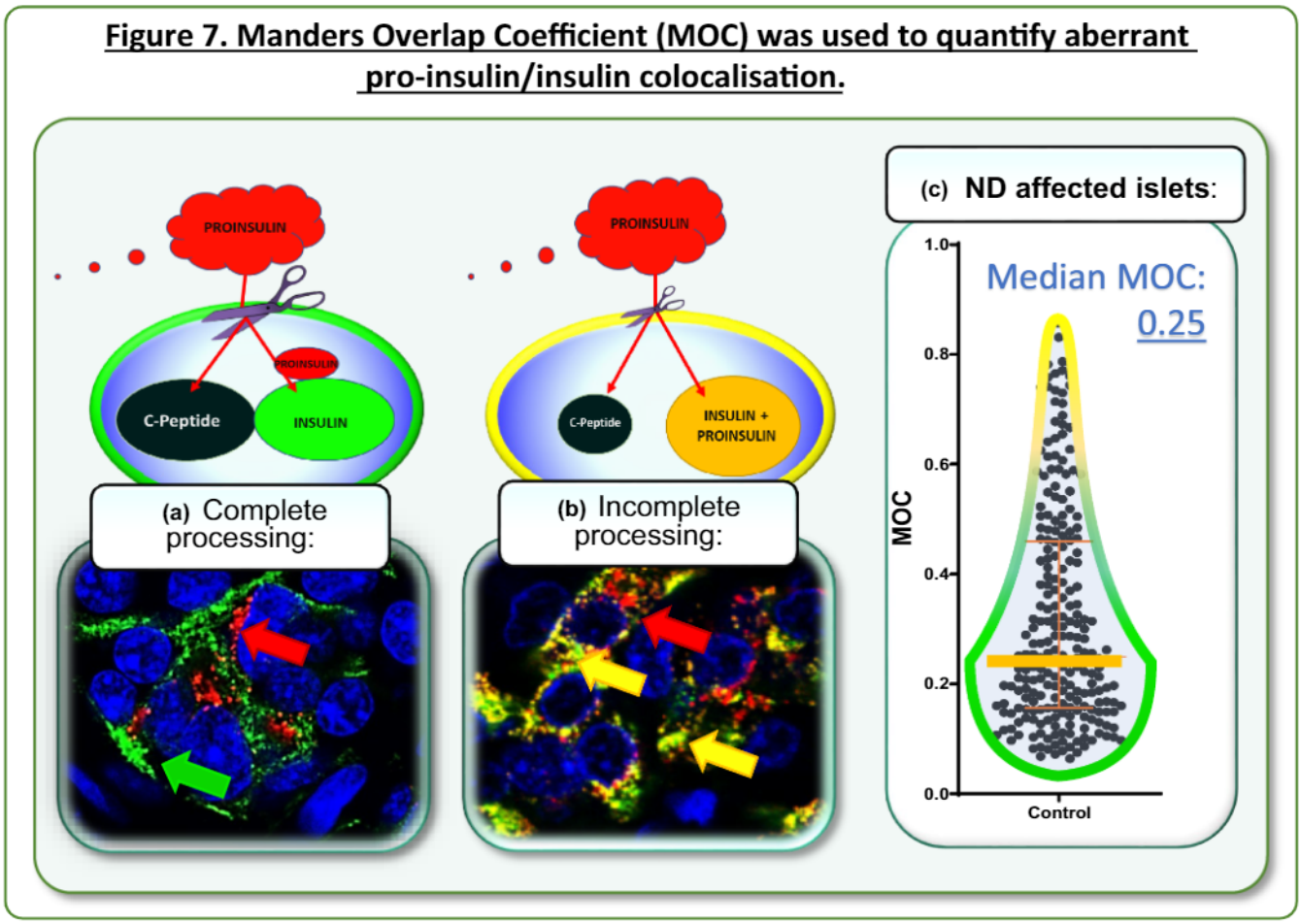
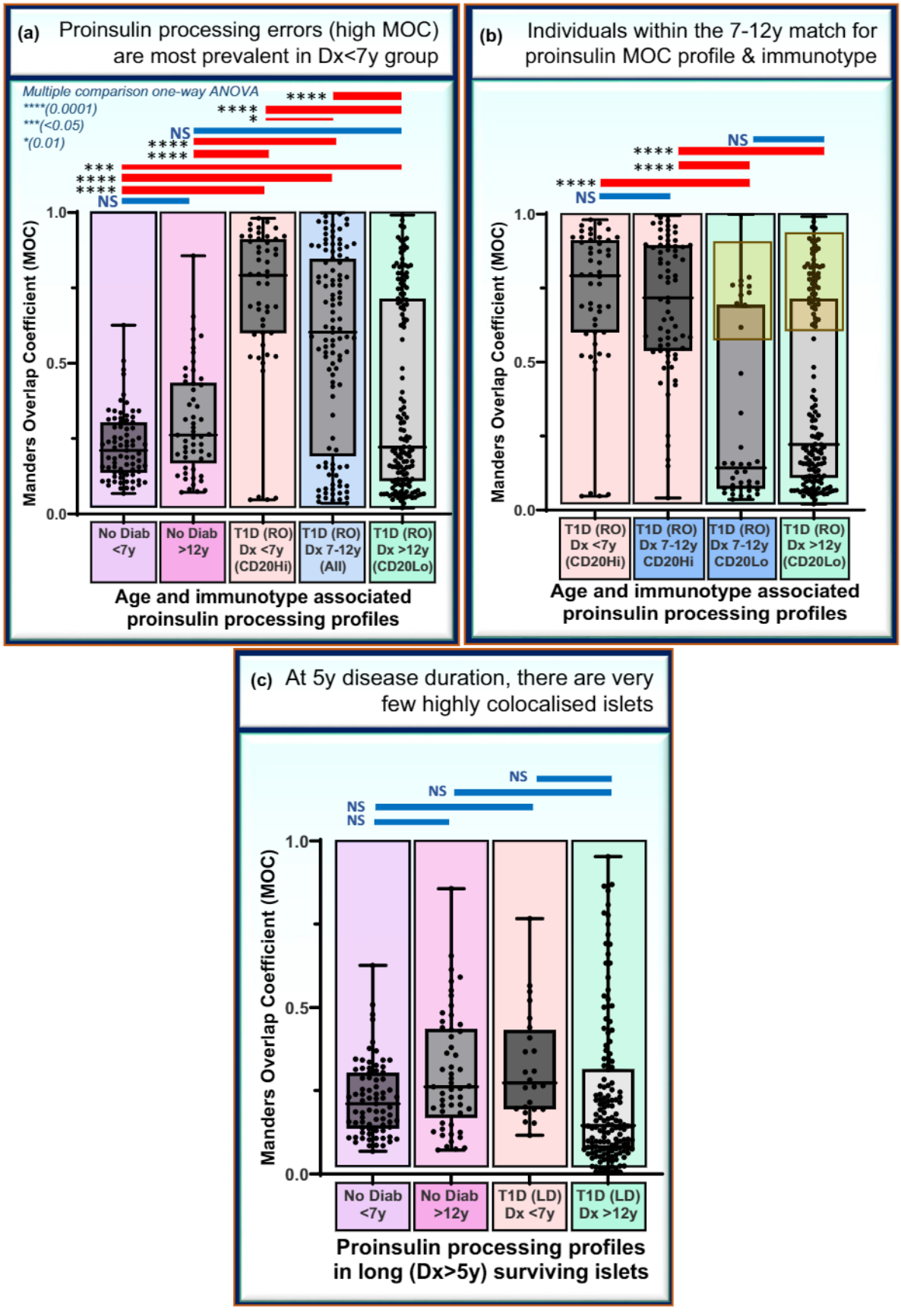


FIGURE 7 Manders Overlap Coefficient (MOC) was used to measure the overlap (or colocalisation) of signal in ß-cells. When dual staining is undertaken, positive staining for insulin (green) and proinsulin (red) should inhabit separate areas of the cell (Figure 6; panel a). (a): In healthy ß-cells, preproinsulin is co-translationally translocated directly into the endoplasmic reticulum (ER) in the perinuclear region, where proinsulin is cleaved from the signal peptide and folded within the Golgi apparatus (stained red in a & b) (red arrow in micrograph). Proinsulin is then packaged into secretory granules. In the majority of ß-cells in non-diabetes-affected individuals, proinsulin is then rapidly cleaved by endopeptidases into equimolar parts of mature insulin (green bubble of the infographic and stained for in green in both a & b) and c-peptide (black bubble; although C-peptide is not stained in the micrographs). The healthy, mature granule (green) is then trafficked towards the ß-cell membrane, where, at this point, there is very little proinsulin remaining in the granule. It docks (green arrow) near the membrane until such a time that glucose signalling dictates that the granule be exocytosed. (b): When proinsulin and insulin co-exist in the same space (b), however, the co-staining appears as the third colour (yellow arrow). Image analysis techniques allow this overlap to be numerically quantified using correlation coefficients such as the Manders overlap coefficient (MOC). (c): The MOC describes the probability that one positive signal (in this case, proinsulin) will overlap and colocalise with another in the same space (i.e. mature insulin). Thus, a low degree of proinsulin:insulin overlap suggests more complete proinsulin processing, whilst a high MOC indicates improper cleavage and suggests proinsulin processing errors. MOC values remain low in the majority of islets in non-diabetes-affected individuals, confirming the probability that the majority of proinsulin is cleaved to mature insulin.

FIGURE 8 Variations in proinsulin:insulin colocalisation profiles reveal age- and immunotype-associated variation in proinsulin processing patterns in T1D. (a): Each data point represents an MOC value of a single islet. Individuals diagnosed <7y (CD20Hi) and with short-duration disease showed consistently high islet colocalisation profiles, with a median MOC of 0.8 (IQR [0.6, 0.9]) vs 0.2[0.1, 0.3] in matched donors without diabetes (Figure 7a). Each of those diagnosed >12y (CD20Lo) showed a bimodal distribution predominated by low colocalising islets (median MOC value 0.2 IQR [0.1/0.9]), likely mediated by the predominance of low MOC islets, versus age-matched>12y group, this did not reach significance. The data from the whole 7-12y-at-diagnosis group revealed an intermediate median MOC (but similar MOC IQR vs the >12y group) (Median MOC 0.6 (IQR [0.2, 0.9])). (b): When the 7-12y group was split by previously determined immunotype profiles, there were no statistically significant differences between the <7y group and the 7-12y-CD20Hi individuals; nor the >12y and the 7-12y-CD20Lo individuals. Suggesting co-occurring phenotypes. (c): After a longer duration of T1D (>5y), no evidence of significant numbers of highly colocalised islets could be found.

Figure 8. Variations in proinsulin:insulin colocalisation profiles reveal age- and immunotype-associated vairation in proinsulin processing patterns in T1D.



MOC for proinsulin:insulin (PI:I), and the majority (~72%) of islets showed very low rates of colocalisation.[8]

Interestingly, 93% of all insulin-containing islets that survive into longer-duration disease have <0.5MOC (unpublished) (Figure 8c); further work to understand this is ongoing.

4 | INTRODUCING ENDOTYPES

If the CD20 and proinsulin processing profiles where independent of each other (albeit heterogeneous age- associated features of T1D), and each was a distinct feature of their biological niche (ie the immune vs endocrine systems) (Figure 9a,b), then one might expect that the "tween-age" group (7-12y at Dx), containing a mixture of immunotypes and examples of both patterns of proinsulin processing might have some individuals with CD20Hi-immunotypes and the majoirty of ilsets expressing low MOC values, and vice versa (Figure 6d; blue box).

However, the strict clustering of these two profiles (where individuals either had one or other combination of 'CD20Hi+ PI: processing error' OR 'CD20Lo+"quiescent islets"') was suggestive of each individual in the study cohort having a version of one of the two distinct co-occurring patterns associated with their T1D (Figure 9C), highlighting the possibility that stratifiable and distinct pathognomic forms of T1D exist in this group of individuals.

Given that it appears that these distinct pancreatic pathologies were so clear cut, the terms T1DE1 (or T1D Endotype 1 (CD20Hi, early-onset, with near ubiquitous evidence of proinsulin processing dysfunction, plus rapid loss of ß-cells after onset)) and T1DE2 (or T1D Endotype 2 (CD20Lo immunotype with later onset, no compelling evidence of proinsulin processing dysregulation, and who retain significant volumes of (potentially quiescent) ß-cell mass)) were adopted (See Figure 9)[8] and the increasing body of evidence to support their existence have recently been comprehensively discussed.[9]

4.1 | The knotty subject of nomenclature

Overall, there is consensus that T1D is heterogeneous, that early age at diagnosis carries a greater disease burden, and that an increasing number of co-lateral distinguishing traits,[9] observable in groups diagnosed at different ages, are emerging.

However, there are concerns that we may be overstating our successes and that the term 'endotype' may be used to subdivide the patient population into ever more complicated subgroups of ever-decreasing size, which may ultimately hamper robust research and deter investment from pharmaceutical companies wishing to avoid fragmented trials.

Caution may be warranted, but this goes both ways. If stratifiable distinct and targetable differences do exist in T1D, then being afraid to study and communicate them decisively may also hamper progress.

Luckily, we stand at a time where emerging technological advances are supporting new ways to explore data, and as we increase our capacity to stratify patients according to distinct molecular, aetiopathological and clinical traits, we are moving ever closer to effective personalised medicine in T1D.

5 | A PROPOSED PIPELINE TO DISTINGUISH THE EFFECTS OF AGE FROM AETIOLOGICAL ENDOTYPE

To address the concerns that research may become increasingly fragmented amongst the near-universal push in the T1D research community to address and categorise the heterogeneity in T1D, it becomes important to build a strategy to stratify meaningful unified methods for endophenotypic delineation of the cohorts.

Over the last few years, I have worked to understand the striking sets of disease-associated patterns that are observable in the pancreas[6,8,31,43,44] and to develop methods to categorise these observations into a meaningful class of either stochastic and isolated observations, or a class of clustered pathognomonic features in T1D (e.g. Endotype 1 and 2). However, whether the pancreatic profiles are called endotype or phenotype is almost immaterial; what is useful is developing a clear, stratified approach to classifying the features of pancreatic heterogeneity so that we can offer clear information and messaging.

The development of my work examining immunotypes in T1D has been possible due to the accessibility of the EADB, nPOD and DiViD cohorts which permitted a wholistic approach to studying examples of the pancreas that (as closely as currently possible) represent people living with T1D, diagnosed at different ages, and having experienced different durations of disease.

Additionally, access to a range of data in the pancreas has provided the opportunity for layering multiple features of the pancreatic histology into a series of interrogations which have facilitated the development of a pipeline (described in Figure 10). This tool helps to test for the occurrence of unrelated features vs co-occurrence of purely age-associated features vs clusters of features that might be suggestive of (ultimately) clinically relevant endotypes.

Thus, if we generate a new finding associated with T1D in the pancreas in my laboratory, our group follows the pipeline in Figure 10 from a–e.

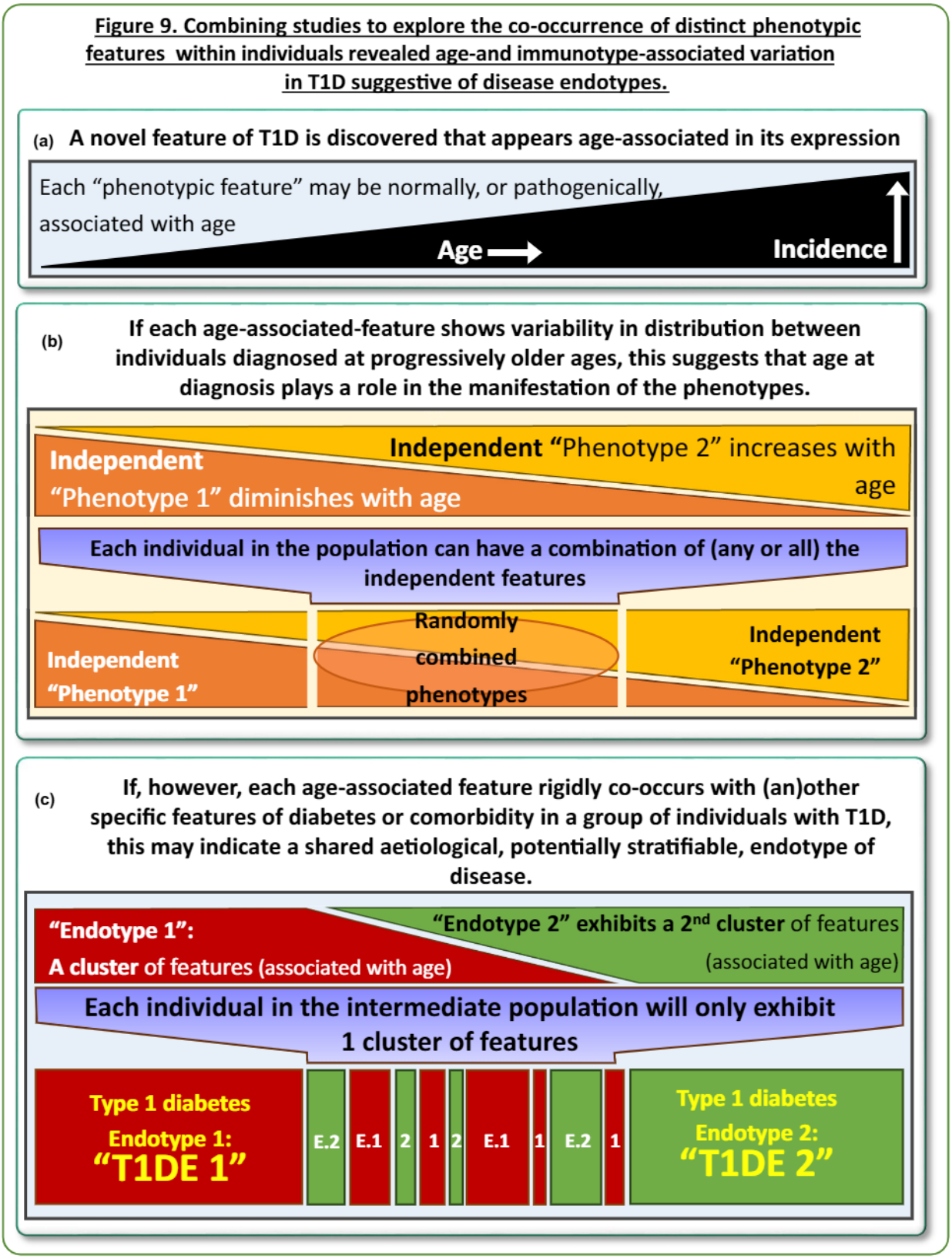


FIGURE 9 Combining studies to explore the co-occurrence of distinct phenotypic features revealed age- and immunotype-associated variation in T1D suggestive of disease endotypes. (a). The black triangle represents a given feature of T1D that is discovered and is distributed incrementally in an age-associated pattern. (b). If each age-associated feature shows variability in distribution between individuals diagnosed at progressively older ages, this suggests that age at diagnosis plays a role in the manifestation of the phenotypes. (c). If, however, each feature rigidly co-occurs with other specific features of diabetes or co-morbidity in a group of individuals with T1D, this may indicate a shared aetiological, potentially stratifiable, endotype of disease.

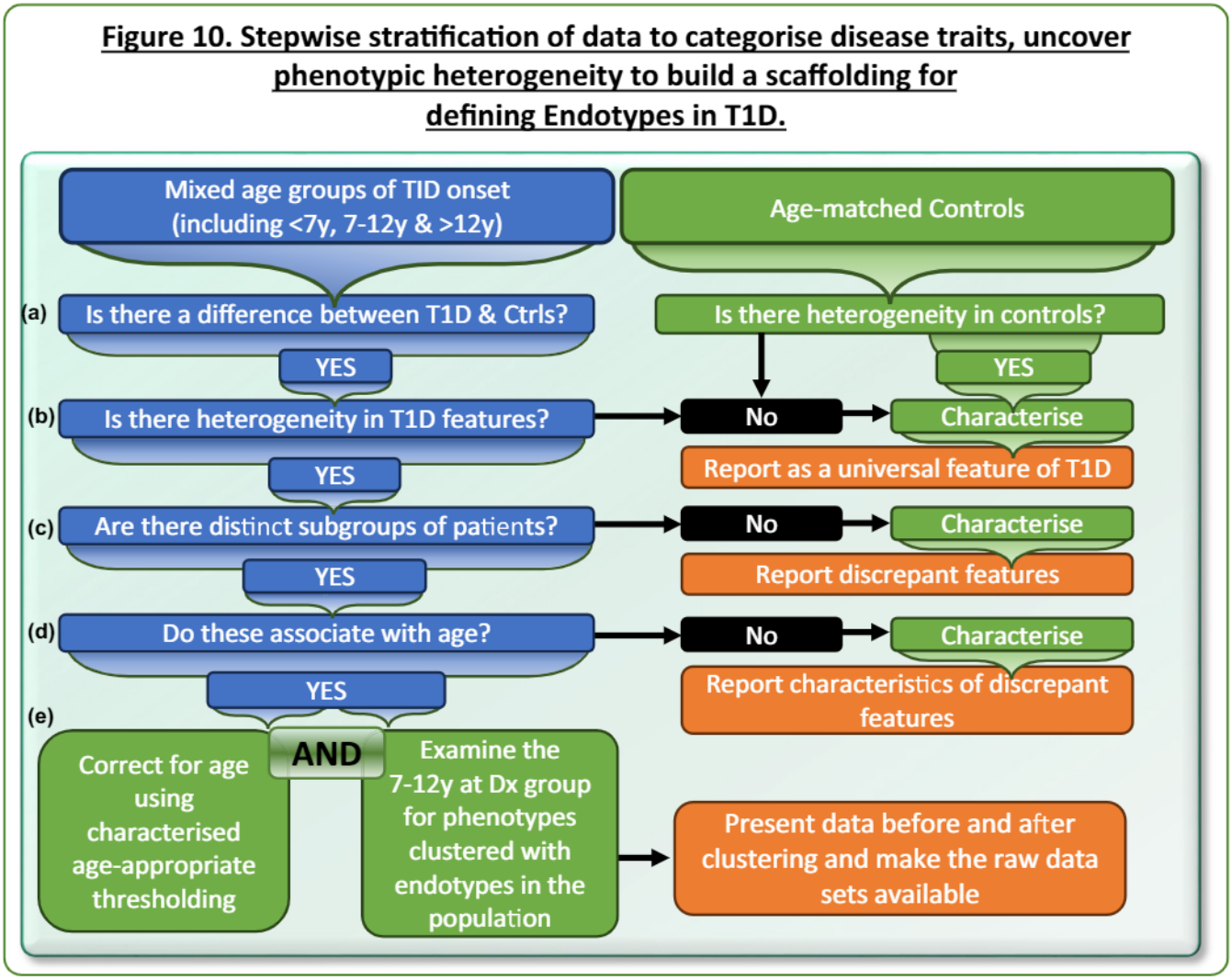


FIGURE 10 Stepwise stratification of data to discover disease traits, uncover phenotypic heterogeneity in early vs late T1D and build scaffolding for defining Endotypes in T1D. A proposed analysis pipeline and reporting framework for studies attempting to understand if a given feature or finding is associated with (a) all people living with Type 1 diabetes; (b) is a feature that is seemingly stochastically distributed amongst those with T1D, (c) appears to suggest stratifiable phenotypes of TID which (d) may align with age and/or (e) the endotypes described herein. In the interest of data access, and future discovery alignment—I also support the proposition of making all raw data available to other researchers for future studies.

Employing the pipeline in planning experiments and analysis then quickly stratifies our thinking into one of the two aetiological possibilities associated with age-aligned phenotypes. These possibilities are: (a) that the discovered trait is associated with a continuum of natural maturation, moving along a trajectory that drifts from infancy through the ages of 6 to 13 years and on towards adulthood; or (b) the trait aligns with one or other of the immuno-endotypes (described above), where either option will then require considerably more validation to confirm (as seen with proinsulin processing).

Critically, even if the differences align with age but not necessarily different disease modalities, individualised treatment might still be needed, especially for the aggressive forms of T1D in those diagnosed under 7y. Therefore, despite the inherent challenges, this implies a strong need to consider how to address aims to protect children diagnosed before 7y when formulating immunotherapy trials.

6 | MOVING FORWARD IN THE PANCREAS: THE EMERGING TECHNOLOGICAL CAPABILITIES AVAILABLE FOR PANCREATIC RESEARCH

At its most basic, microscopy allows for capturing information from a single field of view, employing either simple dyes for gross tissue structure identification, or specific antibodies tagged with a colourimetric reagent

that target a protein of interest, allowing for cellular identification.

However, huge advances in multiplex staining, imaging and computational analysis now allow quantitative analysis and increasingly nuanced interrogation of fixed pancreatic tissue. It is now commonplace to undertake whole-section imaging of multiple targets at high resolution, requiring sophisticated image analysis protocols to capture the myriad of multi-layered information that is now possible[45] (e.g. several cell types can be complex-phenotyped on a single section and, as well as the gathering their frequency, their relational location to each other and relevant regions of interest can also now be analysed).

Furthermore, technologies such as Imaging Mass Cytometry (via Fluidigms Hyperion+ system) and the NanoString nCounter FLEX platform (NanoString Technologies, Inc.) offer high-plex RNA and DNA profiling in fixed tissue and even archival tissue (as recently demonstrated by the discovery that some genes associated with lymphocyte differentiation and migration align with EADB endotypes in the pancreas[46]).

However, histology is also being reimagined: The ability to image whole 200 µm-thick 'living slices' of labelled human pancreatic tissue at the molecular level (maintained in culture medium) in dynamic and experimental settings has also enabled a step-change in learning opportunities about spatial-cellular-temporal relationships in the islet and its surroundings.[47] These living slices allow for in-vitro laboratory-controlled live-imaging of dynamic processes (such as immune cell dynamics within an insulin-containing islet and calcium signalling in response to glucose challenge) in a relatively conserved and spatially relevant context (i.e. connected and within its surrounding exocrine environment).

But even within small laboratories, with the advent of relatively simple protocols using multiplex fluorescent reagents (eg TSA and Akoya Biosciences' OPAL™ dyes) and AI-image analysis platforms (such as IndicaLabs' Halo™ and the freeware, QuPATH), increasingly complex interrogations can be carried out, and in combination with technologies such as those above, giant leaps in our understanding of T1D in the pancreas are increasingly possible.

Multidisciplinary science also offers enormous gains in utilising an ever-growing volume of data, and in addition to supporting basic and clinical science for individual laboratories, global consortia (such as INNODIA, HPAP and nPOD) now offer access to huge swathes of freely available data via new data-sharing platforms. For example, 'Pancreatlas' offers access to view an array of images and data from the pancreas (accessible at https://pancreatlas.org/), and, with the inception of user-friendly interfaces, such as that offered by PancDB, it is now possible for non-data-scientists to deep-dive into data-sharing initiatives (accessible at https://hpap.pmacs.upenn.edu/about-pancdb).

7 | CONCLUSION

The pancreas is an extraordinarily complex and challenging organ to study, but it offers huge opportunities for learning about, and understanding, T1D aetiology. It is also the most sensible place to seek the initiating disease trigger.

In the last century, research in the human pancreas has afforded us considerable gains in our understanding of diabetes—despite the limited availability of material. Nevertheless, there is still work to do. We still have not fully characterised the healthy pancreas and do not know the causal factor(s) of T1D, nor how to translate what we observe in the pancreas into markers detectable in the peripheral circulation. The research community still does not understand the heterogeneity we increasingly find in our studies. And, critically, whilst we still have not learned everything there is to learn from the samples we are already fortunate to study, we have not even begun to characterise the pancreas in diverse populations; for example, T1D presents very differently in Sub-Saharan Africa[48] to European populations, but to the best of my knowledge, there are no pancreas samples from Africa yet available to study.

Thus, with an eye to preventative strategies on the horizon, these are exciting times for the T1D community, but there is still work to do. I look forward to us continuing to emerge from our silos and work together to combine our resources, skills and creativity to build consensus regarding a useful and meaningful route towards stratifying the heterogeneity of T1D, and finding the elusive therapies we need for prevention(s) or cure(s) for those living with, or at risk of developing this pernicious condition.

BULLETED NOVELTY STATEMENT

It is becoming increasingly clear that heterogeneity exists in the immunopathology of type 1 diabetes (T1D), and this may be associated with the different pathological processes that occur in the pancreas in different individuals with T1D. The review gives a historical, current and future perspective of research in the pancreas and offers a simple tool for researchers studying human disease to use to examine if a given research finding might align with the Endotypes of T1D that have been reported.

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CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest to declare.

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