# Evaluation of islet hormone expression profiles and transitional endocrine phenotypes in *post mortem* pancreatic tissue from human donors with type 1 diabetes, cystic fibrosis and cystic fibrosis related diabetes

**Rashmi Rakesh Maheshwari**

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## Declaration

I declare that all work presented in this thesis is entirely my own. All sources of information have been referenced appropriately, and contribution from colleagues has been acknowledged in the respective sections.

-Rashmi Rakesh Maheshwari

## Abstract

All forms of diabetes are characterized by abnormalities in blood glucose regulation due to altered β-cell function of the pancreatic islets. Type 1 diabetes (T1DM) is associated with an autoimmune process targeting the islet β-cells. In cystic fibrosis related diabetes (CFRD), loss of β-cell function is associated with exocrine pancreas fibrosis secondary to duct obstruction.

Until now, apoptosis due to autoimmunity has been considered the primary mechanism underlying insulin loss in T1DM. Prevalence of residual β-cells and thus, insulin-containing islets (ICIs), even in cases of long-standing diabetes suggests that loss of β-cell function, rather than death alone, may contribute to hyperglycaemia in T1DM. In type 2 diabetes, this β-cell dysfunction has increasingly been linked to transitional endocrine cell phenotypes due to loss of end-differentiated protein markers (de-differentiation) and/or expression of other, non-β-cell hormones (trans-differentiation). Evaluation of differences in islet hormone expression in pancreata with and without diabetes is central to elucidating such phenotypic shifts that may be underlying diabetes development.

The studies within this thesis aimed to quantify differences in islet hormone expression profiles and determine transitional endocrine phenotypes in normal and diseased pancreas by immunofluorescence (IF) staining of pancreatic tissue sections derived from deceased donors with T1DM, cystic fibrosis (CF) and CFRD in comparison to control donors.

In Chapter 3, the aim was to quantify changes in pancreatic islet hormone expression profiles and explore any evidence of transitional phenotypes in T1DM pancreas from two distinct cohorts showing differential insulitic (islet immune infiltration) patterns. The two cohorts of T1DM patients were identified based on age of disease onset: Cohort 1 (>13 years old) and Cohort 2 (<7 years old). A significant decrease in the number of ICIs in T1DM compared to their age-matched controls was observed but endocrine cell number in remaining T1DM islets was comparable to age-matched control donors across both cohorts. Reduced β-cell number was mirrored by an increased number of cells expressing non-β-cell hormones in keeping with potential trans-differentiation events. Moreover, polyhormonal and ‘hormone-empty’ cells were

identified in patients with T1DM potentially evidencing β-cell trans- and de-differentiation events.

To overcome the inevitable time restrictions and potential for subjective bias intrinsic to manual quantification of tissue immunostaining phenotypes, an automated method of image analysis for high throughput quantification of islet cell phenotypes was established and validated in Chapter 4. Automated assessment using Vectra slide scanner and analysis by inForm® software was carried out on each T1DM donor to compare with manual analysis. The two methods were shown to be comparable, but validation confirmed that a minimum of 50 islets are required for quantitative sampling to match manual quantification. Moving forward with this approach will enable time-efficient sampling of much larger numbers of islets ensuring that outcomes are representative of the whole organ even when underlying pathology is characterised by its heterogeneity.

In Chapter 5, the aim was to evaluate CF transmembrane conductance regulator (CFTR) expression in normal human pancreata and assess islet hormone expression in CF and CFRD. Whilst it is established that β-cell dysfunction in CF can lead to diabetes, the mechanism by which the CFTR channel influences insulin secretion remains debated. Thus, determination of the localisation of CFTR RNA and protein in normal human pancreata using sophisticated techniques was carried out to determine if CFTR influences β-cell function through cell-intrinsic or extrinsic mechanisms. It was observed that CFTR is absent within β- or any other islet endocrine cell types strongly suggesting that CFTR impacts β-cell function through non-cell autonomous derived factors. On assessment of CF and CFRD pancreata, a decrease in β-cells compared to age-matched controls was observed. Moreover, in CF/CFRD pancreata, abnormal endocrine cell distribution was observed within ducts with apparent budding of islets from the ductal epithelium. Quantification of hormone expression within these ductal regions and ductuloinsular complexes revealed a very high number of non-β-hormone producing cells. Endocrine cells in the ducts were found to be mostly glucagon-positive and ‘hormone-empty’ cells, implying possible attempted β-cell regeneration through intermediate phenotypes.

Together these studies have confirmed maintenance of significant numbers of endocrine cells in T1DM and CFRD and have provided clear evidence of transitional

phenotypes supporting a role for cell plasticity as opposed to death alone in diabetes pathogenesis. This opens the exciting possibility that, by controlling different stressors central to each type of diabetes development, restoration and renewal of β-cells is not impossible but a goal requiring active pursuit towards curative therapies for this devastating disease.

## Dedication

To my parents,

who have been an example of self-sacrificing love, and relentless hard work

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Last, but certainly not the least, my man Gurraj Singh, who was my calm amidst all the chaos, thank you for your patience and undying love even on the worst of my days.

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# List of abbreviations

| | |
|---|---|
| Ano1 | Anoctamin 1 |
| BMI | Body mass index |
| CD | Cluster of differentiation |
| CF | Cystic fibrosis |
| CFRD | Cystic fibrosis related diabetes |
| CFTR | Cystic fibrosis transmembrane regulator |
| ChrA | Chromogranin A |
| $Cl^-$ | Chloride |
| CPHN | Chromogranin-positive hormone-negative |
| DAPI | 4′,6-diamidino-2-phenylindole |
| EADB | Exeter Archival Diabetes Biobank |
| ENaC | Epithelial sodium channel |
| FBS | Foetal bovine serum |
| FOXO1 | Forkhead box protein O1 |
| GIP | Gastric inhibitory polypeptide |
| GLP-1 | Glucagon-like peptide-1 |
| Glut2 | Glucose transporter 2 |
| H & E | Haematoxylin and eosin |
| HLA | Human leucocyte antigen |
| ICI | Insulin-containing islet |
| IFN-γ | Interferon γ |
| IHC | Immunohistochemistry |
| IL-1β | Interleukin-1β |
| iNOS | Inducible nitric oxide synthase |
| ISH | *In situ* hybridisation |
| Jak | Janus kinase |
| $K_{ATP}$ | ATP-sensitive potassium channel |
| KRT7 | Keratin 7 |
| MafA | MAF BZIP transcription factor A |
| MHC | Major histocompatibility complex |
| MODY | Maturity-onset diabetes of the young |
| MSI | Multi-spectral images |

| | |
|---|---|
| NF-κB | Nuclear factor kappa B |
| NGN3 | Neurogenin 3 |
| Nkx6.1 | NK6 Homeobox 1 |
| NO | Nitric oxide |
| PBS | Phosphate buffered saline |
| PDX1 | Pancreatic and duodenal homeobox 1 |
| PP | Pancreatic polypeptide |
| SDF-1 | Stromal cell-derived factor 1 |
| SOX9 | SRY-related HMG-box 9 |
| STAT-1 | Signal transducer and activator of transcription 1 |
| T1DM | Type 1 diabetes mellitus |
| T2DM | Type 2 diabetes mellitus |
| TNF-α | Tumour necrosis factor-α |
| Tris-HCl | Trisaminomethane hydrochloric acid |

## Published work

D Khan, R Kelsey, RR Maheshwari, VM Stone, A Hasib, FM Koivula, A Watson, S Harkin, N Irwin, JAM Shaw, N McClenaghan, V Venglovecz, A Ebert, MF Tullberg, MG White, and C Kelly (2019). Short-term CFTR inhibition reduces islet area in C57BL/6 mice. Scientific Reports 9(1): 11244

RR Maheshwari, CJ Jones, JAM Shaw, MG White (2018). Evaluation of CFTR expression & localization in human pancreas. Diabetes 2018 July; 67 (Suppl. 1), 2166-P; DOI**:** 10.2337/db18-2166-P

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# Chapter 1: Introduction

## 1.1 The human pancreas

The intake of food triggers a physiological response to regulate the digestion and absorption of nutrients from the ingested meal. The pancreas is central to this response as it facilitates the breakdown and use of energy reserves from the food. The pancreas is a glandular organ located in the upper abdomen and forms a part of both the digestive and the endocrine system. The head of the pancreas is situated in the C-shaped curve of the intestinal duodenum (Figure 1.1), and gradually tapers towards the left into the tail of the pancreas which ends just next to the spleen. As this organ is an integral part of glucose metabolism and energy production, it is highly vascularized by major arteries - hepatic, gastroduodenal, splenic, pancreatic, duodenal, and superior mesenteric. The pancreas is made up of lobes which contain a dense network of pancreatic ducts which transport digestive enzymes and bicarbonate-rich juices from the pancreas into the intestine (Röder et al., 2016).

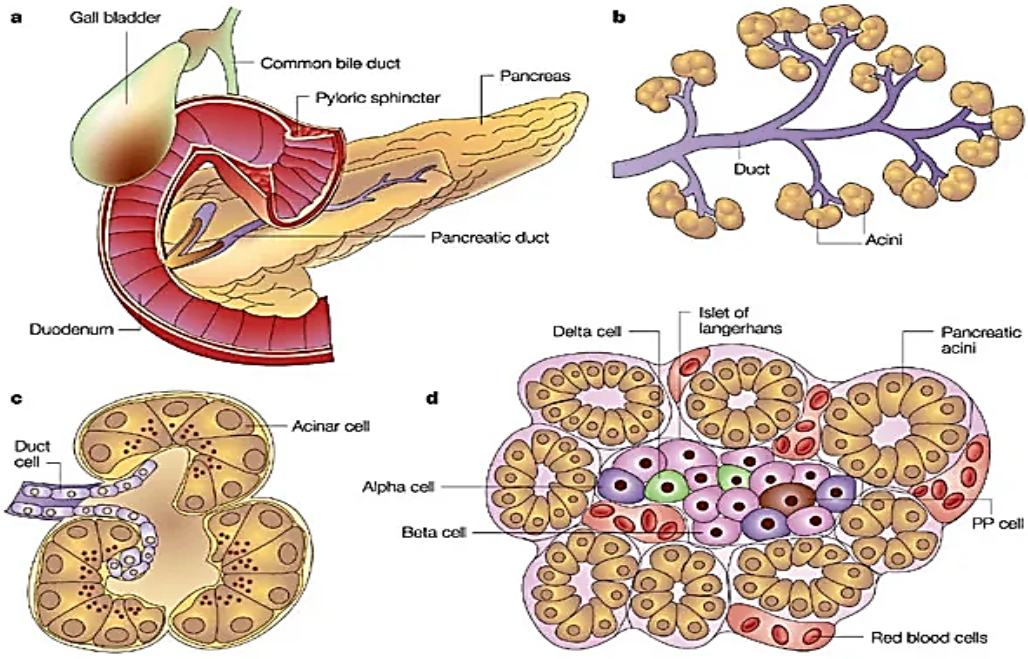


*Figure 1.1: Structure and function of human pancreas (Taken from Bardeesy and DePinho, 2002)*

*The pancreas is situated near the duodenum (a), and has an endocrine (d) and an exocrine compartment (c). The endocrine compartment (d) is made up of islets of Langerhans and is involved in regulation of blood glucose levels. The exocrine compartment is, principally, made up of acinar cells (c) that secrete digestive enzymes into the pancreatic ducts (b).*

The pancreas is divided into two distinct compartments: exocrine and endocrine (Figure 1.1). Over 95% of the pancreas is comprised of the acinar cells that execute the exocrine function of the pancreas. The acinar cells secrete digestive enzymes into the pancreatic ducts. The endocrine cells of the pancreas cluster to form islets of Langerhans that make up about 1–2% of the pancreas mass (Longnecker, 2014). Four different hormone-secreting types of cells constitute the pancreatic islets: α-cells (15-20%), β-cells (65-80%), δ-cells (3-10%), and pancreatic polypeptide (PP) (3-5%) cells (Brissova et al., 2005). Another less-common type of cells i.e. ε-cells are found in the islets that produce ghrelin, also called as the 'hunger hormone'. The functions of the five pancreatic endocrine cells are listed below:

1. α-cells produce glucagon which is released in response to low blood glucose levels and has opposing actions to insulin. Glucagon is released in response to low blood glucose levels. Its main action is on the liver where it breaks down glycogen to glucose. It also stimulates production of glucose from amino acids (gluconeogenesis) (Szablewski, 2014).

2. β-cells produce insulin, a key hormone that regulates blood glucose levels. Insulin is released in response to high glucose following ingestion of a meal. It facilitates uptake of glucose by skeletal muscles and adipose tissue, where it can be stored in the form of glycogen (Aronoff et al., 2004).

3. δ-cells secrete the hormone somatostatin. Its primary function is to inhibit the release of insulin and glucagon (Röder et al., 2016).

4. The PP hormone from the PP cell has a role in the regulation of appetite and regulates somatostatin secretion (Röder et al., 2016).

5. ε-cells which account for less than 1% of islet cells secrete ghrelin. Ghrelin, in conjunction with somatostatin inhibits secretion of insulin (Röder et al., 2016).

Together these hormones, especially insulin and glucagon are involved in maintaining normal physiological levels of glucose in the body, as described below.

## 1.2 Glucose homeostasis

Glucose is the main source of energy for most of the cells and tissues in the body and normal physiological blood glucose levels are central to brain activity (Szablewski, 2014) (Neubauer and Kulkarni, 2006). Low levels of glucose in the blood (hypoglycaemia) can cause complications like unconsciousness, seizures, and even

sudden death (Szablewski, 2014). On the contrary, chronic high blood glucose levels (hyperglycaemia) can lead to blindness, cardiovascular diseases, nephropathies and neuropathies (American Diabetes, 2012). Hence, it is extremely important that the blood glucose levels are strictly maintained within physiological limits (4.0-5.9 mmol/L (72-99 mg/dl) fasting; <7.8 mmol/L (<140 mg/dl) postprandial) at all times (NICE guideline, 2012). This process of maintenance of stable blood glucose levels is called glucose homeostasis and is aided by the fine regulation of hormones responsible for peripheral glucose uptake following meals and production of glucose by liver in between meals (Szablewski, 2014). Postprandial hyperglycemia (excess blood glucose after meals) and fasting hypoglycemia (low blood glucose levels during starvation) are taken care of via the balance between two important hormones produced by the pancreas namely insulin and glucagon (Szablewski, 2014). The following illustration (Figure 1.2) describes the basic mechanism of glucose homeostasis.

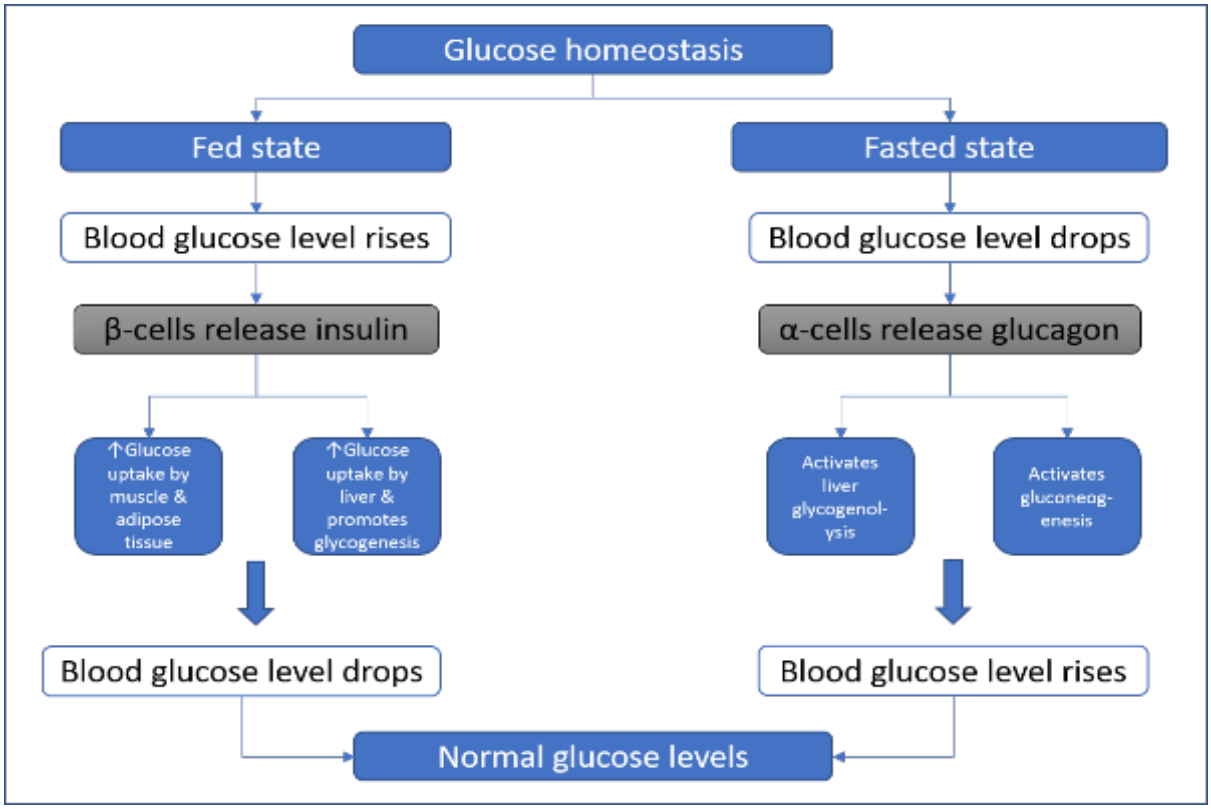


*Figure 1.2: Glucose homeostasis.*

*Following meals (fed state), insulin is secreted from β-cells of pancreatic islets of Langerhans in response to high blood glucose, which facilitates transport of glucose to striated muscles and adipose tissue. Also, hepatic glucose uptake is activated where it is stored in the form of glycogen, thereby reducing blood glucose levels and bringing them back to normal. In between meals or in periods of starvation (fasted state), glucagon is secreted by α-cells and activates glucose production from glycogen in the*

*liver and gluconeogenesis. This eventually leads to an increase in blood glucose levels.*

Beyond insulin and glucagon, many other factors like gut hormones, neuropeptides and hepatokines are also involved in glucose homeostasis (Aronoff et al., 2004, Röder et al., 2016). The fine interplay between all these pancreas intrinsic and extrinsic factors contributes to the regulation of glucose in the body.

## 1.3 Diabetes mellitus: Disease of the endocrine pancreas

Diabetes is a multi-organ disorder that results from inadequate production or response to insulin. It is a life-long, metabolic disorder characterized by loss of function and/or mass of the insulin-producing β-cells (Wang et al., 2010). Insulin is a vital hormone that regulates the uptake of glucose from blood into the body cells where it can be utilized to provide energy. The disruption in insulin production causes hyperglycaemia leading to the symptoms of diabetes (Hameed et al., 2015). Around 422 million people around the globe were diagnosed with diabetes by 2014. In 2017, an estimated 8.8 % of the adult population worldwide had diabetes. This is projected to increase to 9.9 % by the year 2045 (World Health Organization, 2017). In UK, about 1 in 16 people are likely to be affected by diabetes (Diabetes UK Facts and Stats, 2015). In 2015, diabetes was directly responsible for 1.6 million deaths while high blood glucose was the cause of 2.2 million deaths in 2012 (World Health Organization, 2017). Diabetes mellitus manifests itself in two main forms: type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM). Diabetes also occurs in other forms, several of which are described briefly below. Moreover, certain diseases like cystic fibrosis, chronic pancreatitis, pancreatic cancer, also lead to diabetes. According to the American Diabetes Association (2018), DM can be classified into four groups as described below.

### 1.3.1 Type 1 diabetes mellitus

T1DM is caused by autoimmune destruction of the islet β-cells by T cells (cluster of differentiation (CD) CD4+ and CD8+) and macrophages (Rowe et al., 2011). It leads to severe deficiency of β-cell insulin secretion and hence, affected people are dependent on insulin for survival (Foulis et al., 1991). About 10% of the total diabetes cases are attributed to T1DM (Diabetes UK Facts and Stats, 2015). Globally, the incidence of T1DM is increasing at an annual rate of 2.5 – 3 % (DIAMOND project Group, 2006).

### 1.3.2 Type 2 diabetes mellitus

T2DM accounts for approximately 90% (Diabetes UK facts and stats, 2015) of all diabetes cases. T2DM is a complex, multifactorial metabolic disorder resulting in hyperglycaemia through at least relative inadequate secretion of insulin usually with diminished action (insulin resistance) (Hameed et al., 2015). It mostly affects people having genetic predisposition to the disease, however, Lifestyle factors including obesity, physical inactivity, and poor diet, lead to the body's cells developing resistance to the action of insulin (Hameed et al., 2015). As the disease progresses, β-cells are thought to become 'exhausted due to over production of insulin' and eventually become dysfunctional (Lin and Sun, 2010).

### 1.3.3 Gestational diabetes mellitus

Gestational diabetes occurs during pregnancy due to increase in metabolic demand and insulin resistance (Mpondo et al., 2015). It is usually diagnosed in later stages of pregnancy, especially after second trimester (Mpondo et al., 2015).

### 1.3.4 Other forms of diabetes

This category entails any other forms of diabetes like monogenic diabetes e.g. maturity-onset diabetes of the young (MODY), neonatal diabetes, as well as secondary diabetes including cystic fibrosis related diabetes (CFRD), and chronic pancreatitis associated diabetes (American Diabetes, 2018).

## 1.4 Type 1 diabetes mellitus

T1DM appears to be caused by a combination of underlying factors including genetic susceptibility, environmental factors, and viral infections, although the pathogenesis remains incompletely understood (Belle et al., 2011).

### 1.4.1 Factors contributing to development of T1DM

Genetic pre-disposition

The most well-defined risk factor for developing T1DM is genetic susceptibility to the disease. The risk of developing T1DM in a child of a parent with T1DM is around 6 % and amongst siblings is around 7 % (Todd, 1995).

Over the years, more than 50 genes have associated with an increased risk of developing T1DM (Pociot and Lernmark, 2016). As T1DM is an autoimmune disorder, most of these genes associated with the development of T1DM are involved in the immune system, including a number of Human leucocyte antigen (HLA) genes. Other

common susceptibility loci are the cytotoxic T-lymphocyte antigen 4 locus and the insulin locus (Kim and Polychronakos, 2005).

The HLA locus encodes the major histocompatibility complex (MHC) genes. MHCs are involved in binding to antigens and presenting them to the T cell receptors. MHC class I presents the intracellular antigens and MHC class II plays a role in presenting extracellular antigens. The DQ and DR forms of MHC class II are involved in T1DM genetics (Turner, 2004). The DR and DQ forms are involved in antigen presentation to the CD4+ T cells, eliciting an immune response. Infants having the genetic combination of either HLA DR3 or DR4 with HLA DQ2 or DQ8 are at a more than 50 % risk of developing the disease before the age of twelve. The risk of development is enhanced by having a T1DM sibling sharing the same HLA genotype (Aly et al., 2006).

Environmental factors

Although the underlying genetics in identical twins is the same, there is only a 35-40 % congruity in disease development (Olmos et al., 1988). This gave an insight that the development of T1DM is not entirely dependent on genetic pre-disposition. Some of the documented environmental factors implicated in T1DM development are viral infections involving enteroviruses and rotaviruses (van der Werf et al., 2007). Also, seasonal variations have been found (Knip et al., 2005). A study analysing the presence of islet auto-antibodies found that in at-risk groups, islet auto antibodies surfaced in colder months of the year. This is also in accordance with the observation that enterovirus infections are common during these months (Knip et al., 2005). Various studies have reported the presence of Coxsackie virus in the β-cells of children who go on to develop T1DM (Ylipaasto et al., 2004). Also, it has been found that the Coxsackie viruses isolated from diabetic patients can induce diabetes in healthy subjects leading to severe loss of glucose homeostasis (van der Werf et al., 2007).

The modern day lifestyle has been reported to increase the risk of T1DM. Poor diet containing energy-rich and fatty foods, physical inactivity due to sedentary lifestyle, increased stress, irregularity in sleeping patterns, etc. have all been implicated in contributing to disturbances in glucose levels by way of insulin resistance (Vehik et al., 2013). Exposure to sun has decreased in recent times, and so a lot of people have increasingly reported vitamin D deficiency (Vehik et al., 2013). Studies indicate that vitamin D deficiency is associated with higher risk of developing T1DM. This hypothesis

has been supported by some studies that reporting that vitamin D administration in high-risk populations provides protection against T1DM (Hypponen et al., 2001, Stene and Joner, 2003).

### 1.4.2 Immune attack in development of T1DM

T1DM, as described earlier, is characterized by β-cell destruction due to infiltrating CD4+ and CD8+ T cells. Although, the exact aetiology remains unknown, pro-inflammatory cytokines viz. interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) released by the infiltrated macrophages and T cells in the pancreatic islets play a substantial role by causing oxidative stress and impaired mitochondrial function and eventually leading to cell death by caspase-induced apoptosis (Kacheva et al., 2011) or necrosis (Collier et al., 2011). These cytokines have been detected during early islet insulitis in animal studies (Yoon and Jun, 2005).

In disease development, cytokines act in synchronization. *In vitro* studies suggest that while IL-1β has some cytotoxic effects, TNF-α and IFN-γ alone are not capable of inducing β-cell death, but when cytokines are used in combination, β cell death is considerably increased (Cnop et al., 2005). IL-1β and TNF-α act by activating the nuclear factor-kappa B (NF-κB) pathway. NF-κB is an important transcription factor mediating anti- and pro-apoptotic effects (Eizirik and Mandrup-Poulsen, 2001). In β-cells, it is involved in the activation of transcription of various genes regulating cytokine-mediated toxicity (Eizirik and Mandrup-Poulsen, 2001). Inducible nitric oxide synthase (iNOS) is activated by NF-κB signalling, leading to nitric oxide (NO) production, ultimately resulting into nitrosative stress (Darville and Eizirik, 1998, Kacheva et al., 2011). NO has concentration-dependent effects on cell function and apoptosis. IFN-γ acts through the Janus kinase (Jak) and the signal transducer and activator of transcription-1 (STAT-1) cascades (Eizirik and Mandrup-Poulsen, 2001). These pathways augment the production of NO by inducing iNOS. The iNOS promoter molecule holds two binding sites for NF-κB, and one binding site for STAT-1. In rat β cell-lines, NO production is reported to be induced by IL-1β alone (Eizirik and Darville, 2001). However, the concentration of NO increases if IL-1β is used in combination with TNF-α or IFN-γ (Darville and Eizirik, 1998). In human islets, no cytokine can alone induce expression of iNOS, but combinations of IL-1β or TNF-α with IFN-γ can (Eizirik et al., 1994).

### 1.4.3 Loss of β-cells in T1DM

Due to the presence of pro-inflammatory cytokines, cell death by apoptosis is considered to be the primary mechanism of β-cell loss in T1DM (Anuradha et al., 2014). However, observance of apoptotic cells, by way of TUNEL and cleaved caspase-3 staining in the affected islets of T1DM pancreata is a rare phenomenon (Meier et al., 2005). Also, the extent of apoptosis is moderate in comparison to the extent of loss of β-cell phenotype (Butler et al., 2007). While this may suggest the efficiency of macrophages in removing the dead cells from the islets, this may also indicate that non-apoptotic mechanisms contribute to β-cell destruction (Morgan and Richardson, 2018).

The first organ studies of the T1DM pancreata revealed the presence of immune cells in and around the islets of Langerhans (Lecompte, 1958). But it was soon found that not all islets were affected by these immune cells. In recently diagnosed patients, islets in some parts of the pancreas appeared perfectly normal while the other parts suffered from total β-cell loss (Richardson et al., 2014). It has been proposed that most patients with T1DM do not suffer from total β-cell loss and have residual β-cells (Baiu et al., 2011). A similar finding was also observed in a study reporting measurement of endogenous C-peptide levels in about 80% of the 924 patients suffering from T1DM for over 5 years (Oram et al., 2015). This study concluded that majority of those with T1DM have functional β-cells which secrete small amounts of insulin (‘microsecretion’) even years after diagnosis. Also, a study on juvenile diabetic organ donors revealed that even the patients with long-standing history of diabetes had residual β-cells (Gianani et al., 2010). But these islets still retained all other endocrine cells expressing glucagon, somatostatin, and PP. These insulin-deficient islets retaining all other hormones are called ‘pseudoatrophic’ (In’t Veld, 2014). Pseudoatrophic islets orient themselves in a more condensed appearance due to loss of β-cells and look smaller indicating loss of cellular organization (In’t Veld, 2014). These findings indicated a possibility of regeneration of β-cells even in patients affected with diabetes for a long time (Meier et al., 2006b). It has been proposed that islet cells are not entirely helpless when affected by immune cells and they can respond by increasing their rate of replication. This has been reported in both, an older patient (Meier et al., 2006a) and in two independent studies of pancreas samples harvested post-mortem from patients with recent-onset T1DM, where it was shown that islet cell proliferation was increased by as much as 10-fold more than controls (Willcox et al., 2010, Willcox et al., 2011).

Moreover, this increase was most evident in those islets which were inflamed, implying that an inflammation-linked factor, still unknown, was responsible for mediating the effect. Interestingly, this mitotic response was even observed in α-cells, suggesting a general response of islet endocrine cells to a proliferative signal (In't Veld et al., 2007). The rate of endocrine cell proliferation was studied in the inflamed islets of two patients who died without a diagnosis of T1DM but were immunopositive for multiple islet autoantibodies, suggesting that they might have been in a 'pre-diabetic' state. The pancreata of each of these individuals contained islets which showed enhanced rates of endocrine cell replication with the presence of inflammation (In't Veld et al., 2007). Loss of β-cells may, thus, be due to imbalance between regeneration and apoptosis.

However, all these findings suggest the involvement of an alternative mechanism resulting in dysfunction of β-cells. Continuous presence of hyperglycaemia may 'overload' the β-cell's capacity to produce insulin. This, over time, may 'exhaust' the cells and they lose phenotype and function. This phenomenon is well-accepted in T2DM (Dor and Glaser, 2013), and could also be relevant to T1DM.

### 1.4.4 β-cell de-/trans-differentiation as a mechanism of β-cell dysfunction

The most recent theory for loss of β-cell functional mass in T2DM is de-differentiation, describing a change in phenotype of pancreatic β-cells which eventually leads to loss of key transcription factors needed for proper functioning of cells. This phenotypic shift causes disturbances in insulin content and secretion, which is central to pathophysiology of all forms of diabetes (Weir et al., 2013).

De-differentiation is the loss of mature cell identity (Weir et al., 2013) and direct conversion to other cell types is termed trans-differentiation (Kim and Lee, 2016a). The mechanism of β-cell de-differentiation has been studied extensively in T2DM (Talchai et al., 2012, Wang et al., 2014, Brereton et al., 2014, Spijker et al., 2015, Cinti et al., 2016).

β-cell de-differentiation in T2DM was first confirmed by Talchai et al. (2012) in a study using lineage tracing to demonstrate that the decrease in β-cell mass in diabetes is due to this reprogramming and conversion of β-cells to α-cells and not solely due to the death of insulin-producing β-cells (Figure 1.3). FOXO1 is a known transcription factor for the regulation of β-cell mass due to stress. The investigators studied FOXO1 knockout mice and found that induction of physiological stress led to hyperglycemia and decrease in β-cell mass in these mice. Moreover, they

demonstrated that reduced FOXO1 expression and phenotypic conversions was also evident in in rodent models of diabetes, including *db/db* mice.

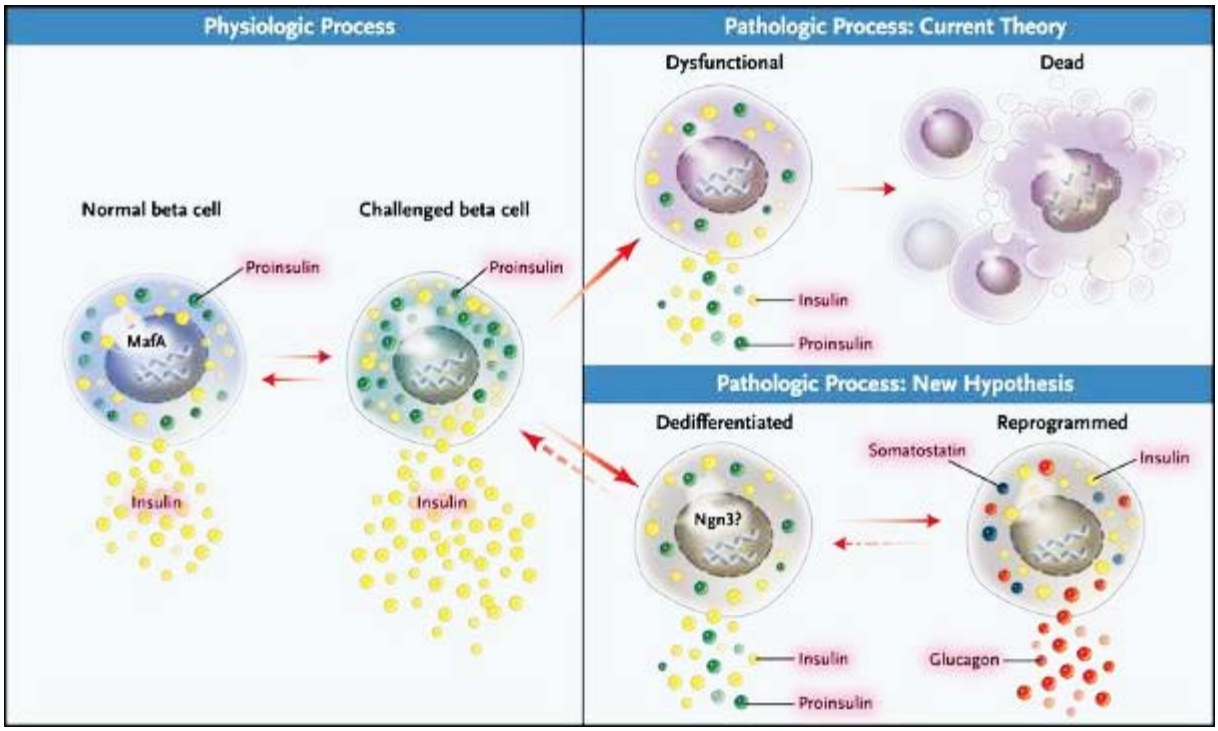


*Figure 1.3: The concept of de-differentiation in T2DM (Taken from Dor & Glaser, 2013)*

*Under normal conditions, pancreatic β-cells produce insulin in response to increase in blood glucose levels. In conditions of hyperglycaemia and insulin resistance (as seen in T2DM), β-cells work harder to produce more insulin to maintain physiological glucose levels. Over time, this imposes ER and oxidative stress on the cells, thereby leading to β-cell dysfunction and eventually death. Recent studies indicate that the dysfunctional β-cells, rather than death, move onto a trans-differentiation phenotype leading to conversion of β-cells to α-cells, which can potentially be reversed to a functional β-cell.*

β-cell de-differentiation was further confirmed by Wang et al. (2014)(Wang et al., 2014), who suggests that β-cells, in T2DM, dedifferentiate to neurogenin-3 (NGN3) positive, insulin-negative cells, which can be restored to their former, fully differentiated β-cell following insulin therapy. Another study has demonstrated the conversion of β-cells to α-cells following hyperglycaemia (Brereton et al., 2014). This transformation was reversible by the restoring normal glucose levels through insulin therapy. Secretion of insulin from β-cells in response to glucose is regulated by ATP-sensitive potassium ($K_{ATP}$) channels (Röder et al., 2016). Loss of insulin secretion machinery due to inexcitability of this channel is implicated in neonatal and T2DM (Brereton et al., 2014). Brereton et al. (2014) demonstrated development of diabetes in adult mice expressing $K_{ATP}$ channel mutation. This disease development led to hyperglycaemia resulting in insufficient insulin and over expression of glucagon within islets. They also

reported co-expression of insulin and glucagon (bi-hormonal)-expressing cells within islets. Using lineage tracing techniques, these polyhormonal cells were found to be β-cells. A study by Cinti et al. (2016) using human pancreatic tissue, confirmed plasticity in T2DM. They demonstrated conversion of β-cells to glucagon producing α-cells and somatostatin producing δ-cells. They also reported de-differentiation in T2DM by assessing pancreata for the presence of Synaptophysin-positive cells (endocrine cells) that are hormone-negative in the islets (non-insulin / glucagon / somatostatin / PP producing). There was a 3-fold increase in dedifferentiated cells in T2DM compared to the non-diabetic cohort (Cinti et al., 2016). Spijker et al. (2015) have also used immunofluorescence staining and double-immunogold labelling to study co-expression of insulin and glucagon in human pancreata from T2DM donors. They reported an 8-fold increase in insulin and glucagon co-expressing cells in T2DM compared to the controls (Spijker et al., 2015).

The mechanism of de-differentiation and plasticity has not been extensively studied in T1DM. Recently, a study by Butler et al. (2016), demonstrated the presence of Chromogranin A (ChrA) – positive / hormone - negative (CPHN) cells (T1DM vs autoantibody positive vs control; 1.11 ± 0.2% vs 0.26 ± 0.6 % vs 0.27 ± 0.1 %) in pancreatic islets. Most of these hormone-negative cells were situated as single endocrine cells around the islets in the exocrine pancreas. They reported that the distribution of hormone-empty cells in T1DM pancreas is similar to the one found in neonatal pancreas, indicating cell-regeneration.

Taken together, these studies indicate that β-cell de-differentiation/plasticity may be a mechanism of β-cell dysfunction in diabetes.

## 1.5 Cystic fibrosis (CF)

Cystic fibrosis (CF) was first described as a disease in 1938 by Dorothy Anderson (Elborn, 2016). CF is an autosomal recessive multi-organ disorder affecting mainly people of North European origin (Lao et al., 2003). It is the commonest hereditary disorder in the UK affecting about 1 in 2,500 new born babies (Davies et al., 2007).

CF is caused by mutations in the CF transmembrane regulator (CFTR) gene located on the long arm of chromosome 7 (Sheppard and Welsh, 1999). Over 2,000 gene defects have been identified and implicated in the pathogenesis of disease and can have varied effects on the manufacture, processing, and function of CFTR protein (VanDevanter et al., 2016). The most common gene defect leading to CF is the deletion

of phenylalanine sequence at position 508, known as F508del (Barrio, 2015). The Table 1.1 below explains the five main classes of CFTR mutations and the risk of CFRD and pancreatic exocrine (digestive juice) insufficiency in each.

*Table 1.1: CFTR gene mutations (Taken from Norris et al., 2019)*

| Mutation class | Effect on CFTR | Example | Disease | Pancreatic insufficiency risk | Diabetes risk |
|---|---|---|---|---|---|
| I | Failed synthesis | G542X | Severe | High | High |
| II | Failed protein processing | F508del | Severe | High | High |
| III | Channel fails to open | G551D | Severe | High | Intermediate |
| IV | Reduced channel function | R117H | Less-severe | Low | Low |
| V | Reduced synthesis or processing | A455E | Less-severe | Low | Low |

CFTR is an epithelial, anion channel regulating rate of chloride ($Cl^-$) flow leading to controlled $Cl^-$ ionic movement across the epithelium (Bellin et al., 2013). The CFTR protein facilitates passage of chloride ions out of the cells which is followed by osmotic passage of water leading to thinning of mucus to maintain optimum composition of surface liquid in the airways, digestive tract and sweat glands (Rafeeq and Murad, 2017). CFTR also causes inhibition of the neighbouring epithelial sodium channel (ENaC) (Elborn, 2016). When the CFTR gene becomes mutated, transport of Cl- out of the cell is interrupted followed by loss of inhibition of EnaC leading to escalated Na+ absorption, thereby resulting into thick, sticky bicarbonate-rich mucus secretions in the affected organs (Elborn, 2016). Figure 1.4 below describes CFTR function in normal and diseased conditions (Elborn, 2016).

While CF has deleterious effects on different parts of the body, organs of the respiratory and the gastrointestinal system are the most severely affected. CF affects the lung the most and is distinct from other organ system manifestation in CF because lung function failure is the cause of premature deaths in about 95% of the patients and the lung is the only organ that develops a chronic infection phenotype with an intense inflammatory response (Donaldson and Boucher, 2006). The extent of severity is very subjective and differs from patient to patient, but chronic lung infection resulting into progressive decline of lung function is the major cause of death in CF patient (Davies et al., 2007).

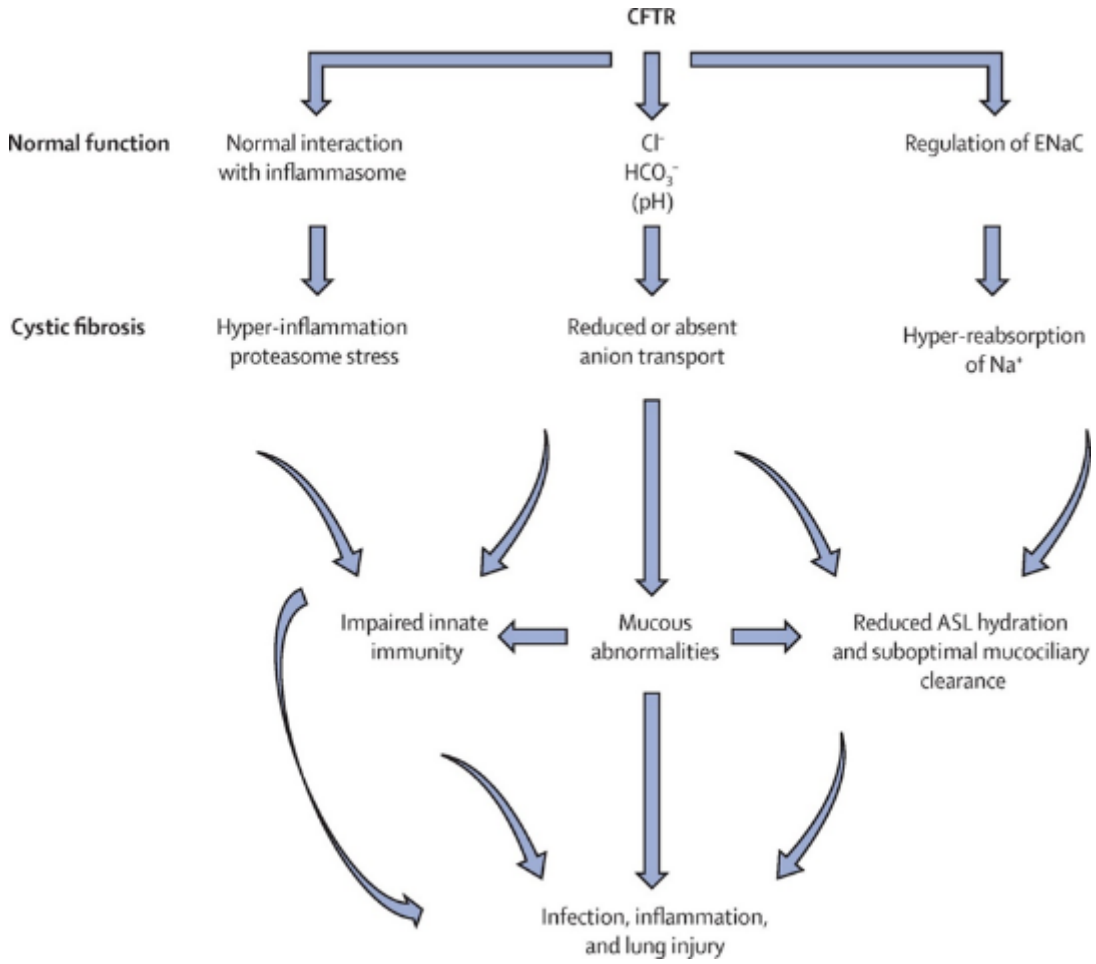


*Figure 1.4: Effects of CFTR in normal and diseased states (Taken from Elborn, 2016)*

### 1.5.1 Cystic fibrosis related diabetes (CFRD)

Diabetes is the commonest co-morbidity of CF (Barrio, 2015). As CF-related diabetes (CFRD) has unique features of its own, it doesn't fall into T1DM or T2DM categories. CFRD is considered as a different clinical entity and is a form of so-called type 3c diabetes (pancreatogenic diabetes) which is secondary to diseases of the exocrine pancreas (Makuc, 2016). However, it also shares some common features with both T1DM and T2DM viz. gastric disorders including malnutrition and malabsorption, multi-organ pathophysiology, pancreatic insufficiency, chronic infection, and insulin resistance (Hart et al., 2018). While most CF patients have compromised pancreas at birth (Davis, 2006), classical signs of CFRD are usually prevalent in the second decade of life, but are preceded by pre-diabetic state leading to glucose abnormalities even in CF patients under 10 years of age (Ode and Moran, 2013). Post puberty, CFRD prevalence increases 5% each year with advancing age, affecting about 15% of adolescent and 50% of adult CF patients (Moran et al., 2009).

Moreover, CFRD has deleterious effects on lung function and nutritional status leading to increased morbidity and mortality in CF patients (Ode and Moran, 2013, Barrio, 2015). Hence, early diagnosis and pro-active treatment of CFRD is central to improving CF outcomes.

### 1.5.2 Pancreatic pathology in CF

About 85% of patients with CF suffer from exocrine pancreatic insufficiency and need pancreatic enzyme replacement for growth (Wilschanski and Novak, 2013). The risk of pancreatic insufficiency is more common in class I-III mutations (Barrio, 2015). Development of pancreatic disease in CF starts in the antenatal stage and 23% of CF patients under 1 year of age and 75% between 1 and 4 years of age show signs of severe exocrine loss of pancreas (Bogdani et al., 2017). Moreover, the severity of early exocrine pancreatic pathology may drive CFRD and associated endocrine pancreas pathology later on in life (Norris et al., 2019).

One of the central functions of pancreatic ducts is to facilitate the absorption of $Cl^-$ and release of $HCO^{3-}$ secretions in the ducts. The CFTR gene is expressed on the epithelial layer of these pancreatic ducts and helps in the transportation of $HCO^{3-}$ and $Cl^-$ that lead to the production of alkaline fluid in the ducts (Wilschanski and Novak, 2013). These bicarbonate secretions play an important role in neutralizing the gastric acid and providing optimal pH for digestive enzymes to function effectively (Park and Lee, 2012). It is hypothesized that in CF-affected pancreas, composition of ductal secretions is altered due to low pH, reduction in volume secreted, and excess protein content (Durie and Forstner, 1989). All this is believed to disturb zymogen secretions leading to pancreatic duct obstruction (Gibson-Corley et al., 2016b). This results in the early signs of pancreatic changes that include obstruction of small ducts and acinar tissue, but soon progresses to plugging of acini and dilation of ducts leading to disruption of epithelial layer. Pancreatic obstructions result primarily from altered enzyme compositions, however, with progression of disease lead to mucus aggregation from faulty CFTR in epithelial cells in the ducts (Tucker et al., 2003). This is then followed by inflammation, exocrine fibrosis, and ultimately fatty infiltration continuing until total replacement of exocrine tissue with fat (Gibson-Corley et al., 2016a). The endocrine compartment i.e. islets, appear remarkably spared (although altered/remodelled) in spite of extensive exocrine fibrosis (Norris et al., 2019)

### 1.5.3 Development of CFRD

The precise cause of CFRD remains unclear. However, it is understood that patients homozygous for F508del type of genetic mutation are the most susceptible to developing CFRD (Koivula et al., 2016). The main driver of CFRD is insulin deficiency and is associated with disturbances in first-phase insulin response (Koivula et al., 2016). Abnormal glucose tolerance is present earlier in life in CF patients (possibly even from birth) as is demonstrated in CF ferrets (Olivier et al., 2012), pigs (Uc et al., 2015), and young children aged between 3 months and 5 years (Yi et al., 2016a).

Two main mechanisms for the development of CFRD are proposed in the literature (Barrio, 2015):

1. Loss of β-cell mass

Loss of β-cell mass has been shown secondary to exocrine fibrotic damage and lipoatrophy (Litvin and Nwachukwu, 2016). Examination of post mortem CF pancreas suggests a reduction in number of islets (Lohr et al., 1989) and approximately 50% decline in number of β-cells. Also, overall islet architecture is severely disrupted due to presence of fibrosis and amyloid deposits (Iannucci et al., 1984, Couce et al., 1996). Lohr et al. (1989) also reported that β-cell loss was associated with an increase in non-β-cells in the islets. Moreover, β-cell area is also reduced in CF patients (without CFRD) by 11 to 52% (Norris et al., 2019). Endoplasmic reticulum stress, and low antioxidant level leading to oxidative stress have also been shown to impact β-cell mass contributing to CFRD development (Litvin and Nwachukwu, 2016). Ferret models of CF have also shown decline of β-cells at birth (Olivier et al., 2012). These β-cells further decline progressively due to inflammation and exocrine fibrosis (Bridges et al., 2018). Collectively, these studies point towards a low reservoir of β-cells that pre-disposes CF patients at higher risk of hyperglycaemia (Norris et al., 2019).

2. β-cell dysfunction

Large islets and/or occasional ducts in a bed of fatty adipose tissue have been observed in severely affected CFRD pancreas (Olivier et al., 2015). Also, pancreatic insufficiency is diagnosed within months of birth, but CFRD is not usually diagnosed until the second decade of life. Moreover, almost 50 % of the β-cell mass is retained in CFRD patients, which is insufficient to cause diabetes (Sun et al., 2017). Thus, the loss of β-cells cannot be attributed to exocrine fibrosis alone (Litvin and Nwachukwu, 2016). CF patients have reduced β-cell function in comparison to normal (non-CF)

(Sheikh et al., 2017, Nyirjesy et al., 2018). Proinsulin secretion is higher in CF patients with impaired glucose response suggesting early defects in β-cell function (Sheikh et al., 2017, Nyirjesy et al., 2018). This indicates a mechanism for β-cell dysfunction, due to inflammatory stress and fatty infiltration (Litvin and Nwachukwu, 2016).

β-cell dysfunction in CFRD is thought to be driven by various mechanisms, some of which are listed below:

Islet inflammation

Studies in young CF pancreas obtained from children under four years of age have demonstrated presence of immune infiltrate, usually rich in cytotoxic T-cells, within the islets (Hart et al., 2018, Hull et al., 2018). Moreover, islet IL-1β immunoreactivity is an early feature in CF and CFRD patients, especially in children younger than 10 years of age (Hull et al., 2018). As already discussed before and as demonstrated in literature (Wilcox et al., 2016), presence of pro-inflammatory cytokines within islets may induce β-cell death and dysfunction.

Lipotoxicity

A mechanism by which exocrine lipoatrophy may affect insulin secretion and β-cell function is lipotoxicity. The lipotoxicity hypothesis highlights the relationship between tissue damage and metabolic abnormalities associated with ectopic fat deposition in the tissue (Saisho, 2016). A feature of the CF pancreas is lipoatrophy of the exocrine tissue (Lohr et al., 1989). Fatty infiltration in pancreas is associated with β-cell dysfunction (loss of glucose-stimulated insulin secretion) and subsequent development of non-insulin dependent diabetes in rats (Lee et al., 1994). A study in 52 human participants with impaired glucose tolerance or impaired fasting glucose reports inverse relationship between pancreatic fat and insulin secretion, thereby highlighting a mechanism for β-cell dysfunction (Heni et al., 2010). Free fatty acids have also been shown to disrupt β-cell function and induce apoptosis (Cnop, 2008). Also, inflammatory cells recruited as a result of adipocytes (adipocytokines) in the exocrine pancreas, may induce β-cell dysfunction by paracrine interactions (Saisho, 2016).

Pancreas extrinsic defects

Gastrointestinal diseases affecting the levels of incretin hormones central to normal functioning of islets is prevalent in CF (Norris et al., 2019). Hormones like glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP) are decreased

in CF patients (Sheikh et al., 2017) with faster gastric emptying (Kayani et al., 2018). β-cell dysfunction is considered to be a result of disturbances in entero-insular axis, i.e. impaired incretin signalling. Perano et al. (2014) demonstrated a reduction in postprandial hyperglycaemia by improving incretin secretion and increasing the time of gastric emptying. Even factors like low immunity, and vitamin D deficiency are implicated in β-cell dysfunction (Barrio, 2015).

Paracrine effects of CFTR

CFTR in the human pancreas is usually expressed in the epithelium lining of the ducts and is central to their normal physiology (Wilschanski and Novak, 2013). Ductal cells, through paracrine interactions, are shown to influence islet function (Bertelli and Bendayan, 2005). Thus, pancreatic duct obstruction in CF secondary to abnormal CFTR may influence islet function via paracrine mechanisms.

Role of CFTR in β-cells

Studies also indicate a direct role of CFTR in regulation of insulin secretion from pancreatic β-cells (Litvin and Nwachukwu, 2016). Various hypotheses exist to explain the role of CFTR in the normal functioning of the endocrine pancreas and the mechanisms by which CFTR mutations may lead to the development of CFRD (Sun et al., 2017).

Abnormalities in chloride conductance (like the one mediated by CFTR channel) have been shown to affect β-cell function (Kayani et al., 2018). A study by Edlund et al. (2014) shows a novel function of CFTR channel in islet β-cells as regulator of insulin secretion and exocytosis. They have also demonstrated the role of CFTR in regulation of Anoctamin 1 (Ano1) which facilitates insulin release from β-cells (Edlund et al., 2014). In another study, this group reported presence of CFTR channels in human and rodent α-cells and that faulty CFTR leads to disturbances in glucagon secretion (Edlund et al., 2017). A study by Boom et al. (2007), also reported expression of CFTR in α-cells of rat islets. Guo et al. (2014), also reported similar conclusions demonstrating absence of membrane depolarization in mouse β-cells in presence of CFTR channel inhibitors like CFTRinh-172 and glyH-101. Absence of glucose-stimulated membrane depolarization leads to faulty insulin secretion due to increase in concentration of calcium in the cytoplasm (Guo et al., 2014). Another study reported that islets from CFTR-deficient mice are more susceptible to injury compared to controls. A low-dose streptozotocin was administered that resulted in impaired glucose

regulation (Stalvey and Flotte, 2009). Moreover, as CFTR-deficient mice failed to develop exocrine fibrosis and scarring, they concluded that exocrine pathology could not be the only mechanism leading to CFRD in CF patients (Stalvey and Flotte, 2009).

However, in contrast to this, CFTR mRNA (by *in situ* hybridisation (ISH)) and protein (by immunohistochemistry (IHC)) was found to be undetectable in animal CF models like rats and ferrets as well as human pancreas (Hart et al., 2018, Norris et al., 2019). Further, RNA sequencing of β-cells (mouse and human) showed extremely low level expression of CFTR mRNA in about 5 % of β-cells (Hart et al., 2018). CFTR is thought to affect insulin secretion by disrupting β-cell electrophysiology. Hence, it is important to address if (and the levels) CFTR is expressed within β-cells or not.

Due to such contrasting hypotheses, further research is needed to address the question if CFTR influences β-cell function by cell-intrinsic or extrinsic pathways. Also, examination of acinar-islet and ductal-islet interactions is essential to understand if and how exocrine atrophy and ductal obstruction affect islets.

## 1.6 Rationale of the studies comprising thesis

The role of β-cell dysfunction as opposed to solely β-cell death in the development and progression of diabetes is being widely accepted. β-cell dysfunction is associated with loss of insulin secretion as a result of stressors like hyperglycaemia, glucotoxicity, inflammation, etc. and often precedes diabetes diagnosis by several years. Further, recent studies have reported the presence of transitional phenotypes associated with de-/trans-differentiation of pancreatic β-cells which is hypothesized to be a ‘hideaway’ from the ongoing insult. This phase of dysfunctional β-cells (if present) represents a potential therapeutic target, wherein if this switch is reversed or paused, may lead to a pool of functional β-cells capable of normal insulin response in T1DM and CFRD.

## 1.7 Project aims

Most of the knowledge in human diabetes, T1DM or CFRD, is limited by the availability of pancreatic tissue samples fit for appropriate study. The studies comprising this thesis aimed to characterize changes in non-diabetic and diabetic pancreas by immunostaining of pancreatic tissue sections derived from deceased T1DM, CF and CFRD affected donors.

The T1DM study (Chapter 3) aimed to assess changes in islet hormone expression profiles between non-diabetic and T1DM pancreas and explore the

possibility of β-cell de-differentiation and/or plasticity in T1DM using human pancreatic sections. It was hypothesized that loss of β-cell identity and associated dysfunction may be a contributory mechanism to β-cell loss in T1DM.

Further, in Chapter 4, evaluation of the effectiveness and accuracy of automated analysis of cell counting compared to the manual analysis undertaken in Chapter 3 was performed.

The CFRD study (Chapter 5) in this project was funded by the CF trust (UK) as part of the CFRD Strategic Research Centre (CFRD-SRC). First, determination of localisation of CFTR mRNA and protein in normal (control, no known pancreatic pathology) human pancreata using ISH and IHC respectively was carried out, to address the question of whether CFTR influences β-cell function through cell-intrinsic or extrinsic mechanisms. Similarly, to the T1DM study, Chapter 5 aimed to examine changes in islet hormone expression in CF and CFRD pancreas.

The specific aims of the work comprising this thesis were as follows:

1. To quantify changes in islet hormone expression and determine β-cell transitional phenotypes in pancreas of 2 clinically distinct cohorts with T1DM.
2. To assess validity of automated image analysis by Vectra 3.0 and PerkinElmer inForm® software compared to manual counting for tissue analysis.
3. To evaluate CFTR expression in control human pancreas
4. To characterize islet changes in CF and CFRD-affected pancreas associated with ductal pathology in CF.

# Chapter 2: Methods

## 2.1 Materials

All reagents were purchased from Sigma-Aldrich Company (Saint Louise, Missouri, USA), unless otherwise stated.

## 2.2 Pancreatic tissue sampling

Pancreatic tissue sections were obtained from the Exeter Archival Diabetes Biobank with the help of Prof Noel Morgan and Dr Sarah Richardson. Appropriate ethical approval was obtained for use and transport of the tissues. All tissue was pre-cut into 4 µm thick sections from formalin-fixed or mercuric-chloride fixed paraffin-embedded blocks. These slides were then provided 'ready-to-use' for immunofluorescence staining. The details of each donors are provided in the respective chapters.

## 2.3 Immunofluorescence (IF) staining

Depending on the study, pancreatic tissue sections were stained to assess the expression of insulin, glucagon, somatostatin, PP, chromogranin A (ChrA), CFTR, keratin 7 (KRT7) and keratin 19 (KRT19). Formalin-fixed tissue sections were first dewaxed for 10-minutes in Histoclear (National Diagnostics, Atlanta, Georgia, USA) followed by 3-minutes each in 100 %, 90 %, and 70 % ethanol. Where the sections were fixed in mercuric chloride, dewaxing was carried out first in 0.5 % iodine in xylene and then xylene alone for 5-minutes each. This was then followed by deparaffinization in graded alcohols as described above. Antigen retrieval was performed by microwave cooking for 20-minutes at 900 W in 0.01 M sodium citrate pH6 buffer. Following this, slides were washed with 1 x PBS (phosphate buffered saline) then blocked for a certain time with appropriate blocking buffer depending on the antibody species (Table 2.1). Primary antibody diluted in suitable antibody diluent (Table 2.2), was added and incubated overnight at 4 °C (Table 2.3). No primary antibody controls were always used to ensure staining is developed due to antigen detection by the primary antibody and not due to other reasons like non-specific binding of secondary antibody. Slides were then washed in 1 x PBS thrice for 5-minutes each before the addition of the appropriate Alexa Fluor® conjugated secondary antibody (Table 2.4). After 1-hour incubation in the dark, slides were washed thrice before mounting with 4, 6-diamidino-2-phenylindole (DAPI) Vectashield (Vector labs, Burlingame, California, USA) mounting medium.

*Table 2.1: Blocking buffers for IF staining*

| Blocking buffer | Incubation time |
|---|---|
| 5% goat serum (Abcam, Cambridge, UK) in 1xPBS | 5 minutes |
| 5% donkey serum (Abcam, Cambridge, UK) in 1xPBS | 5 minutes |
| 20% foetal bovine serum (FBS) in 1xPBS | 1 hour |

*Table 2.2: Primary antibody diluents*

| Solution | Manufacturer |
|---|---|
| Antibody diluent® | DAKO (Carpinteria, USA) |
| Signal Enhancer HIKARI for Immuno-stain Solution B | Nacalai tesque Inc. (Kyoto, Japan) |
| 0.05% goat serum in 1xPBS | Made in-house using goat serum |

*Table 2.3: Primary antibodies for IF staining*

| Antibody | Raised in | Dilution of stock | Supplier/ Catalogue number | Diluent used | Blocking buffer |
|---|---|---|---|---|---|
| Anti-insulin | Guinea pig | 1/100 | Abcam (Cambridge, UK), #ab7842 | DAKO Antibody diluent/ 0.05% goat serum in 1xPBS/ solution B | 5% goat serum in 1xPBS |
| Anti-glucagon | Mouse | 1/100 | Sigma- Aldrich (Saint Louise, Missouri, USA), #G2654 | DAKO Antibody diluent/ 0.05% goat serum in 1xPBS | 5% goat serum in 1xPBS |
| Anti-somato-statin | Mouse | 1/500 | Thermofisher scientific (Waltham, Massachusetts, USA), #14-9751-80 | DAKO Antibody diluent/ 0.05% goat serum in 1xPBS | 5% goat serum in 1xPBS |
| Anti-PP | Mouse | 1/250 | R&D systems (Abingdon, UK), #MAB62971 | DAKO Antibody diluent/ 0.05% goat serum in 1xPBS | 5% goat serum in 1xPBS |
| Anti-ChrA | Rabbit | 1/250 | Abcam (Cambridge, UK), #ab15160 | DAKO Antibody diluent/ 0.05% goat serum in 1xPBS | 5% goat serum in 1xPBS |
| Anti-CFTR 596 | Mouse | 1/2000 | CF foundation (Chapel Hill, North Carolina, USA) | Solution B | 20% FBS in 1xPBS |
| Anti-KRT7 | Rabbit | 1/8000 | Abcam (Cambridge, UK), #ab218440 | 0.05% goat serum in 1xPBS/ solution B | 20% FBS in 1xPBS |
| Anti-KRT19 | Guinea pig | 1/100 | Progen (Heidelberg, Germany), #GP-CK19 | 0.05% goat serum in 1xPBS/ solution B | 20% FBS in 1xPBS |

| | | | | | |
|---|---|---|---|---|---|
| Anti-somato-statin | Rat | 1/100 | R&D systems (Abingdon, UK), #MAB2358 | 0.05% goat serum in 1xPBS/ solution B | 5% goat serum in 1xPBS |
| Anti-insulin | Guinea-pig | 1/100 | DAKO (Carpinteria, California, USA), #a0564 | 0.05% goat serum in 1xPBS/ solution B | 5% goat serum in 1xPBS |

*Table 2.4: Secondary antibodies for IF staining*

| Antibody | Raised in | Dilution | Conjugate | Supplier |
|---|---|---|---|---|
| Anti-guinea pig | Goat | 1/1000 | Alexa Fluor® 488 | Thermofisher scientific (Waltham, Massachusetts, USA) |
| Anti -rabbit | Donkey | 1/1000 | Alexa Fluor® 568 | Thermofisher scientific (Waltham, Massachusetts, USA) |
| Anti-mouse | Rabbit | 1/1000 | Alexa Fluor® 488 | Thermofisher scientific (Waltham, Massachusetts, USA) |
| Anti-mouse | Donkey | 1/1000 | Alexa Fluor® 568 | Thermofisher scientific (Waltham, Massachusetts, USA) |
| Anti-mouse | Goat | 1/1000 | Alexa Fluor® 647 | Thermofisher scientific (Waltham, Massachusetts, USA) |

## 2.4 Haematoxylin & eosin (H & E) staining

H & E staining was performed to determine the basic morphology/pathology of the pancreatic tissues. Tissue slides were first dewaxed in Histoclear for 10-minutes and then immersed in 100 %, 90 % and 70 % alcohol for 3-minutes each. Tissue was rehydrated by immersing in deionized water for 5-minutes. Slides were then put in a haematoxylin containing pot for 3-minutes and then washed with deionized water. To enable development of stain, slides were then transferred to Scott's tap water for 5-minutes. Following this, slides were dipped quickly in 0.3 % acid ethanol 8 - 10 times, before washing them in Scott's tap water for 2-minutes. Slides were then counter-stained with eosin for 30-seconds followed by tissue dehydration in 95 % and 100 % ethanol for 3-minutes each. They were then transferred to xylene before mounting them with DPX mounting medium.

## 2.5 Microscopy and image analysis

Stained tissue sections were imaged using a Nikon A1R confocal microscope (Nikon, Melville, New York, USA). All images of either islets or ducts were obtained either at 10x or 20x magnification and used for further analysis in NIS Elements AR software (Nikon, Melville, New York, USA, version 4.6).

## 2.6 Manual counting of islets

50 islets per section were imaged at 20x magnification for analysis. An islet was defined as a cluster of 10 or more cells. Islet cells were manually counted with the help of 'cell counter' function on the NIS Elements AR software. The number of ChrA$^+$, insulin$^+$, hormone cocktail$^+$ (glucagon / somatostatin / PP) cells were determined. β-cell plasticity/trans-differentiation was characterized by insulin$^+$ cells co-expressing non-β-cell hormones (polyhormonal cells) and was determined by cells co-expressing insulin and hormone cocktail. The number of hormone-empty (CPHN) cells (negative for all four hormones) was also counted as a marker of β-cell de-differentiation/regeneration. All cells were counted with help of DAPI on individual channels and data was entered into MS Excel spreadsheets. Manual counting of 50 islets took roughly 6-7 hours depending on the quality of staining.

## 2.7 Automated counting using Vectra

PerkinElmer Vectra 3.0 (Akoya Biosciences, Menlo Park, California, USA) Automated Quantitative Pathology Imaging system was used for automated assessment of immunofluorescence staining. This was carried out by the Newcastle Molecular Pathology Node, Department of Cellular Pathology, Royal Victoria Infirmary (RVI), Newcastle-upon-Tyne. A typical, multi-step, work-flow involving automated analysis is described below:

### 2.7.1 Whole-slide scanning

Slides were loaded onto the slide rack in Vectra 3.0 and whole slide-scans were obtained for each slide. Figure 2.1 below indicates an example of a slide scan of a whole section of pancreatic tissue.

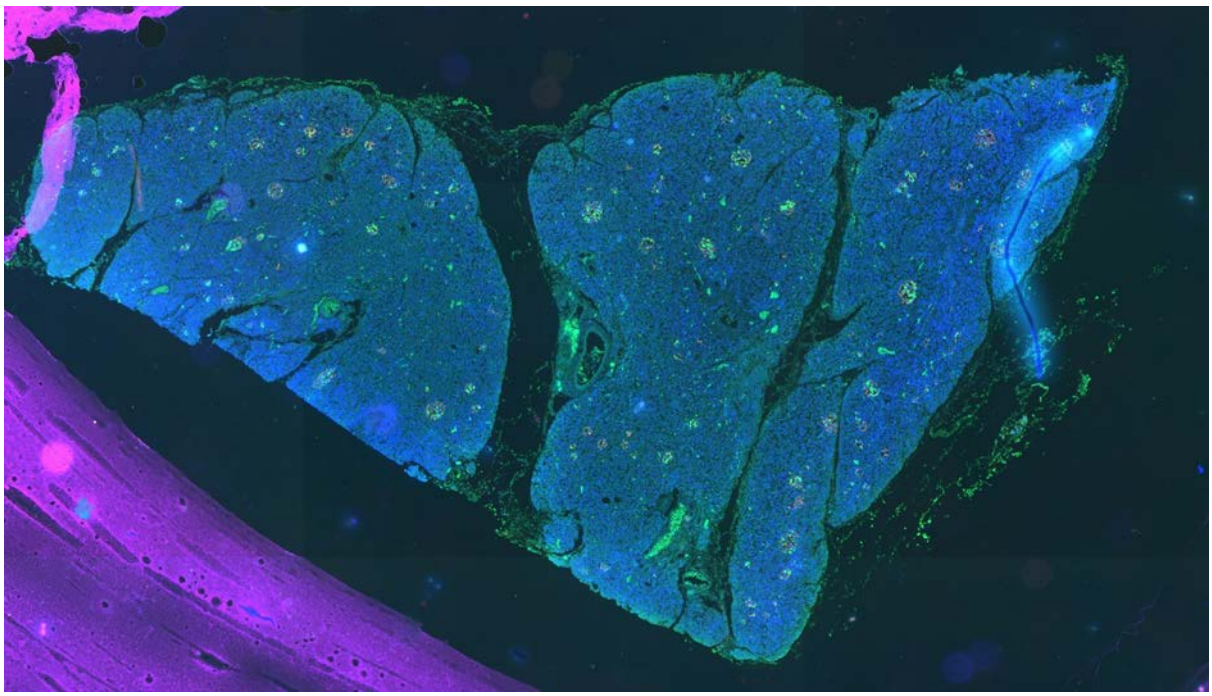

*Figure 2.1: Whole slide scan on Vectra*

### 2.7.2 Using Phenochart™ software to identify regions of interest

Phenochart™ (Akoya Biosciences, Menlo Park, California, USA) software was then used to mark regions of interest i.e. islet-containing regions, as is seen in the Figure 2.2 below. This would enable Vectra machine to lock co-ordinates of the tissue section so further image acquisition can be performed.

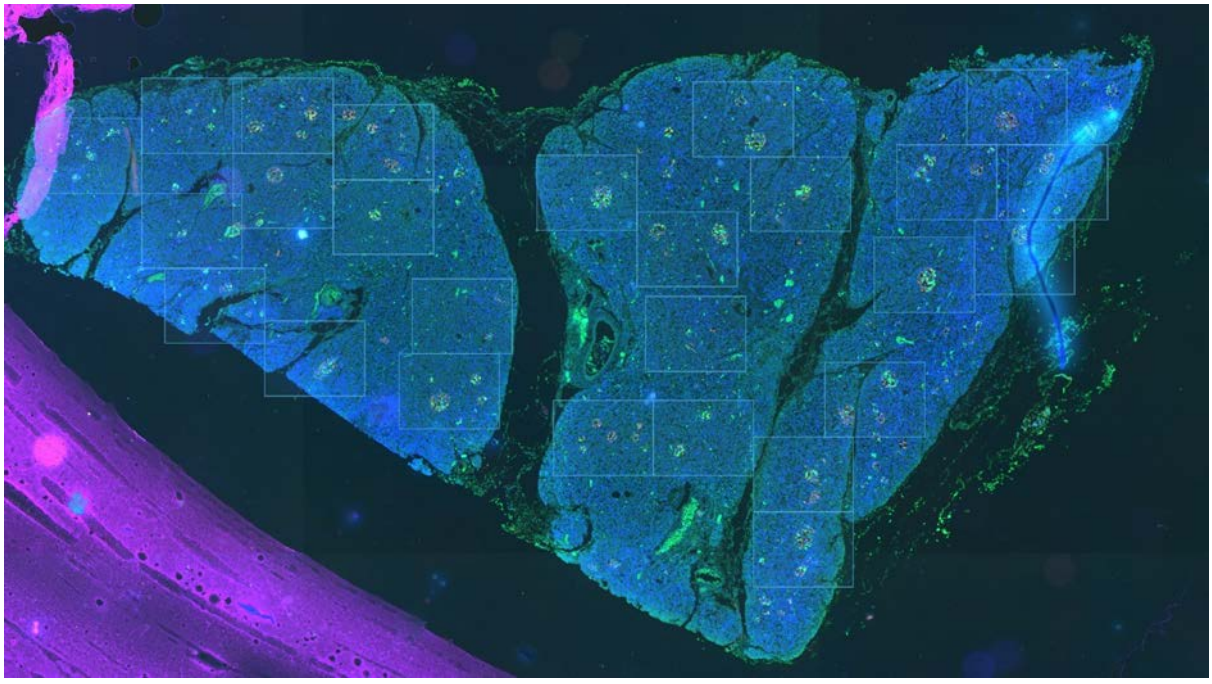

*Figure 2.2: Marked regions of interest on Phenochart™*

### 2.7.3 Acquiring multi-spectral images (MSI) of marked regions of interest

The regions of interest marked using Phenochart™ software were then sent to Vectra in order to acquire high-powered MSI images of each area, which can be used for further analysis.

### 2.7.4 Using a set algorithm in inForm® software to identify different cell phenotypes

The MSI images of islets are then used in inForm® (Akoya Biosciences, Menlo Park, California, USA) software for quantitative assessment of staining. A standard algorithm is developed with the desired steps for image-processing, cell-identification, and phenotype training, which is applied uniformly to all the cases in the study. A step-by-step brief of the algorithm is described below:

#### *2.7.4.1 Preparing images*

Images were initially prepared with selection of appropriate channels and corresponding colours to suit the study.

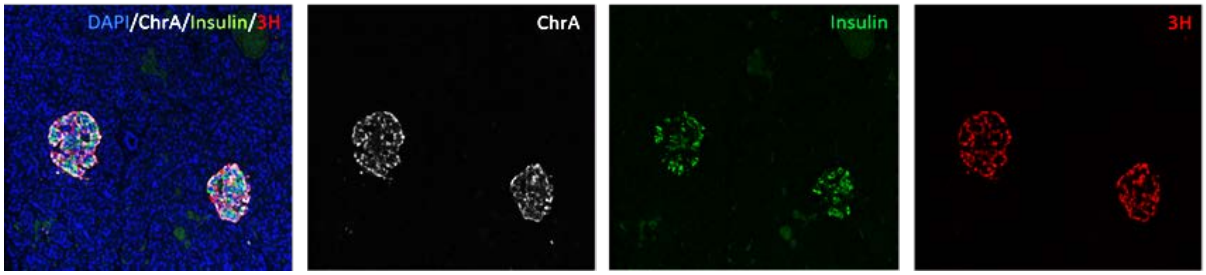


*Figure 2.3: Prepare images on inForm® software*

#### *2.7.4.2 Manual classification*

Individual islets in each image were selected as regions of interest by manually drawing around each.

#### *2.7.4.3 Cell segmentation*

Cell segmentation is required to identify individual cells and the nuclei, cytoplasm, and membrane within each. Cells within the region of interest were segmented using the standard, pre-determined parameters in the software. Minimum and typical pixel size for nuclei was set at 80 and 200 respectively to allow the nuclei of each cell to be identified. ‘Split’ and ‘grow’ nucleus settings, which enable visual segmentation of cell nuclei, were used to finalise nuclei size as this helps to set the outer boundary of the nucleus.

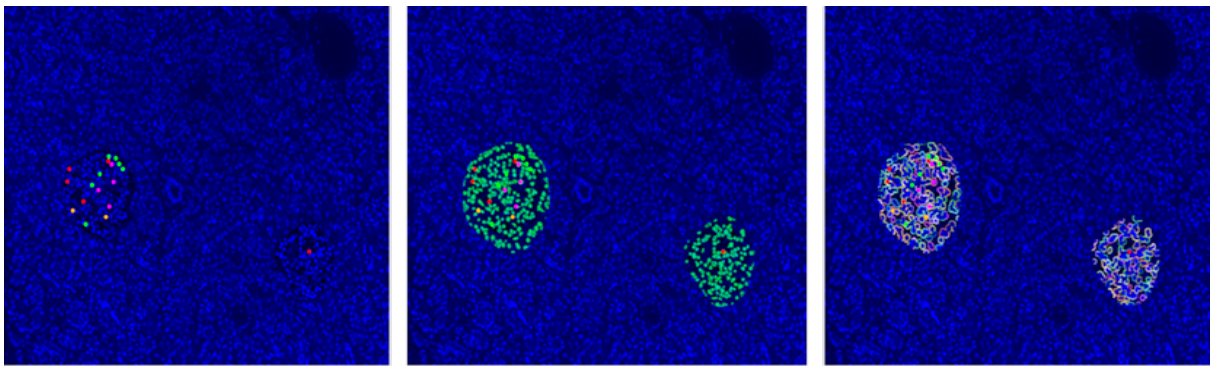

*Figure 2.4: Cell segmentation for identification of nuclei*

#### *2.7.4.4 Phenotyping*

Cells were then assigned phenotype manually so the software can be 'trained'. The phenotypes assigned to the cells were one of the following: ChrA only, Ins ChrA, 3H ChrA, Polyhormonal and DAPI only, as explained below.

1. ChrA only: cells expressing ChrA, but no hormones i.e. insulin, glucagon, somatostatin or PP.
2. Ins ChrA: cells expressing ChrA and insulin (β-cell).
3. 3H ChrA: Glucagon, somatostatin and PP were used together as a hormone cocktail to be detected using a single secondary antibody. 3H ChrA was assigned to cells expressing ChrA and the hormone cocktail (non-β-cell).
4. Polyhormonal: cells expressing ChrA, insulin, as well as hormone cocktail (3H).
5. DAPI only: cells expressing no markers i.e. ChrA, insulin, or hormone cocktail. This was used to identify non-endocrine cells on the islet periphery and inside the islet structure.

At least five cells need to be assigned to each phenotype before the software is ready for 'learning'. After enough cells were selected for 'training', the software was run to assign all other cells with appropriate phenotype. Following this, manual confirmation of whether the software-assigned phenotype is right or not, was done on various number of cells. This step helps to improve the accuracy and confidence level of data. The software was again left to run for automatic assignment of phenotype and this process was repeated until desired level of accuracy of phenotype was achieved. After slide scanning, and MSI capture, the process of automated processing and phenotyping takes about at least 3 hours per 50 islets. Finally, data were exported and processed in Matlab software (Mathworks, Natick, Massachusetts, USA) to get final counts per islet for each phenotype.

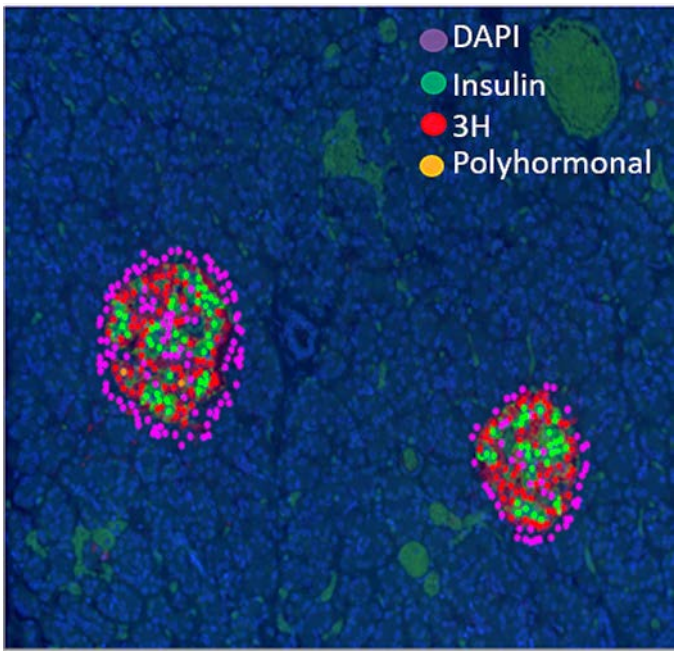


*Figure 2.5: Cell phenotyping*

## 2.8 Antibody stripping and re-staining of tissue

The slides were soaked in 1 x PBS overnight to gently dislodge coverslip and ensure complete removal of mounting medium. In order to strip the primary antibody, slides were incubated in 50 ml of stripping buffer (10 ml 10 % sodium dodecyl sulphate, 3.125 ml 1M Tris-HCl pH 7.5, 400 µL β-mercaptoethanol, and 36.475 ml deionized water) for 2-hours at 50 °C with gentle shaking. After 2 hours of stripping, slides were thoroughly washed in running water for 30 minutes, followed by a brief rinse (1-2 minutes with gentle shaking) in 50 % ethanol to complete removal of β-mercaptoethanol. Slides were then washed twice with 1 x PBS for 5-minutes each. To test for antibody removal, slides were blocked with 5 % goat serum in 1 x PBS and further incubated with the secondary antibody (the same ones used for the first round of staining), before mounting with DAPI Vectashield mounting medium. The confocal microscope was then used to examine if all primary antibodies are removed (all channels negative).

Once successful stripping was achieved, slides were washed with 1 x PBS to remove mounting media and coverslip. Following this, antigen retrieval and subsequent steps for immunofluorescence staining were carried out using appropriate primary and secondary antibodies.

## 2.9 RNAscope®

RNAscope® Technology is a novel, sophisticated *in situ* hybridization (ISH) technique from Advanced Cell Diagnostics Biotechne (ACDBio, Abingdon, UK) which facilitates signal amplification and background reduction through use of highly-specific patented probes against target RNA. RNAscope® was carried out to assess expression of CFTR in control, non-diabetic pancreas. This was carried out by the Pathology Node in RVI. Briefly, the technique (RNAscope®) begins with a permeabilization step using pre-treatment kits, followed by a hybridisation step that enable RNAscope® probes to detect and attach to target RNA site. Finally, an amplification step allows amplification of RNA signal, which can then be visualized by light microscope.

## 2.10 Graphical representation and statistical analysis

All acquired data from manual and automated counting of cells were analysed using GraphPad Prism version 8 software for Windows (GraphPad, San Diego, California, USA), to generate appropriate graphs. Data are either shown as mean ± standard error of the mean (SEM) or mean ± standard deviation (SD). Different statistical tests including one-way ANOVA, Student's t-test, linear regression, correlation, etc. were applied as appropriate, with $p<0.05$ accepted as statistically significant. To compare if one dataset was statistically different to the other, Student's t-test (paired or unpaired) was used. Unpaired t-tests were used to compare data between control and diseased groups in Chapters 3 and 5. One-way ANOVA along with post-hoc analysis was used when comparing between 3 or more groups (control vs T1DM groups based on disease duration) in Chapter 3. Paired t-tests were used to compare data obtained from manual and automated quantification in Chapter 4. Bland-Altman plots were used to compare quantitative data obtained by methods of automated and manual counting. In this, limits of agreement are determined on the basis of mean and standard deviation of the difference between the two values. As per Bland-Altman recommendation, if 95% of the points lie between these limits of agreement, then the two methods provide comparable results (Giavarina, 2015).

# Chapter 3: Quantification of islet hormone expression profiles and evidence for transitional phenotypes in two separate cohorts of T1DM based on age-of-disease-onset

## 3.1 Introduction

### 3.1.1 β-cell dysfunction

T1DM is classically characterized by insulin deficiency due to loss of β-cells (detected by positive insulin immunostaining) in the pancreatic islets (Meier et al., 2005). Cell death due to autoimmunity (insulitis) has been considered as the primary mechanism underlying β-cell loss in T1DM (Tomita, 2017). It is considered that between 70 and 90 % of β-cells are lost at disease diagnosis (Morgan and Richardson, 2016). While the loss of β-cell mass is evident due to decreased insulin expression observed in post-mortem tissue, there are various studies that suggest that insulin deficiency cannot be attributed to β-cell apoptosis alone.

Firstly, T1DM is associated with a short 'honeymoon period' in which insulin function improves after diagnosis and following initiation of insulin therapy (Moole et al., 2015). Although this phase usually wears off quickly, it points towards an existing pool of potentially functional β-cells. Moreover, β-cell loss at diagnosis seems to present itself in a lobular way with some areas of the pancreas containing no insulin-containing islets whereas islets appear normal in the other regions of the pancreas (Morgan and Richardson, 2018). Insulitis, defined as immune cell infiltration in the islets (Vov Meyenburg, 1940) containing majorly CD8+ lymphocytes along with mixture of CD4+ lymphocytes, B lymphocytes and macrophages, is a key lesion in T1DM (In't Veld, 2014). According to the consensus guideline published in 2013 (still widely accepted), insulitis is confirmed by the combined observation of ≥15 CD45+ cells in three or more islets combined with presence of pseudoatrophic islets (islets devoid of β-cells) (Campbell-Thompson et al., 2017). Insulitis is usually only present in less than 10% of islets in T1DM pancreata (In't Veld, 2011). The majority of patients with T1DM never suffer from absolute loss of β-cells (Baiu et al., 2011). The process of loss of β-cells happens slowly, starting before diagnosis and continues until most of the β-cells are lost (Cnop et al., 2005). Clinically significant residual insulin secretion has been found in one out of three patients even after over 3-years of T1DM diagnosis (Davis et al., 2015). A study conducted in 924 patients having T1DM for more than 5-years

found detectable C-peptide 'microsecretion' levels in approximately 80 % of the patients (Oram et al., 2015). This group concluded that insulin microsecretion is a feature observed in most patients, with a considerable number of patients sustaining clinically significant levels of endogenous insulin (Oram et al., 2015). A study in patients with long-standing T1DM demonstrated proinsulin secretion, a prohormone precursor to insulin, in almost 50 % of 98 patients (Steenkamp et al., 2017). Results from the DiViD study reported an improvement of β-cell function in islets obtained from recent-onset T1DM patients after three and six days of *in vitro* culture (Krogvold et al., 2015). Studies on donors from the Juvenile Diabetes Research Foundation (JDRF) Network for Pancreatic Organ Donors with Diabetes (nPOD) program indicate that β-cell dysfunction, and not death alone, might be the cause of hyperglycaemia at the time of T1DM diagnosis (Pugliese et al., 2014). Another study reports that β-cell dysfunction exists at least five years before T1DM diagnosis in autoantibody–positive individuals (Evans-Molina et al., 2018). These studies collectively indicate a residual pool of β-cells which fail or are insufficient to keep up with the increasing demand of insulin due to hyperglycaemia suggesting a loss of a β-cell function. It is proposed that glucotoxicity due to chronic hyperglycaemia causes an overload on β-cells leading to β-cell dysfunction and loss of insulin secretion (Cernea and Dobreanu, 2013).

### 3.1.2 De-differentiation/plasticity as a mechanism of β-cell dysfunction

β-cell dysfunction is widely established in studies in T2DM (Cernea and Dobreanu, 2013). An evolving concept for loss of β-cell function in T2DM is β-cell de-differentiation, describing a change in phenotype of pancreatic β-cells which eventually leads to loss of key transcription factors needed for proper functioning of cells (Weir et al., 2013). The three main mechanisms thought to drive phenotypic shift are:

1. De-differentiation: De-differentiation is the loss of end-differentiated proteins causing the cell to regress back to a 'progenitor-like' state, e.g. insulin expression is lost and expression of endocrine progenitor cell marker Neurogenin 3 (NGN3) is gained (Weir et al., 2013, White et al., 2016).
2. Trans-differentiation: Trans-differentiation implies a direct fate switch from one differentiated cell type to another cell type e.g. β-cell to α-cell conversion (Kim and Lee, 2016b).
3. Loss of identity: Loss of identity is decrease in expression of proteins and/or phenotypic markers central to a type of cell, e.g. in the case of β-cells, insulin

and β-cell transcription factors like Pancreatic and duodenal homeobox 1 (PDX1) (Hunter and Stein, 2017).

These faulty phenotypes cause disturbances in insulin content and secretion, central components in the pathophysiology of all forms of diabetes (Weir et al., 2013).

### 3.1.3 Evidence for de-differentiation and plasticity in human T2DM

β-cell de-differentiation as a mechanism of β-cell failure was first described by the Accili group (Talchai et al., 2012). Specifically, the role of FOXO1, an important transcription factor involved in β-cell adaptation to stress was explored. β-cell specific ablation of FOXO1 and lineage tracing analysis demonstrated that the decrease in β-cell mass following a range of metabolic stressors, including ageing and pregnancy, was due to β-cell de-differentiation and conversion of β- cells to α- cells, rather than β-cell death. In this study, chromogranin A (ChrA) was used as a marker of endocrine cells, NGN3 was used as endocrine cell progenitor marker, and SRY-related HMG-box 9 (SOX9) served as a pre-endocrine progenitor marker. The group found abundant $ChrA^{+}/SOX9^{-}$ cells representative of an endocrine pre-β-cell phenotype. Moreover, they demonstrated that reduced FOXO1 expression and phenotypic conversions was also evident in rodent models of T2DM. In T2DM mice, Talchai et al. (2012) found a maintenance of endocrine cell mass demonstrated by ChrA and synaptophysin IF staining, in spite of high reductions in the expression of insulin, Pdx1 and MAF BZIP transcription factor A (MafA). These cells were called 'hormone-empty' cells ($ChrA^{+}/insulin^{-}/glucagon^{-}/somatostatin^{-}/PP^{-}$) that represented a population of dedifferentiated cells (Talchai et al., 2012). β-cell de-differentiation was further confirmed by Wang et al. (2014), who suggests that β-cells, in T2DM, dedifferentiate to NGN3-positive, and insulin-negative cells, which can be restored to their former, fully differentiated β-cell following insulin therapy (Wang et al., 2014).

Another study by the Accilli group using human pancreatic tissue (Cinti et al., 2016) provides circumstantial evidence for β-cell plasticity in T2DM. They reported conversion of β-cells to glucagon producing α-cells and somatostatin producing δ-cells suggesting β-cell trans-differentiation. They demonstrated this by presence of transcription factors central to β-cell activity in glucagon and somatostatin-expressing cells. They also reported de-differentiation in T2DM by assessing pancreata for the presence of Synaptophysin-positive cells (endocrine cells) that are hormone-negative in the islets ($Synaptophysin^{+}/insulin^{-}/glucagon^{-}/somatostatin^{-}/PP^{-}$). There was a 3-fold increase in dedifferentiated cells in T2DM as compared to the non-diabetic cohort (Cinti

et al., 2016). Spijker et al. (2015) have also used IF staining and double-immunogold labelling to study co-expression of insulin and glucagon in human pancreata from T2DM donors. They reported an 8-fold increase in insulin and glucagon co-expressing cells in T2DM compared to the controls. These cells represent an intermediate phase in the transition of cell phenotype from β to α. They report a loss of β-cell identity demonstrated by insulin-negative cells that expressed glucagon and β-cell transcription factor NK6 homeobox 1 (Nkx6.1) (Spijker et al., 2015).

Another study has demonstrated the direct conversion of β-cells to α-cells (trans-differentiation) following hyperglycaemia as a mechanism of β-cell dysfunction (Brereton et al., 2014). This transformation was reversible by restoring normal glucose levels through insulin therapy. Secretion of insulin from β-cells in response to glucose is regulated by ATP-sensitive potassium ($K_{ATP}$) channels. Loss of insulin secretion machinery due to inexcitability of this channel is implicated in neonatal and T2DM pancreas (Ashcroft and Rorsman, 2013). Brereton et al. (2014) demonstrate development of diabetes in adult mice on activation of $K_{ATP}$ channel mutation. This disease development led to hyperglycaemia resulting into insufficient insulin and over-expression of glucagon within islets. They also reported co-expression of insulin and glucagon-expressing cells (polyhormonal) within islets. When used lineage tracing techniques, these polyhormonal cells were found to be β-cells. These changes were associated with chronic hyperglycaemia as they were totally reversed by restoring normal blood glucose levels by insulin therapy and sulphonylureas. This study supports a direct role of glucotoxicity in driving β-cell trans-differentiation as a potential mechanism underlying β-cell dysfunction and loss.

The above studies each provide accumulative evidence of de-differentiation, trans-differentiation and loss of identity as a mechanism of β-cell dysfunction or failure in T2DM. However, studies from the Butler group argue against the existence or at least significance of such mechanisms (Butler et al., 2016). While they found the presence of 'hormone-empty' cells ($ChrA^+$/$insulin^-$/$glucagon^-$/$somatostatin^-$/$PP^-$) in human T2DM pancreata, they propose that the amount of these cells is not enough to account for loss of endocrine mass. Moreover, they found that these 'hormone-empty' cells are usually found scattered in the exocrine pancreas, rather than in islets. Such a distribution of 'hormone-empty' endocrine cells is found in foetal and neonatal pancreas, thereby suggesting that they may be a source of (β-) cell-regeneration in T2DM (Butler et al., 2016).

Finally, cytokines have also been implicated in contributing to β-cell dysfunction in T2DM. Nordmann et al. (2017) reported that inflammation mediated by pro-inflammatory cytokines including IL-1β, TNF-α and IL-6 trigger *β*-cell de-differentiation and dysfunction in *in vitro* cultures of mouse and human islets (Nordmann et al., 2017). The core presence of autoimmunity and associated cytokine release in T1DM, suggests that these mechanisms may also occur in this form of diabetes.

### 3.1.4 Evidence for de-differentiation and plasticity in human T1DM

Although the idea of β-cell dysfunction in T1DM is gaining acceptability, little work has been done to demonstrate direct evidence of de-differentiation and plasticity in human T1DM. Nevertheless, conclusions from studies of different nature, suggest the possible existence of such mechanisms in T1DM.

Firstly, an increasing number of studies have been reporting residual β-cells and insulin (micro) secretion even in long-standing diabetes (Cnop et al., 2005, Keenan et al., 2010, Oram et al., 2015, Davis et al., 2015). Also, some suggest that the loss of β-cells cannot be accounted by apoptosis alone due to absence of apoptotic cells in the islets. While this could be attributed to the efficiency of macrophages to clear the apoptotic cells from the islet structure, this could also suggest absence of high numbers of apoptotic cells altogether (Morgan and Richardson, 2016).

A study by Powers and colleagues has demonstrated abnormal glucagon secretion in T1DM (Brissova et al., 2018). They did not find presence of cells co-expressing insulin and glucagon in human T1DM pancreata but found low level expression of Nkx6.1 (a key β-cell transcription factor) in glucagon-expressing cells. Moreover, they even found a 2-fold increase in glucagon expression in isolated islets from T1DM compared to their controls (Brissova et al., 2018). Hyperglucagonaemia is also observed in children with recent-onset T1DM (Brown et al., 2008). In such cases, there was a 37 % increase in glucagon secretion post meals, compared to the age-match controls, suggesting a possible role of imbalance between insulin and glucagon secretion in T1DM aetiology.

Recently, a study by Butler et al. (2016), demonstrated the presence of ChrA-positive/hormone-negative (CPHN) ($ChrA^{+}$/$insulin^{-}$/$glucagon^{-}$/$somatostatin^{-}$/$PP^{-}$) cells in pancreatic islets of adult T1DM patients. Most of these hormone-negative cells were situated as single endocrine cells around the islets. They reported that the distribution of hormone-negative cells in T1DM pancreas is similar to the one found in neonatal

pancreas, indicating cell regeneration. However, 10 – 20 % of these CPHN cells were found to express Nkx6.1, indicative of β-cell lineage. Moreover, they found a 50 % increase in expression of non-β-cell hormones (glucagon, somatostatin and PP) per islet in T1DM pancreata compared to controls (Md Moin et al., 2016). Another study by the same group in children with T1DM demonstrated no difference in the number of CPHN cells compared to age-matched controls, but an increase compared to the above adult T1DM cohort. A substantial proportion of these CPHN cells (about 6%) were also expressing the cell proliferation marker Ki67 indicating a population of replicating cells (Md Moin et al., 2017).

Studies from the Levine group (Chung and Levine, 2010, Piran et al., 2014b) have provided evidence of trans-differentiation in pharmacologically-induced models of T1DM. First, they demonstrated β-cell neogenesis by trans-differentiation of pre-existing *α*-cells by partial duct ligation (PDL) in a high dose alloxan-induced (β-cell toxin) model of T1DM (Chung et al., 2010). In another study, they used a combination of drugs, caerulein (pancreatitis inducer) and alloxan, to induce trans-differentiation of α-cells to β-cells. These newly-formed β-cells then converted to somatostatin-expressing δ-cells, thereby demonstrating islet cell plasticity in murine T1DM (Piran et al., 2014b).

Together these studies indicate presence of transitional β-cell phenotypes in T1DM that may contribute to β-cell dysfunction. However, further work to describe de-differentiation and/or trans-differentiation in T1DM is needed to assess the contribution of each in driving β-cell failure in T1DM.

### 3.1.5 Aims

The potential contribution of de-differentiation and trans-differentiation in the aetiology of T1DM has not been explored in detail. The biggest challenge in T1DM research is the availability of suitable (fit for study) pancreatic samples. In the last 50 years, less than 600 human T1DM pancreatic specimens have been studied (Morgan and Richardson, 2018). The Exeter Archival Diabetes Biobank (EADB) is home to one of the world-wide largest collection of autopsy samples from young T1DM patients under the age of 20-years. Studies from these samples have revealed reduction and/or total absence of β-cells in different T1DM patients (Morgan and Richardson, 2018). However, a more tantalising finding was that about 50 % of patients retain varying levels of insulin-producing cells even in long standing diabetes of over 50-years (Lohr

and Kloppel, 1987, Keenan et al., 2010), suggesting that the response of β-cells to aggressive immune attack may differ on a case-by-case basis.

A key feature studied in human T1DM pancreas is insulitis - the process of immune cell infiltration in islets. The mechanism of insulitis in T1DM involves the recruitment of immune cells, importantly cytotoxic T-cells (CD8+), to the inflamed islets which bring about the destruction of β-cells (Willcox et al., 2009). However, variable extents of immune cell infiltration have been observed in patients (Morgan and Richardson, 2016). Another type of immune cell, CD20+ B cells are also involved in the insulitis process. On examination of human pancreas from deceased donors, it was observed that the patients diagnosed before the age of 7 years have a CD20Hi profile of insulitis, containing of a high number of both T-cells and B-cells, while the patients diagnosed after 13 years of age have a milder insulitic profile (CD20Lo) consisting of T-cells, but very low amount of B-cells (Leete et al., 2016). This may suggest that the disease is less aggressive in patients that are diagnosed in their teenage years and the rate of loss of β-cells may be slower. Moreover, there is a direct relationship between residual insulin-containing islets (ICIs) and the age at disease onset. Approximately 40 % islets containing insulin were observed at diagnosis in patients with CD20Lo profile. This may suggest β-cell dysfunction, in addition to β-cell loss may play an important role in disease development at least in this group (Leete et al., 2016).

Using immunohistochemical techniques, the study aimed to determine islet hormone expression profiles and evidence of any transitional phenotypes in T1DM pancreata obtained from deceased donors within two distinct cohorts based on age-of disease-onset.

### 3.1.6 Specific objectives

1. To determine the number of insulin containing islets (ICIs) and islet endocrine (ChrA+) cells in each T1DM cohort and age matched controls.
2. To assess differences in hormone expression profiles between each T1DM cohort compared to age-matched controls.
3. To examine the presence of polyhormonal cells co-expressing insulin and other non-β-cell hormones in each T1DM patient cohort.
4. To assess the presence of ‘hormone-empty’ (CPHN) cells in each T1DM patient cohort.

## 3.2 Study design

Based on age-of-disease-onset, two clinically distinct cohorts were used for the study:

Cohort 1: Older-onset T1DM (>13-years of age)

Cohort 2: Young-onset T1DM (<7-years of age)

Formalin-fixed, pancreatic tissue sections from the EADB were studied to assess changes in islet endocrine composition in T1DM.

## 3.3 Results

### 3.3.1 Tissue sampling and donor characteristics of Cohort 1

Pancreatic tissue sections were obtained from the Exeter Archival Diabetes Biobank, UK. Appropriate ethical clearance, from the respective institutions, was obtained for use and transport of the tissues.

16 T1DM pancreatic tissue sections from deceased donors over the age of 13 years (Cohort 1) and eight age-matched control, non-diabetic pancreatic donors were selected for the study. T1DM donors were categorized into three groups based on the disease-duration:

i. Short-duration (<1-month); comprising recently diagnosed patients requiring insulin therapy.
ii. Mid-duration (1-month – 2-years); comprising patients on insulin therapy post-diagnosis but can be in remission/honeymoon phase in which their existing β-cells secrete significant levels of insulin (Moole et al., 2015).
iii. Long-duration (>2-years); comprising patients with established T1DM, outside remission period and requiring constant insulin treatment.

The Table 3.1 below provides information on the Cohort 1 T1DM donors and their non-diabetic controls.

*Table 3.1: Donor information on Cohort 1 T1DM and non-diabetic cases*

| Group | Donor ID | Age (years) | Sex | Age at onset | Disease duration | Insulitis |
|---|---|---|---|---|---|---|
| Non-diabetic controls | 65/71 | 40 | Male | - | - | - |
| | 191/67 | 25 | Male | - | - | - |
| | 12142 | 17 | - | - | - | - |
| | PAN1 | 22 | - | - | - | - |
| | PM146 | 18 | Female | - | - | - |
| | PM329 | 24 | Male | - | - | - |
| | PM333 | 16 | Male | - | - | - |
| | PM447 | 32 | Female | - | - | - |
| Short-duration T1DM (<1-month) | E168 | 18 | Male | 18 | recent | Yes |
| | E396 | 14 | Female | 14 | 1 day | Yes |
| | E431 | 14 | Female | 14 | <1 week | Yes |
| | SC57 | 18 | Female | 18 | <1 week | Yes |
| | SC76 | 20 | Male | 20 | 3 weeks | Yes |
| Mid-duration T1DM (-1 month – 2-years) | E260 | 15 | Female | 13 | 2 years | No |
| | E385 | 19 | Female | 17 | 1.5 years | Yes |
| | E386 | 15 | Male | 16 | 6 months | Yes |
| | E556 | 18 | Male | 18 | 4 months | Yes |
| | E560 | 42 | Female | 40 | 1.5 years | Yes |
| Long-duration T1DM (>2 years) | E557 | 22 | Male | 18 | 4 years | No |
| | SC100 | 27 | Male | 20 | 7 years | Yes |
| | SC107 | 18 | Female | 13 | 5 years | No |
| | SC109 | 20 | Male | 14 | 6 years | No |
| | SC112 | 22 | Male | 13 | 9 years | No |
| | SC116 | 35 | Male | 20 | 15 years | No |

### 3.3.2 Optimization of primary antibodies for IF staining

To assess changes in islet endocrine constitution, IF staining was conducted for ChrA, insulin, glucagon, somatostatin and PP. ChrA was used as a general endocrine cell marker that is contained within the secretory granules of all hormone-producing cells. Insulin was used as a β-cell marker, and the hormone cocktail that comprised of glucagon, somatostatin, and PP was used to mark α-cells, δ-cells and PP cells respectively. Glucagon, somatostatin and PP (three different primary antibodies) were all detected using a single secondary antibody so all the three non-β-hormones could be detected on a single fluorescence channel on the microscope.

As all antibodies were added simultaneously on the tissue section, it was vital that they were optimized to give good signal for the given buffering conditions (Ref Methods 2.2). For this, all above antibodies were tested at five dilution factors (1:50, 1:100, 1:250, 1:500, and 1:1000) in parallel with no primary antibody controls. An example of ChrA optimization at 5 dilution factors is shown below (Figure 3.1). Based on the fluorescence imaging, the dilution factor with the best quality and least background staining was selected for use (1:250 dilution used for ChrA to achieve best signal to noise ratio).

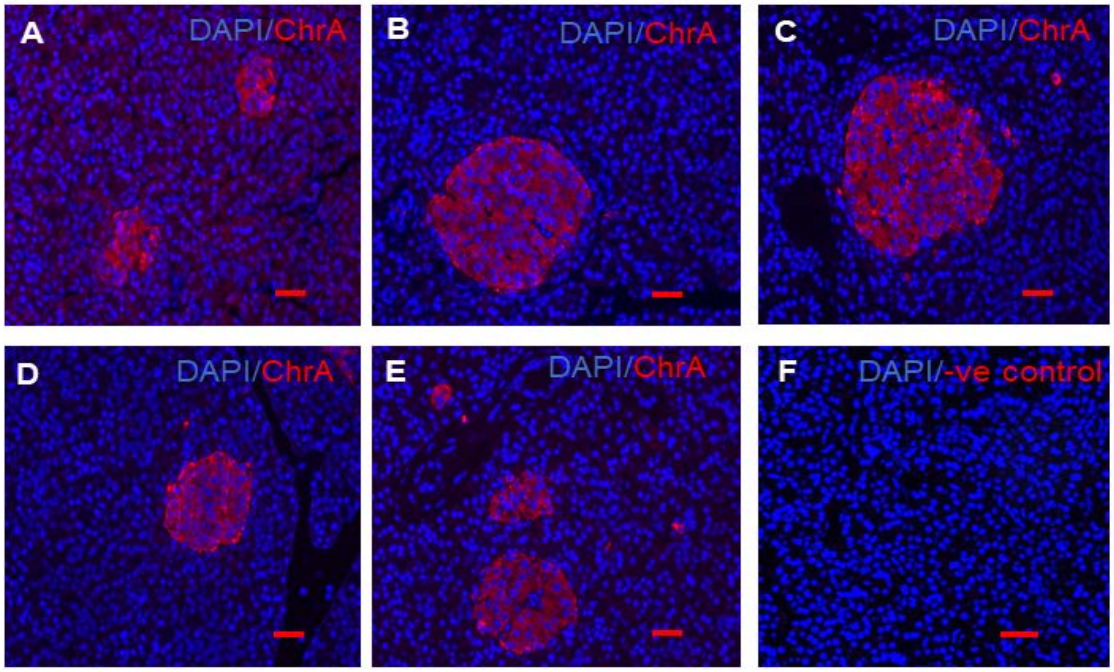


*Figure 3.1: Optimization of anti-ChrA primary antibody concentration*

*Anti-ChrA primary antibody optimization achieved by testing at five different dilutions of the stock. Scale bars represent 25 µm. Tested dilutions were 1:50 (A), 1:100 (B), 1:250 (C), 1:500 (D) and 1:1000 (E), along with a no-primary negative control (F).*

As the anti-glucagon, anti-somatostatin, and anti-PP antibodies were all raised in mouse and expression detected using the same fluorophore conjugated secondary antibody (Alexa Fluor 647), optimization was performed so that the selected concentration for the three primary antibodies produced similar signal intensities.

### 3.3.3 Assessment of changes in insulin-containing islets (ICIs) in T1DM and non-diabetic pancreata

As T1DM is classically characterized by loss of insulin-producing β-cells, the number of ICIs was determined to study the extent of β-cell loss in the pancreatic tissue sections. Insulin expression in 50 islets (wherever possible) was assessed in each non-diabetic and T1DM donor to determine the percentage of islets containing insulin. Figure 3.2 demonstrates representative IF images of islets (4x magnification) showing $ChrA^+$ endocrine cells in islets (red) and its insulin expression (green). The number of ICIs appear to decline with disease progression. Quantification of ICIs in control and T1DM donors is shown in Figure 3.3.

All islets (100%) in the control group contained insulin, whereas ICIs in T1DM were decreased by approximately 75 % as compared to non-diabetic donors (Figure 3.3A), and this decline was found to be dependent on the length of disease duration (short vs mid vs long: 54.1 ± 13 % (n = 5) vs 27.4 ± 7.9 % (n = 5) vs 1.8 ± 1.2 % (n = 6) (Figure 3.3B), with only 1 donor (SC100) in the long-duration T1DM group showing ICIs. Also, a lobular distribution of ICIs was found within the T1DM pancreata, with islets rich in insulin confined to certain lobes and not uniformly distributed throughout the pancreas.

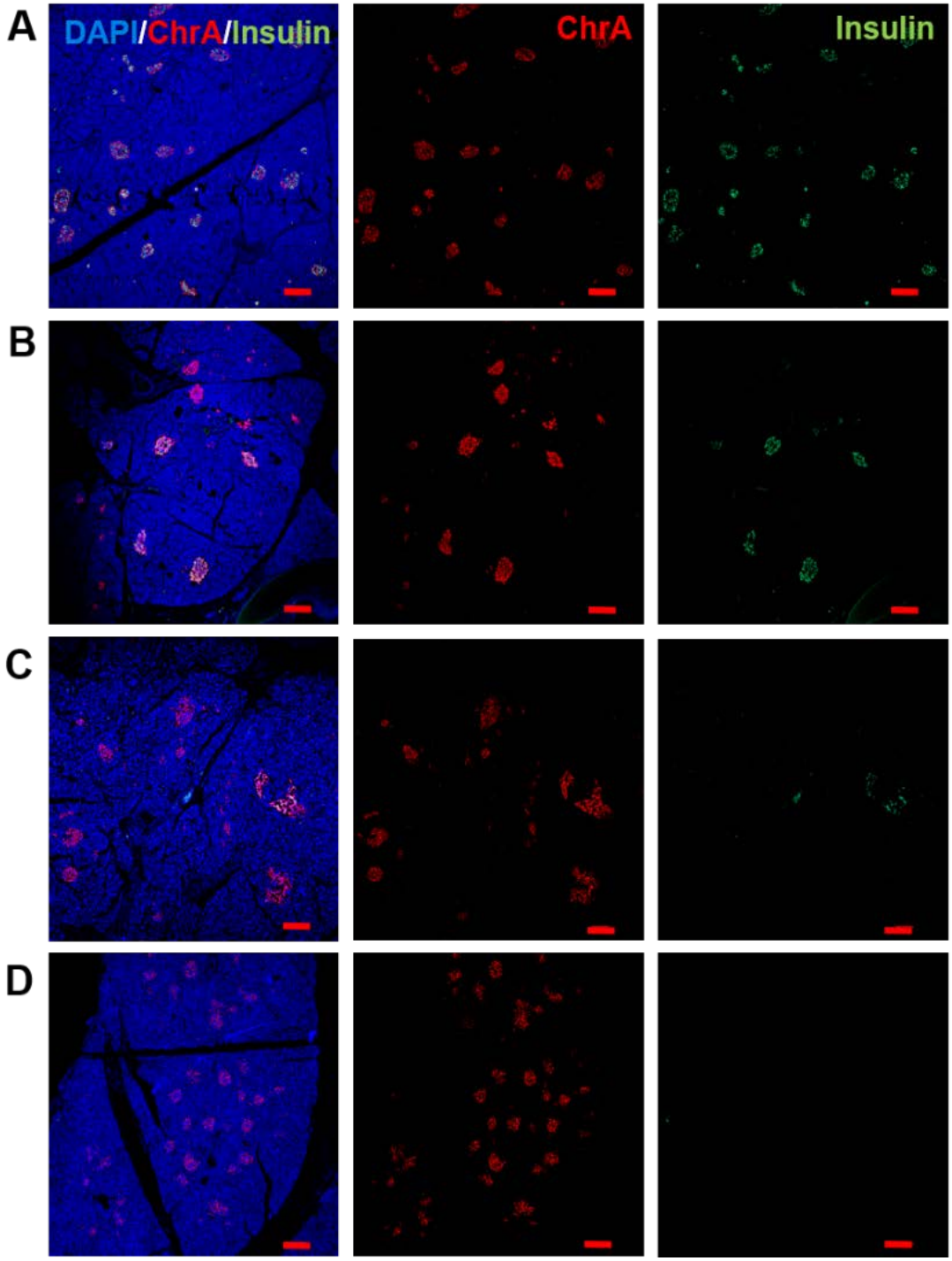


*Figure 3.2: Representative IF images showing reduction in ICIs with progression in disease-duration*

*Representative IF images, taken at 4x magnification on Nikon A1 confocal microscope, showing islets from a non-diabetic donor PM333 (A), short- duration T1DM donor E396 (B), mid-duration T1DM donor E386 (C), and long-duration T1DM donor SC112 (D). Scale bars represent 200 µm.*

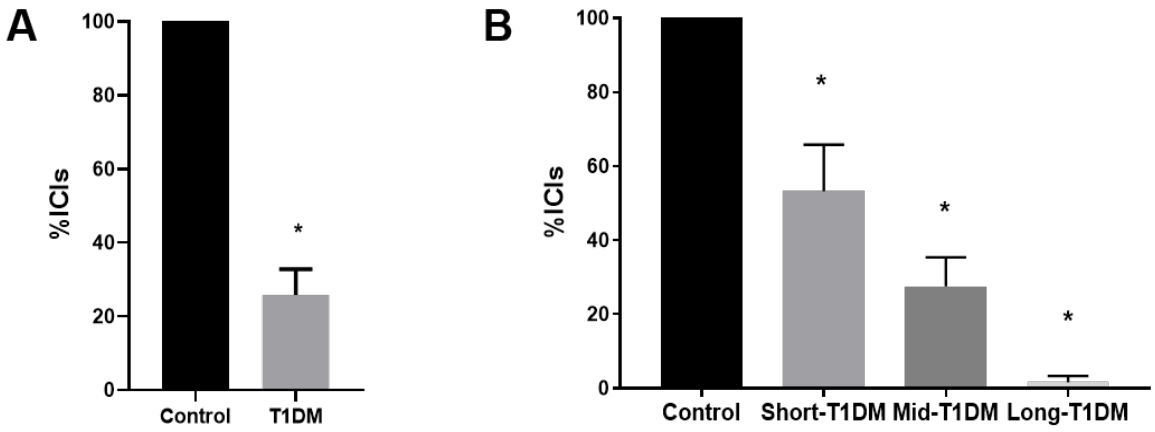


*Figure 3.3: Decline in number of ICIs with progression in disease-duration.*

*(A) Number of ICIs in T1DM (n = 16) was significantly reduced compared to non-diabetic Cohort (n = 8). (B) When categorized on the basis of disease duration, there was a gradual, significant decrease in percentage of ICIs with disease progression. Bars represent percentage of ICIs denoted as mean ± SEM (*p<0.001 compared to controls, unpaired Student's t-test (A), one-way ANOVA with post hoc analysis (B)).*

### 3.3.4 Representative images of islets in T1DM and non-diabetic donors

50 islets from each donor section were captured using Nikon A1 confocal microscope. Figure 3.4 indicates representative images of islets from non-diabetic and diabetic donors. All islets are marked by ChrA$^+$ cells (red). Within the islets, β-cells are marked by insulin (green), and non-β-cells are marked by hormone cocktail (3H, white). A key observation is that as the disease advances, there is a clear visual loss of insulin expression.

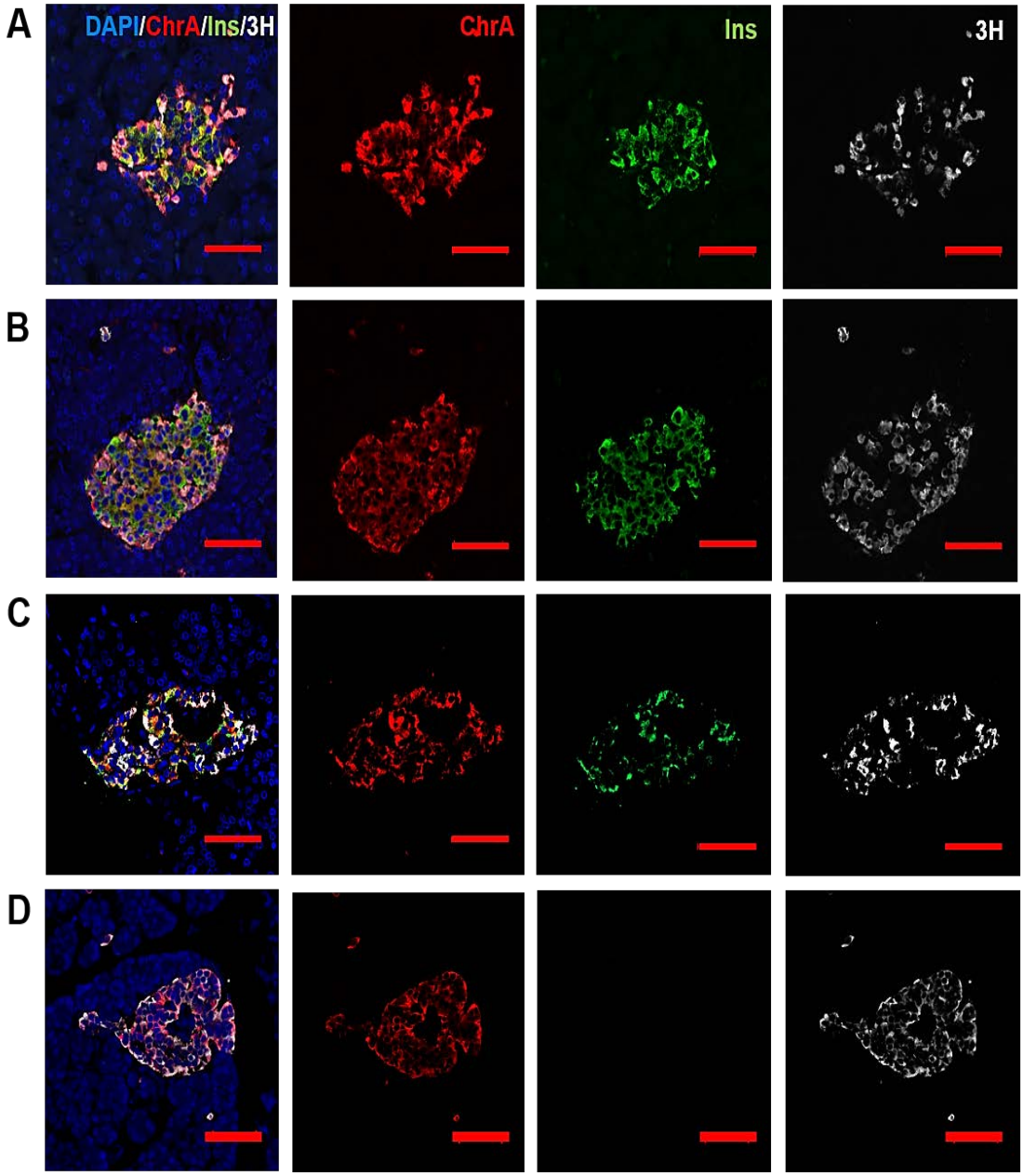


*Figure 3.4: Representative images of islets in normal and T1DM donors*

*Representative images of an islet in non-diabetic control donor (A), short-duration T1DM donor (B), mid-duration T1DM donor (C), and long-duration T1DM donor (D). Images taken at 20x magnification on Nikon A1R confocal microscope. Scale bars represent 50 µm.*

### 3.3.5 Quantification of ChrA$^+$ endocrine cells per islet in T1DM and non-diabetic pancreata

To examine whether there is loss of endocrine cells within islets of patients with T1DM, the number of ChrA$^+$ cells was determined by manual counting. 50 islets (wherever possible) were counted on each section and a mean number of ChrA$^+$ cells

per islet was obtained. Figure 3.5 indicates the maintenance of number of ChrA$^+$ cells per islet in non-diabetic and T1DM cohorts.

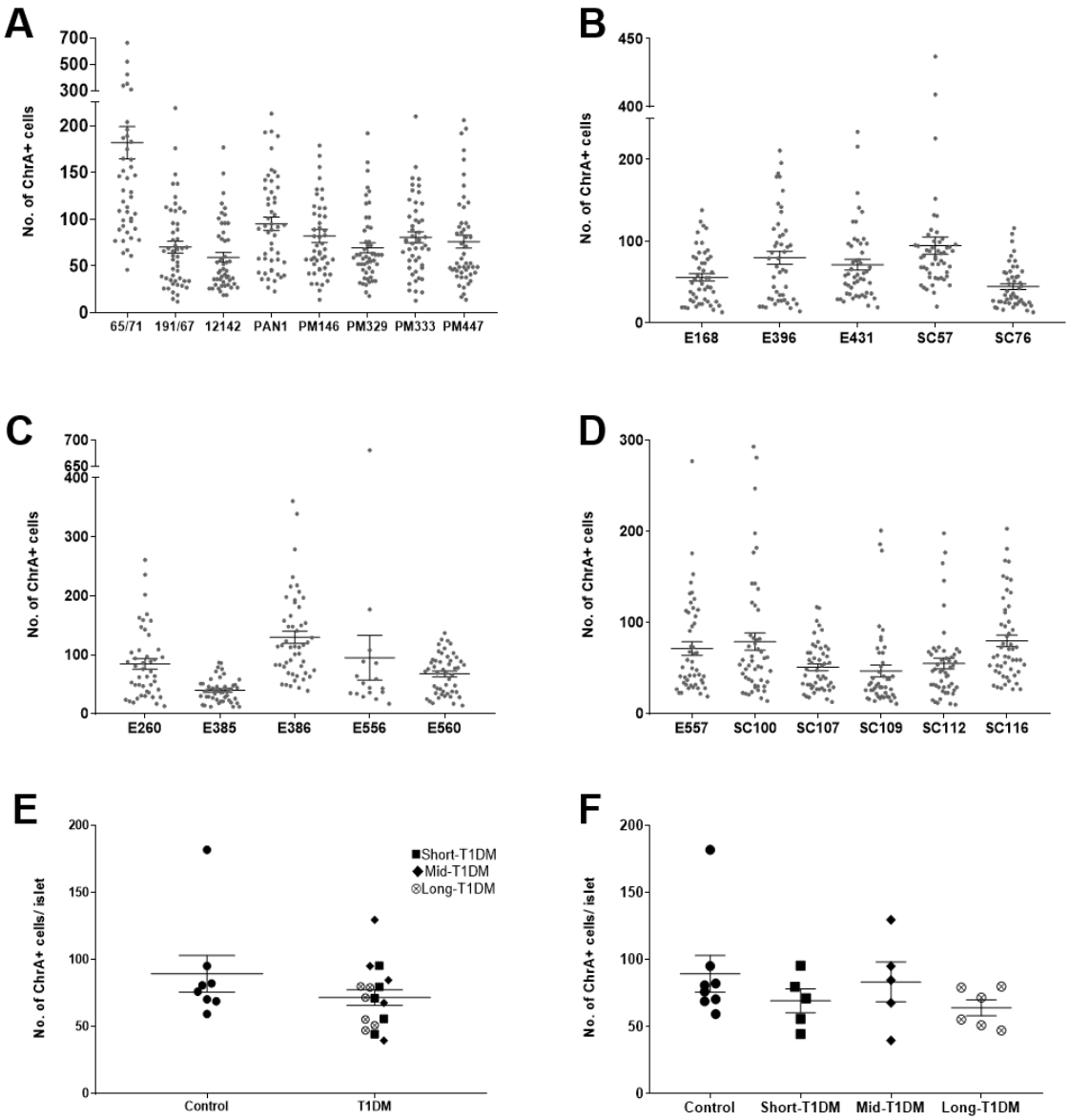


*Figure 3.5: Maintenance of ChrA$^+$ endocrine cell number in T1DM and non-diabetic states.*

*Quantification of endocrine cells (ChrA$^+$) per islet in non-diabetic and diabetic donors. Data represent mean ± SEM. (A-D) Graphical representation of the number of ChrA$^+$ cells in individual islets in controls (A), short-duration T1DM (B), mid-duration T1DM (C), and long-duration T1DM (D) (each dot represents a single islet). E) Dot-plot of the mean number of ChrA$^+$ cells per islet in each control (n = 8) and T1DM (n = 16) donor (denoted as separate dots). F) Dot-plot of the mean number of ChrA$^+$ cells categorised on the basis of disease duration.*

Figure 3.5C indicates while there is trend towards reduced mean number of endocrine cells per islet in T1DM, this is not significant compared to controls. Number of ChrA cells per islet cross-section in controls (89.3 ± 13.7 %) was not significantly

different from T1DM cohort (71.7 ± 5.2 %). When compared on the basis of disease duration (Figure 3.5), this number still remained insignificantly different from controls (short vs mid vs long: 69.2 ± 8.9 % (n = 5) vs 83.3 ± 14.9 % (n = 5) vs 64.5 ± 4.4 % (n = 6)).

Because T1DM is strongly associated with β-cell death, a larger decline of endocrine cells from islets was expected. However, this is not observed, supporting involvement of other mechanisms for loss of β-cells.

### 3.3.6 Determination of islet endocrine area in T1DM and non-diabetic pancreases

To determine endocrine area per islet, area of ChrA$^+$ staining was quantified using the NIS Elements software. To do this, desired region of interest i.e. areas of ChrA$^+$ staining were selected manually on the NIS Elements software. The software then gives an automated measurement of area in μm$^2$. Figure 3.6 below indicates the mean area of ChrA per islet (from 50 islets) in each donor.

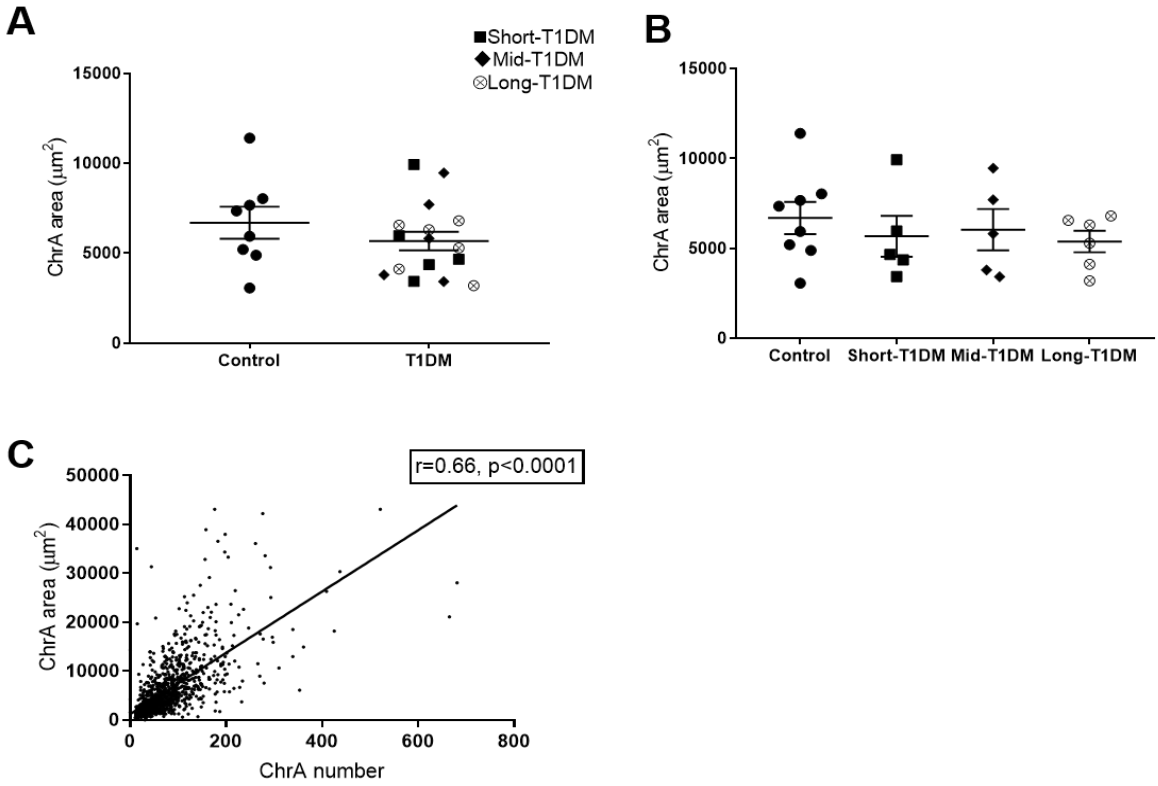


*Figure 3.6: Maintenance of endocrine area in T1DM and non-diabetic states.*

*Automated assessment of endocrine area (ChrA staining) per islet in non-diabetic and diabetic donors. Data represents mean ± SEM. (A) Dot-plot representing mean ChrA area per donor (denoted as separate dots) in control (n = 8) and T1DM (n = 16) cohorts. (B) Further characterization of endocrine area based on disease duration also revealed*

*no significant changes in between the control and diabetic groups. (C) The correlation analysis of ChrA number with corresponding ChrA area (per islet) indicated a significant positive correlation between the two variables.*

The mean ChrA area per islet of control donors was 6690 ± 892.1 $\mu m^2$ (mean ± SEM) and that of T1DM donors was 5673 ± 517.8 $\mu m^2$ (mean ± SEM). Figure 3.6A indicates a trend towards decrease in ChrA area in T1DM group compared to controls. However, this change was not found to be significant according to unpaired Student's t-test. Also, no difference in the ChrA area was observed with disease progression (Figure 3.6B) A positive correlation was found between ChrA number and area indicating that mean endocrine cell size did not change with diabetes regardless of disease duration (Figure 3.6C).

### 3.3.7 Assessment of islet hormone expression profiles in T1DM and non-diabetic pancreata

To assess islet hormone expression profiles between control and diabetic groups, all $ChrA^+$ cells that were insulin-positive ($ChrA^+/Ins^+$) and $ChrA^+$ cells that were hormone cocktail-positive ($ChrA^+/3H^+$) in the islets were counted manually. Figure 3.7 demonstrates the mean number of insulin and non-β-cells per islet from each donor.

Figure 3.7 describes striking changes in islet hormone expression (denoted as number of β or non-β cells per islet) in non-diabetic and diabetic states. A significant decrease in insulin expression (number of insulin-expressing cells per islet) in the total T1DM cohort (11.5 ± 4.1) was observed in comparison to the control, non-diabetic donors (56.0 ± 6.04). Moreover, expression of non-β-cell hormones (number of non-β-hormone-expressing cells per islet) was significantly increased in the T1DM cohort (60.9 ± 4.7) compared to controls (36.5 ± 8.5). As is expected in diabetes, insulin expression is highly affected by disease duration, as the number of insulin-positive cells decreased with disease progression i.e. the longer the disease duration, the less insulin-positive cells. This phenomenon was evident in our data in which insulin expression significantly decreased with the length of disease duration (short vs mid vs long: 25.6 vs 14.7 vs 0.3). Only one donor in the long-duration T1DM cohort showed insulin-expressing islets. Hence, the number of insulin expressing cells is as low as 0.3 (mean of 50 islets) in that group. A more interesting finding was the increase in number of non-β-cells. Expression of non-β islet hormones increased with increase in disease duration (short vs mid vs long: 46.2 vs 71.6 v 63.4). While the increase was gradual

with increasing duration, it was statistically significant between the control and mid-duration T1DM groups.

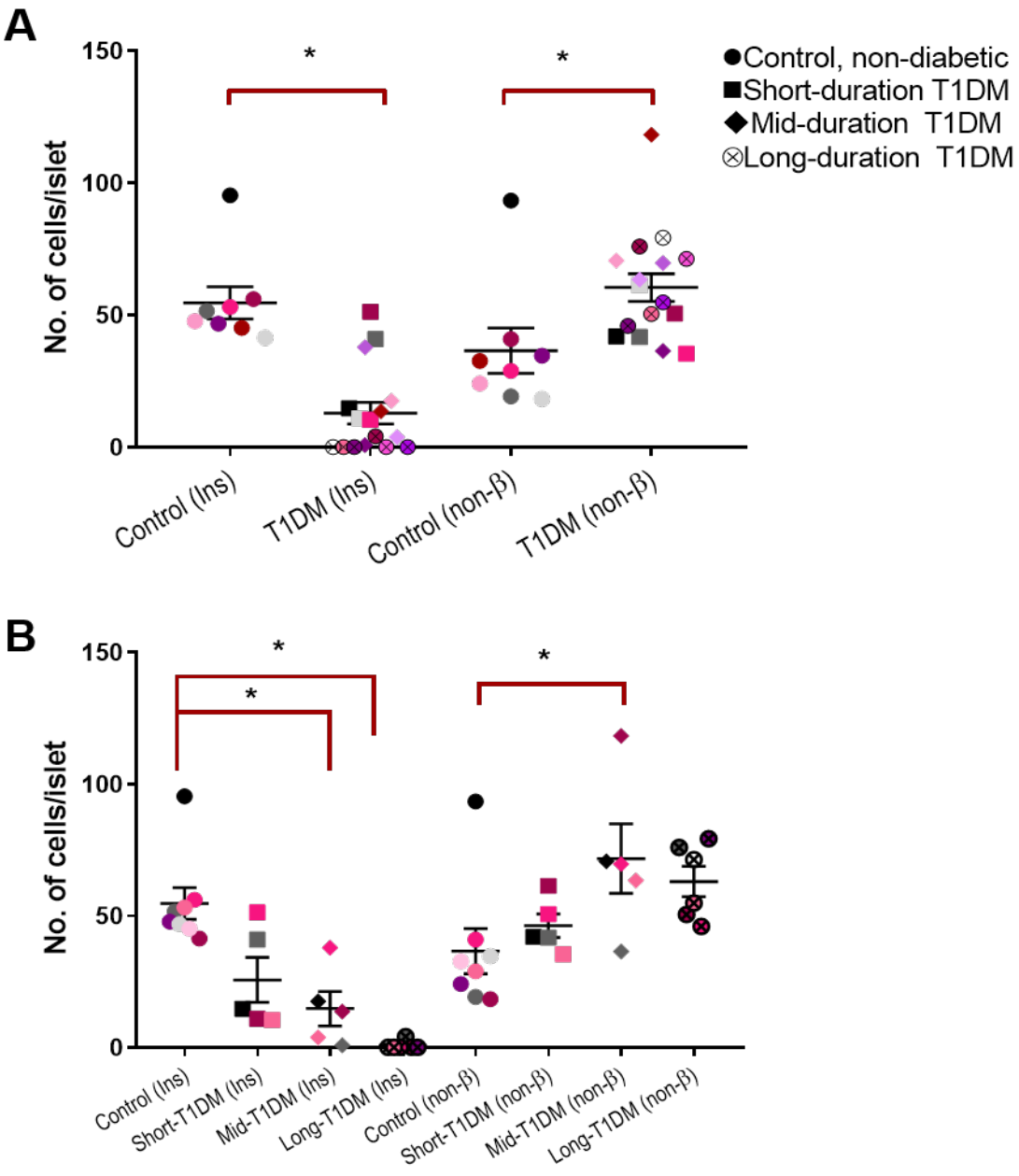


*Figure 3.7: Changes in islet hormone expression profiles in T1DM and non-diabetic donors.*

*(A) Quantification of hormone expression demonstrates a significant decrease in insulin expression in the total T1DM (n = 16) cohort when compared to the control, non-diabetic cohort (n = 8). In parallel, significant increase in expression of non-β-cell hormones was evident in the T1DM cohort compared to controls. (B) When categorized on the basis of disease duration, these changes in hormone expression profiles were associated with the duration of disease. Insulin expression significantly decreased with the length of disease duration, whereas expression of other non-β-cell hormones increased with increase in duration. Data represents mean ± SEM (*p<0.05 compared to controls, one-way ANOVA with post hoc analysis)*

### 3.3.8 Evidence of β-cell trans-differentiation in T1DM

Studies in mice and human T2DM have reported trans-differentiation of insulin-producing β-cells to glucagon-producing α-cells in response to chronic hyperglycaemia (White et al., 2013, Brereton et al., 2014, Cinti et al., 2016), demonstrated by cells that co-express insulin and glucagon or by cells that express β-cell specific transcription factors and glucagon (Brereton et al., 2016). This suggests that this switch of β-cells to α-cells might contribute to the overall loss of β-cells from the islets and also may be a potential cause of hyperglucagonaemia that is observed in T2DM (Brereton et al., 2016). Abnormal glucagon secretion is also observed in T1DM (Brissova et al., 2018). Moreover, Levine group has reported conversion of β-cells to δ-cells in a drug-induced model of T1DM (Piran et al., 2014b). These studies, therefore, led to the hypothesis that such a phenotypic shift in β-cell identity might even be relevant to T1DM.

Thus, one of the aims of the study was to determine whether there was evidence for β-cell trans-differentiation or plasticity i.e. conversion to alternative endocrine (non-β) cell types in T1DM. This fate conversion would be associated with a transitional phenotype in which the cell may express β-cell as well as non-β-cell hormones

To assess this, IF staining to determine expression of ChrA, insulin and hormone cocktail (3H) consisting of glucagon, somatostatin and PP. For the assessment of β-cell trans-differentiation, $ChrA^+$ cells co-expressing insulin and non-β hormone cocktail were evaluated and quantified. Representative images of these polyhormonal cells (Figure 3.8) and their quantification in non-diabetic and T1DM donors (Figure 3.9) is shown below.

Figure 3.8 shows images of islets from a non-diabetic and diabetic donor. In Figure 3.8A, a spectral profile plot showing fluorescence intensities of a typical (non-diabetic) β-cell and non-β-cell is depicted. In Figure 3.8B, an islet containing polyhormonal cells from a diabetic donor is shown. A polyhormonal cell is defined as a $ChrA^+$ cell that expresses insulin and the hormone cocktail as indicated in the figure. The spectral profile plot (Figure 3.8B) confirms co-localisation of the three markers in a single cell, providing evidence of endocrine cell trans-differentiation in T1DM.

Polyhormonal cells were observed in the islets of both, non-diabetic and diabetic donors. Not all islets contained polyhormonal cells, and the number of polyhormonal cells in each islet in non-diabetic and diabetic donors is shown in Figure 3.9A-D.

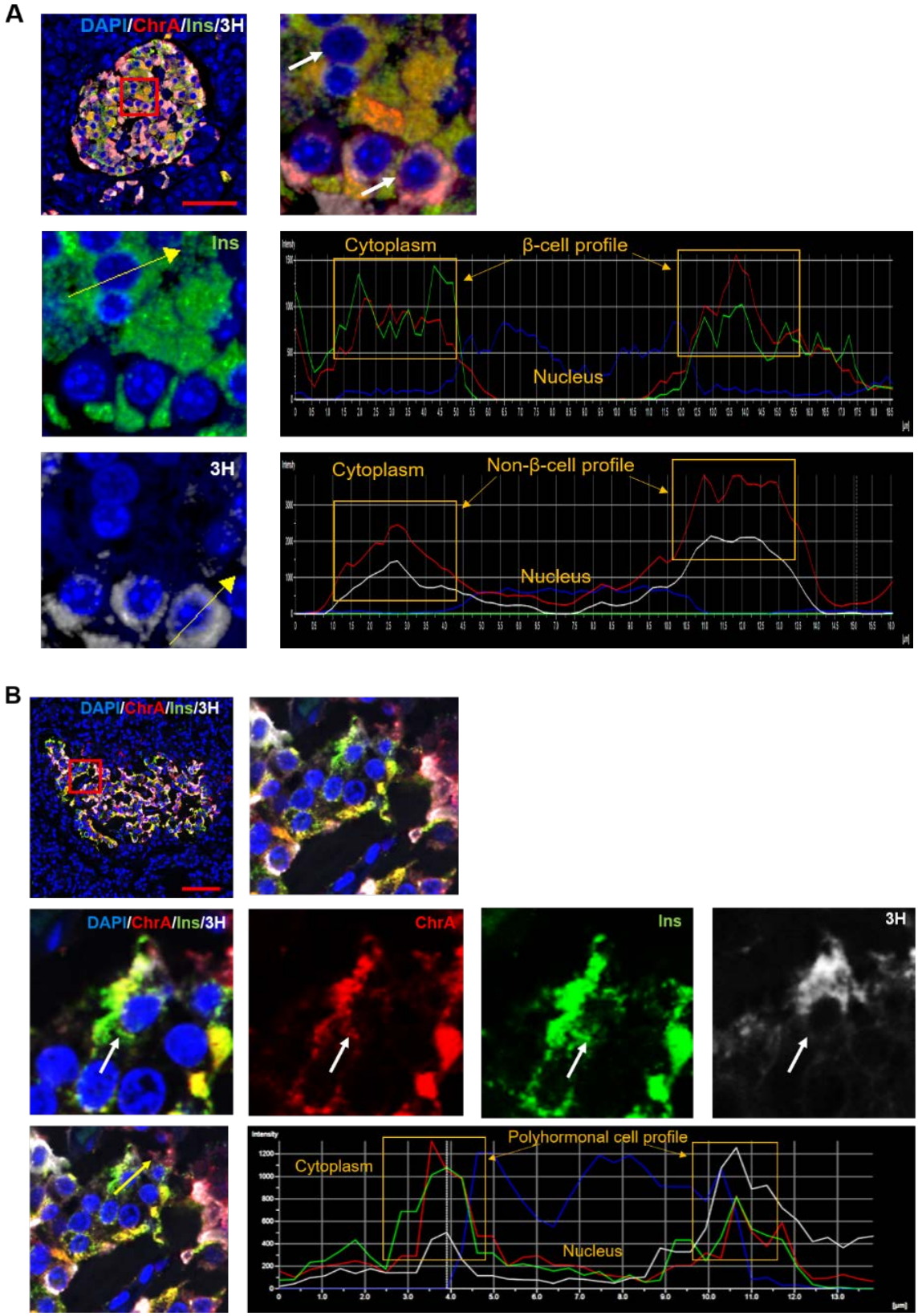


*Figure 3.8: Evidence of β-cell trans-differentiation in T1DM*

*(A) Representative images of hormone expression in an islet (at 20x magnification) from non-diabetic donor. Red box represents magnified region. The arrows indicate a β-cell and a non-β-cell. A spectral profile plot of the two cells is done using NIS Elements AR software to confirm expression of insulin (for β-cell) and hormone cocktail*

*(non-β-cell) on a ChrA⁺ cell. (B) Representative images of hormone expression in an islet (at 20x magnification) from short-duration T1DM donor E396. Red box represents magnified region. The arrow indicates a polyhormonal cell co-expressing insulin and hormone cocktail. To further confirm co-expression, we performed a spectral profile plot using NIS Elements AR software which demonstrates the excitation of all three channels at the same point on the cell (yellow boxes). Scale bars represent 50 µm.*

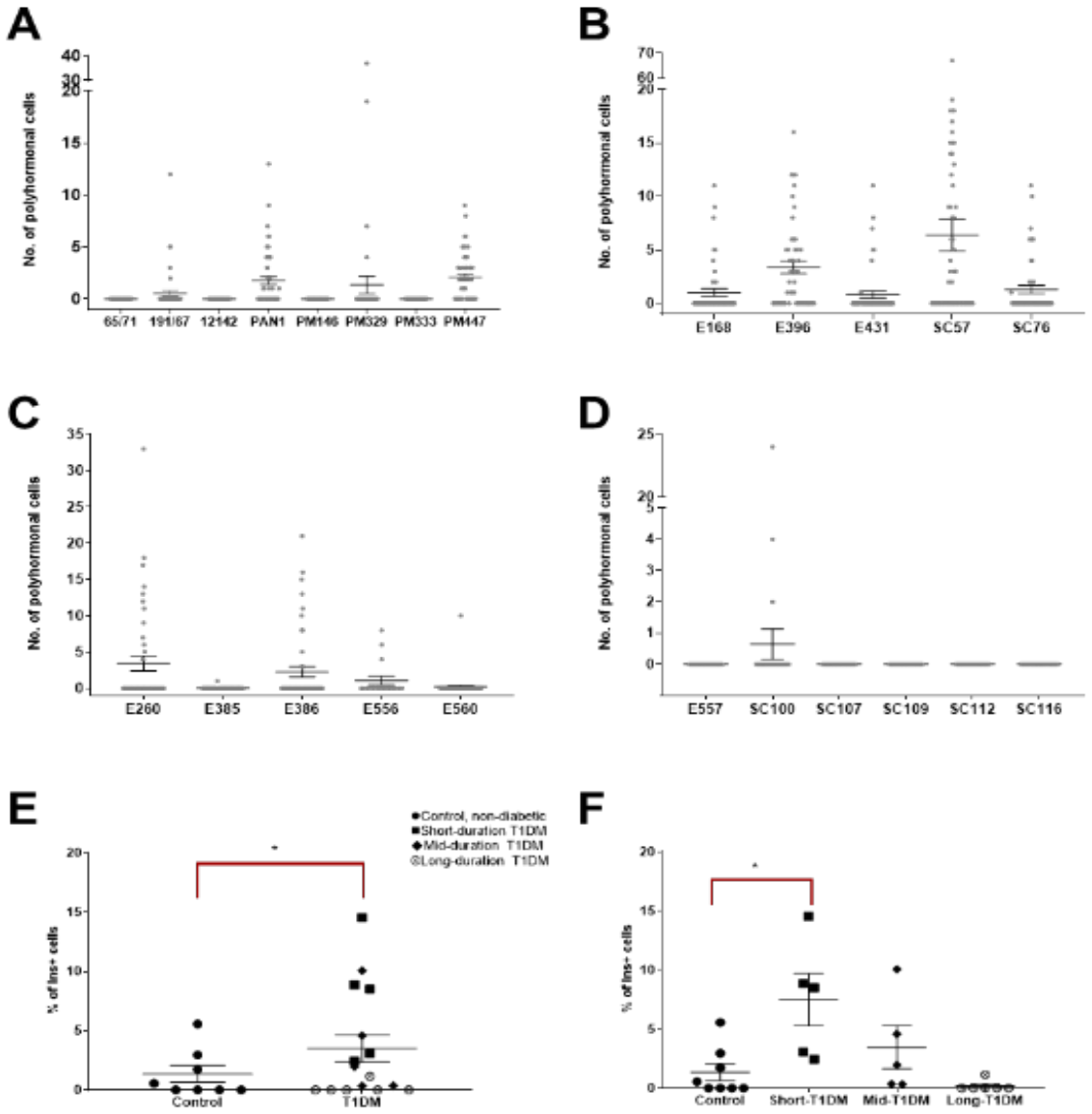


*Figure 3.9: Quantification of polyhormonal cells in T1DM and non-diabetic pancreata*

*Quantification of polyhormonal cells was assessed by measuring the number of insulin-positive cells co-expressing non-β-cell hormones (3H). (A-D) Dot-plot of the number of polyhormonal cells per counted islet in 8 non-diabetic donors (A), 5 short-duration T1DM donors (B), 5 mid-duration T1DM donors (C), and 6 long-duration T1DM donors (D). (E) Percentage of insulin⁺ cells co-expressing non-β-cell hormones was significantly greater in T1DM than in control donors. (F) Further, polyhormonal cells were significantly higher in the short duration cohort compared to the controls. Data represents mean ± SEM (*p<0.05, Student's unpaired t-test (E), one-way ANOVA with post hoc analysis (F)).*

As polyhormonal cells can only be present in ICIs, polyhormonal cells were calculated as a percentage of insulin-positive cells per donor (Figure 3.9E-F). This revealed that percentage of polyhormonal/insulin cells was significantly greater (over two times) in T1DM (3.5 ± 1.1 %, n = 16) than in control pancreas (1.4 ± 0.7%, n = 8). Moreover, disease duration was also a factor in the presence of polyhormonal cells. Polyhormonal cells in short-duration T1DM (7.5 ± 2.2 %) were nearly twice more frequent than the mid-duration T1DM (3.5 ± 1.8 %) and were significantly more commonly observed than in the non-diabetic group. As insulin-expressing cells were only observed in one of the long-duration T1DM donors, polyhormonal cells were very rare in this group. This finding indicates that presence of polyhormonal cells in T1DM group is associated with the number of insulin-positive cells and hence, evidence of β-cell trans-differentiation by this method is restricted to the early stages of the disease when there are still a substantial number of insulin-producing β-cells in the islets.

### 3.3.9 Quantification of ChrA$^+$/hormone$^-$ (CPHN) cells in T1DM as a measure of de-differentiation in T1DM

As described earlier, cells expressing ChrA but lacking insulin, glucagon, somatostatin and PP have been previously described as dedifferentiated or regenerating cells in human T1DM and T2DM pancreata (Talchai et al., 2012, Md Moin et al., 2016, Butler et al., 2016, Md Moin et al., 2017). The same approach was employed in this study to determine if such ChrA$^+$/hormone$^-$ (CPHN) cells were observed in the islets of T1DM pancreata. Figure 3.10 shows representative images and quantification of CPHN cells in T1DM pancreata.

Figure 3.10A shows a representative image of an islet from a T1DM donor containing CPHN cells. CPHN cells were calculated as a percentage of ChrA$^+$ cells per donor. Although the frequency of CPHN cells in the islets of T1DM donors (1.3 ± 0.4 %) was very low, these cells were absent in non-diabetic islets. Also, CPHN cells were the highest in the mid-duration T1DM donors (1.7 ± 1.1 %) compared to long-duration T1DM donors (1.4 ± 0.3 %) and the short-duration T1DM cohort (0.6 ± 0.3 %). Even if in small numbers, these cells seem specific to T1DM and may be representative of a transitional phenotype in diabetes.

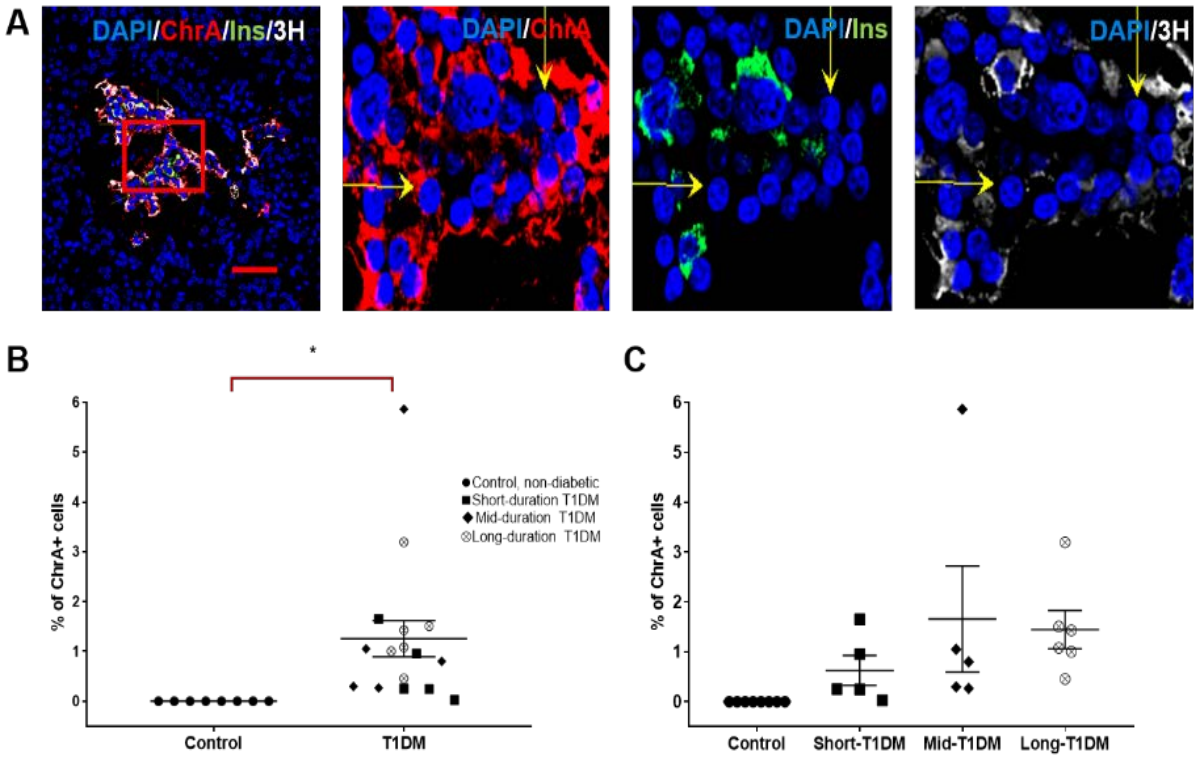


*Figure 3.10: Presence of CPHN cells in T1DM*

*Representative images of hormone expression in an islet from T1DM donor. Box represents magnified area. The red arrows indicate ChrA+ cells not expressing insulin and hormone cocktail (3H). Scale bars represent 100 µm. (B) Quantification of CPHN cells was done by measuring the percentage of ChrA⁺ cells not expressing any islet hormones. The extent of hormone-empty cells was significantly greater in T1DM than in control pancreas. (C) CPHN cells, in comparison to non-diabetic controls, were more abundant in the mid duration cohort and long duration and the least in the short-duration cohort. Data represents mean ± SEM (*p<0.05 compared to controls, Student's unpaired t-test)*

### 3.3.10 Tissue sampling and donor characteristics of Cohort 2

T1DM is characterized by loss of β-cells due to cytotoxic T-cell mediated immune attack. This autoimmunity leads to leucocyte infiltration in and around the islets by a process called as insulitis (In't Veld, 2011). As described earlier, owing to differences in the pattern of insulitis and immune infiltrate in the islets, two distinct groups of T1DM patients have been identified. These differences in insulitic lesions dictate the extent of β-cell loss and age of disease presentation. Principally, the biggest difference between the two groups is the presence of CD20+ B-cells. When high number of CD20+ B-cells are present in the immune infiltrate in the islets (CD20Hi), a more aggressive attack on islet β-cells occurs resulting into a faster loss of β-cells and consequently a smaller number of ICIs at disease diagnosis. Patients having such an

insulitic profile are diagnosed with T1DM before the age of 7 years. On the other hand, fewer number of CD20+ B-cells (CD20Lo) in the islet infiltrate are associated with a milder (less aggressive) form of immune attack. Patients having a CD20Lo profile of insulitis are usually diagnosed beyond their teenage years (>13 years) (Leete et al., 2016).

Due to these differences between the two patient groups, it is important to determine if these transitional events occur in younger T1DM patients who are diagnosed before 7 years of age. However, characterisation of insulitis was outside the scope of this project due to unavailability of H & E stained slides.

As with the previous Cohort 1, pancreatic tissue sections were obtained from the EADB. Appropriate ethical clearance was obtained for use and transport of the tissues. Four T1DM pancreatic tissue sections from deceased donors under the age of 7 years (young-onset T1DM) and four age-matched control, non-diabetic pancreatic donors were selected for the study. Table 3.2 below describes the details of the T1DM donors and their non-diabetic controls in Cohort 2.

*Table 3.2: Donor information on Cohort 2 T1DM and non-diabetic cases*

| Group | Donor ID | Age (years) | Sex | Age at onset | Disease duration | Insulitis |
|---|---|---|---|---|---|---|
| Non-diabetic controls | 240/90 | 2 | Male | - | - | - |
| | 242/89 | 3 | Male | - | - | - |
| | 2189 | 4 | Female | - | - | - |
| | 27491 | 6 | Male | - | - | - |
| T1DM donors | E254 | 6 | Female | 6 | recent | Yes |
| | E308 | 3 | Female | 3 | recent | Yes |
| | SC41 | 4 | Female | 4 | 3 weeks | Yes |
| | SC115 | 1.5 | Female | 1 | 1 week | Yes |

### 3.3.11 Assessment of changes in insulin-containing islets (ICIs) in T1DM and non-diabetic pancreata

As before, the number of ICIs was determined to study the extent of β-cell loss in the pancreatic tissue sections. Insulin expression in 50 islets (wherever possible) was assessed in each non-diabetic and T1DM donor to determine the percentage of

islets containing/deficient in insulin. Figure 3.11 indicates the decline in number of ICIs in T1DM compared to non-diabetic donors.

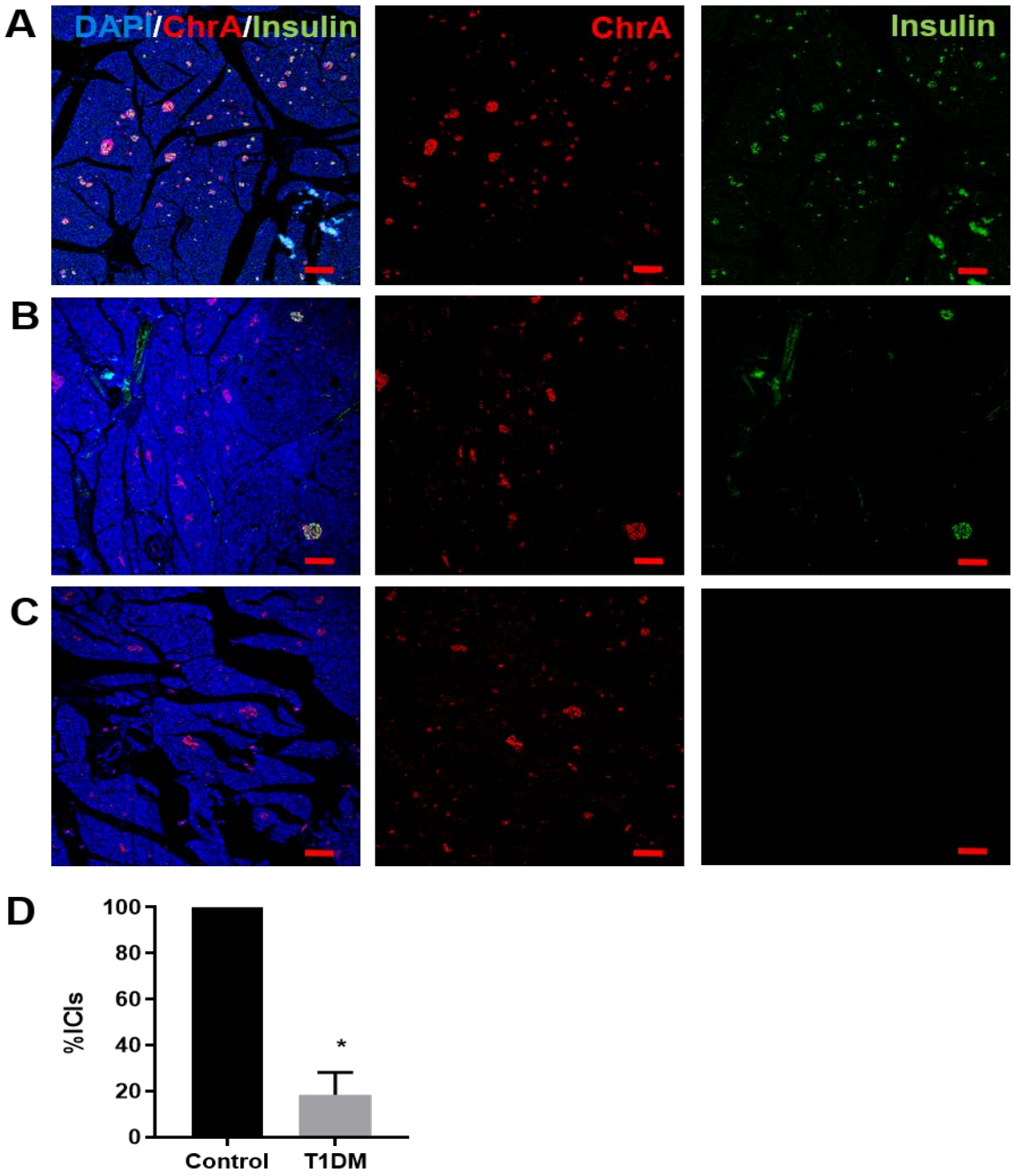


*Figure 3.11: Decline in number of ICIs in T1DM.*

*Representative IF images, taken at 4x magnification on Nikon A1 confocal microscope, showing islets from a non-diabetic donor 240-90 (A), and T1DM donors E254 (B) and E308 (C). Scale bars represent 200 µm. (D) Number of ICIs in T1DM (n = 4) was significantly reduced compared to non-diabetic cohort (n = 4). Bars represent percentage of ICIs denoted as mean ± SEM (*p<0.001 compared to controls, Student's unpaired t-test).*

As in Cohort 1, number of ICIs in T1DM was decreased as compared to the non-diabetic donors. However, this decrease was more exaggerated in the young

Cohort with over 80 % loss of ICIs (Figure 3.11). All islets (100 %) in the control group contained insulin whereas there was a significant decline in T1DM to an average 18 ± 10 % Percentage of ICIs between the four T1DM donors varied from 0 to 44 %.

### 3.3.12 Representative images of islets in T1DM and non-diabetic donors

50 islets from each donor section were captured using Nikon A1 confocal microscope.

Figure 3.12 indicates representative images of islets from non-diabetic and diabetic donors. All islets are marked by ChrA+ cells (red). Within the islets, β-cells are marked by insulin (green), and non-β-cells are marked by hormone cocktail (3H, white). In the T1DM islets, there was a more pronounced loss of insulin compared to T1DM Cohort 1.

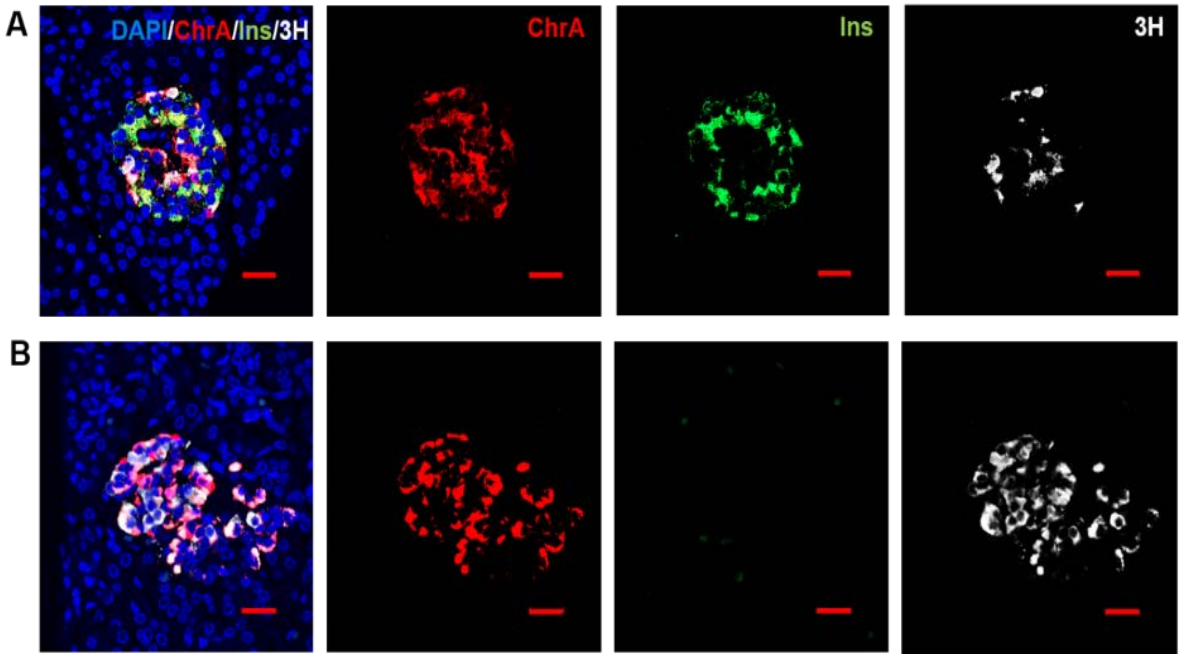


*Figure 3.12: Representative images of islets in normal and T1DM donors*

*Representative images of an islet in non-diabetic control donor (A), and a T1DM donor (B). Images taken at 20x magnification on Nikon A1R confocal microscope. Scale bars represent 50 µm.*

### 3.3.13 Quantification of ChrA+ endocrine cells per islet in T1DM and non-diabetic pancreata

To examine whether there is loss of endocrine cells within islets of patients with T1DM, the number of ChrA+ cells was determined by manual counting. 50 islets

(wherever possible) were counted on each section and a mean number of ChrA+ cells per islet was obtained. Figure 3.13 indicates the maintenance in number of ChrA$^+$ cells per islet in non-diabetic and T1DM cohorts.

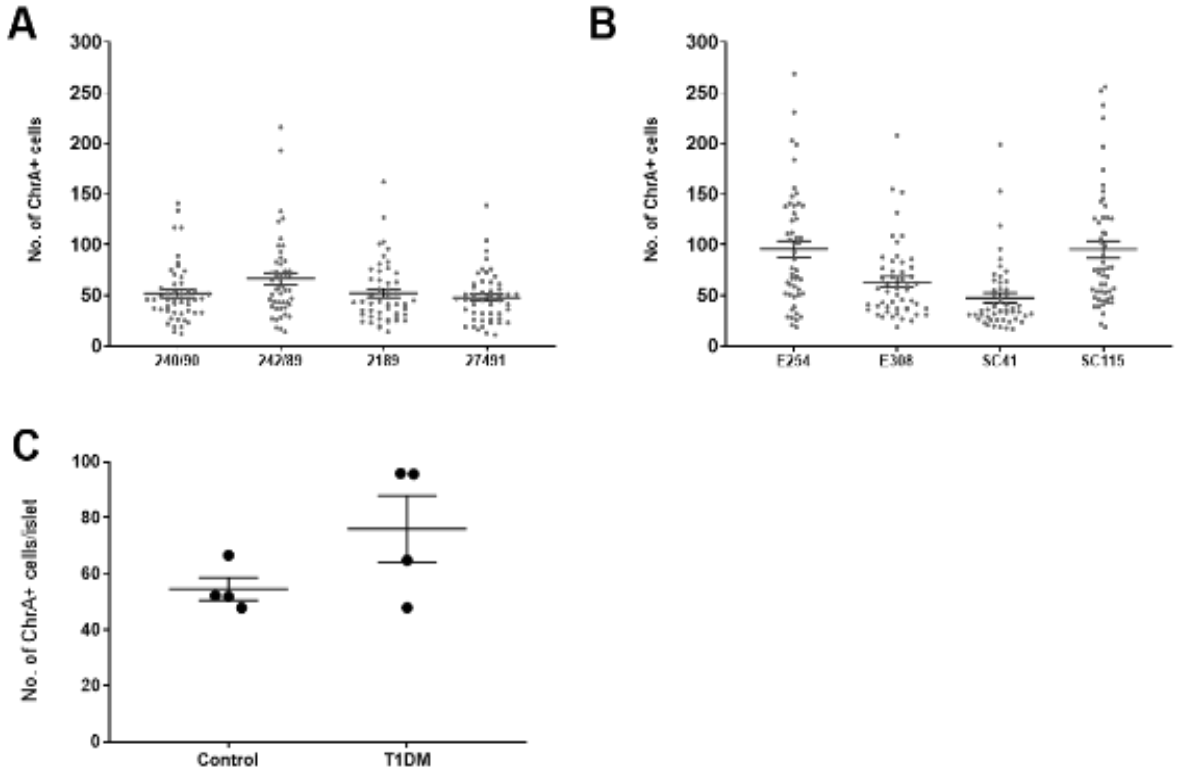


*Figure 3.13: Maintenance of ChrA$^+$ endocrine cell number in T1DM and non-diabetic states.*

*Quantification of endocrine cells (ChrA$^+$) per islet in non-diabetic and diabetic donors. Data represent mean ± SEM. (A-B) Graphical representation of the number of ChrA$^+$ cells in individual islets in non-diabetic (A), and T1DM donors (B). (C) Dot-plot of the mean number of ChrA+ cells per islet in each control (n = 4) and T1DM (n = 4) donor (denoted as separate dots).*

Figure 3.13C indicates that there is trend towards slight increase in ChrA$^+$ endocrine cells in T1DM (75.99 ± 11.87 %) compared to controls (54.54 ± 4.1 %). However, this increase was not found to be significant ($p>0.05$) and could be attributed to the variations in the mean number of ChrA+ cells between the four T1DM donors.

### 3.3.14 Determination of islet endocrine area in T1DM and non-diabetic pancreata

Automated measurement of ChrA staining was performed to determine islet endocrine area. Figure 3.14 below indicates the mean area of ChrA per islet in each donor.

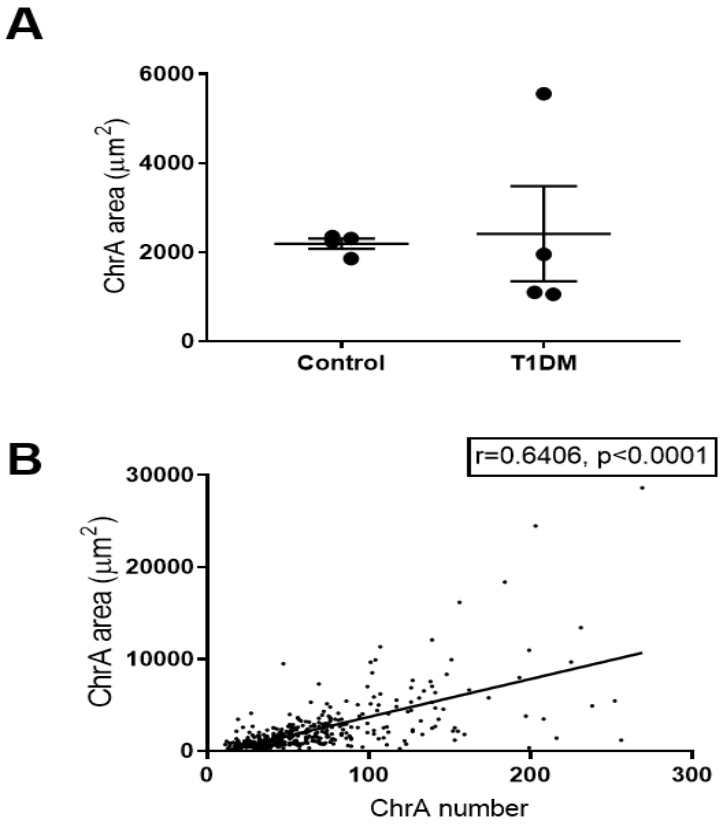


*Figure 3.14: Maintenance of endocrine area in T1DM and non-diabetic states.*

*Automated assessment of endocrine area (ChrA staining) per islet in non-diabetic and diabetic donors. Data represents mean ± SEM. (A) Dot-plot representing mean ChrA area per donor (denoted as separate dots) in control (n = 4) and T1DM (n = 4) cohorts. (B) The correlation analysis of ChrA number with corresponding ChrA area (per islet) indicated a significant positive correlation between the two variables.*

Figure 3.14A indicates no significant change in ChrA area in T1DM group (2410 ± 1067 µm$^2$), compared to controls (2184 ± 113.8 µm$^2$). As seen in Cohort 1, a positive correlation was found between ChrA area and number (Figure 3.14B) suggesting no change in cell size in T1DM.

### 3.3.15 Assessment of islet hormone expression profiles in T1DM and non-diabetic pancreata

To assess islet hormone expression profiles between control and diabetic groups, all ChrA+ cells that were insulin-positive (ChrA$^+$/Ins$^+$) and ChrA$^+$ cells that were hormone cocktail-positive (ChrA$^+$/3H$^+$) in the islets were counted manually. Figure 3.15 demonstrates the mean number of insulin and non-β-cells per islet from each donor.

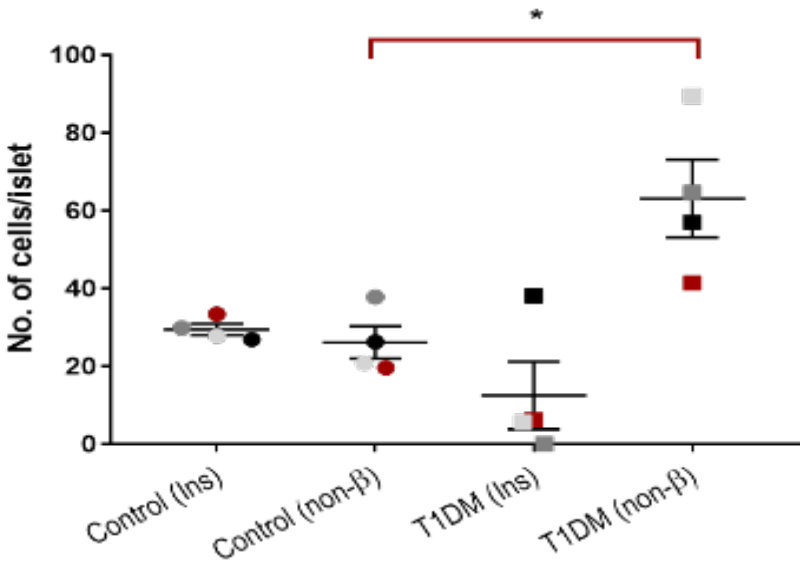


*Figure 3.15: Changes in hormone expression profiles in T1DM and non-diabetic donors.*

*Quantification of hormone expression demonstrates a decrease in insulin expression in the T1DM cohort when compared to the control, non-diabetic cohort. In parallel, significant increases in expression of non-β-cell hormones was evident in the T1DM cohort compared to controls. Data represents mean ± SEM (*p<0.05, Welch's t-test).*

Figure 3.15 clearly describes the changes in hormone expression in non-diabetic and diabetic states. Almost 50 % decrease in insulin expression, however insignificant, is evident in T1DM (12.45 ± 8.7) compared to controls (29.44 ± 1.44). Donor E254 (6 years old) had significantly higher number of insulin$^+$ β-cells compared to other T1DM donors, and hence loss of insulin in T1DM compared to controls was not statistically significant. Also, a significant increase is expression of other non-β-cell hormones was observed in the T1DM cohort (63.1 ± 10.02) compared to controls (26.1 ± 4.15).

### 3.3.16 Evaluation of β-cell trans-differentiation

For the assessment of β-cell trans-differentiation, ChrA$^+$ cells co-expressing insulin and non-β hormone cocktail were evaluated and quantified, as done in Cohort 1. The quantification of polyhormonal cells in non-diabetic and T1DM donors of Cohort 2 is demonstrated in Figure 3.16.

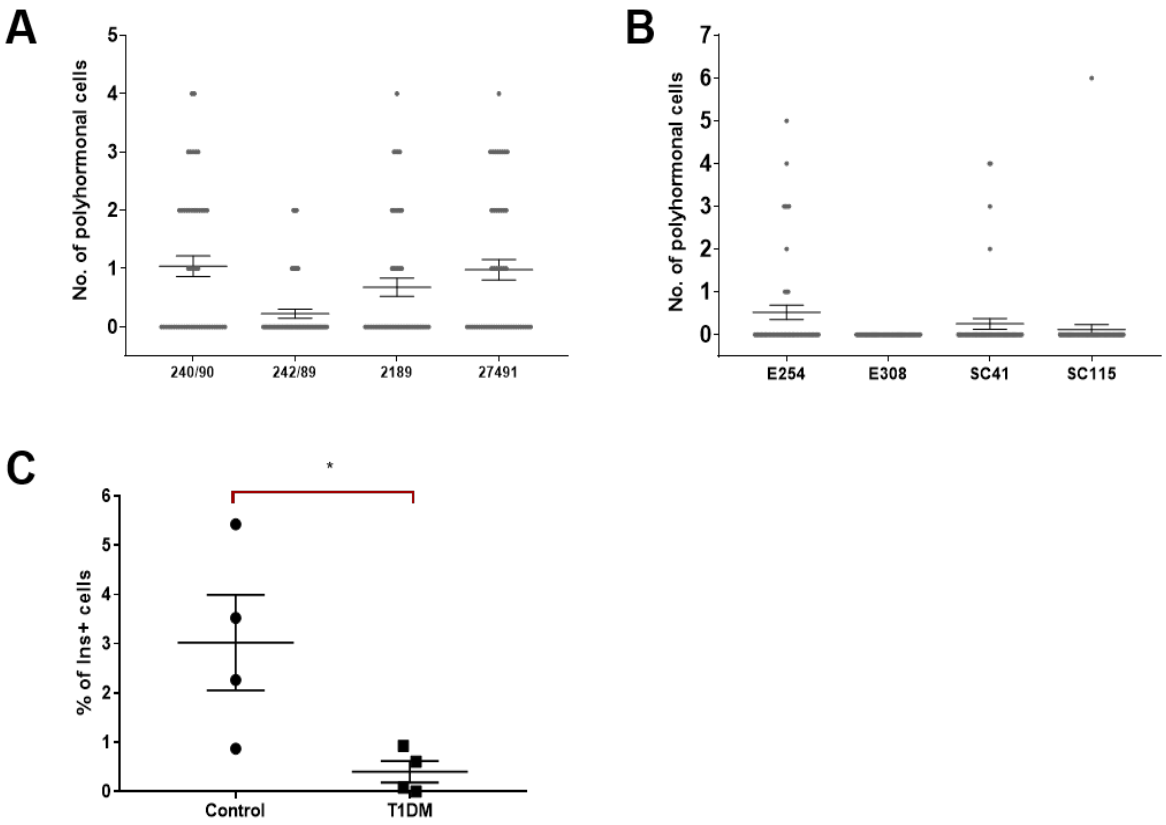


*Figure 3.16: Quantification of polyhormonal cells in T1DM and non-diabetic pancreata*

*Quantification of polyhormonal cells was done by measuring the number of insulin-positive cells co-expressing non-β-cell hormones. Data represents mean ± SEM. (A-B) Dot plot of the number of polyhormonal cells per counted islet in four non-diabetic donors (A) andfour4 T1DM donors (B). (C) Co-expression of insulin and non-β-cell hormones was significantly greater in control than in T1DM pancreas (p<0.05, Student's unpaired t-test).*

Contrary to Cohort 1, Figure 3.16 demonstrates significantly higher (~7 times) co-expression of insulin with other hormones in the non-diabetic donors (3.02 ± 0.97 %) compared to T1DM donors (0.4 ± 0.22 %). This indicates negligible presence of polyhormonal cells in T1DM which correlates with greater loss of insulin-producing β-cells in this group. Moreover, the number of polyhormonal cells in non-diabetic donors of Cohort 2 was two times greater than the non-diabetic donors of Cohort 1 (1.4 ± 0.7 %), suggesting that the presence of such 'transitional' cells is higher in younger pancreas.

### 3.3.17 Quantification of CPHN cells in T1DM

CPHN cells in the islets were assessed as a representative of de-differentiated cells.

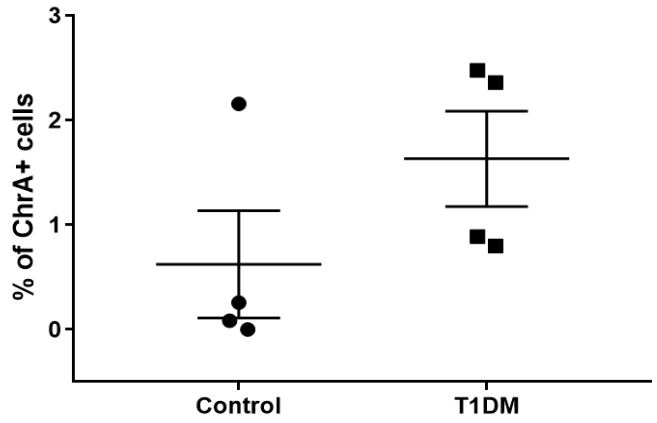


*Figure 3.17: Quantification of CPHN cells in non-diabetic and T1DM pancreata*

*Quantification of CPHN cells was done by measuring the percentage of ChrA$^+$ cells not expressing any islet hormones. The percentage of hormone-empty cells was greater in T1DM than in control pancreas. Data represents mean ± SEM.*

CPHN cells were calculated as a percentage of ChrA$^+$ cells per donor. Figure 3.17 indicates the percentage of hormone-negative ChrA$^+$ cells in the islets of T1DM donors (1.6 ± 0.46 %) was greater compared to non-diabetic donors (0.6 ± 0.5 %). Contrary to the older Cohort, CPHN cells were also observed (in low numbers) in the non-diabetic controls in the young donors. The percentage of CPHN cells in the T1DM donors across both cohorts was similar (1.3 ± 0.4 % in T1DM Cohort 1). These results indicate the presence of such 'hormone-empty' cells in both cohorts of T1DM and also the younger, non-diabetic donors.

### 3.3.18 Antibody stripping and re-staining of pancreatic tissue sections

For IF staining of the pancreatic sections, a cocktail of non-β-hormone antibodies consisting of glucagon, somatostatin and PP together were used. A lot of polyhormonal cells co-expressing insulin and non-β-hormones were found in T1DM and non-diabetic donors. To determine which specific hormone is co-expressed with insulin, further IF staining with individual non-β-hormone i.e. either glucagon, somatostatin or PP, was required. Due to limited availability of tissue, a pre-optimized stripping and re-staining protocol (refer Methods 2.8) was used to do insulin, glucagon, somatostatin IF-staining on the same pancreatic sections used for the earlier staining.

Firstly, the previous antibodies (ChrA, insulin, glucagon, somatostatin, and PP) were removed with the help of the antibody stripping protocol. Whether there was complete removal of antibodies was confirmed by the addition of the same secondary

antibodies used for the first round of staining. Once absolute removal was ensured, the tissue was re-stained using insulin, glucagon and somatostatin.

While the stripping protocol was efficient in complete removal of insulin and hormone cocktail, some problems were encountered which included lifting off of tissue from the glass slides, loss of tissue section and non-removal of ChrA antibody. Extended stripping times led to complete removal of ChrA but resulted in higher chances of lifting off tissue sections from the slides. To overcome this problem, use of antibody raised in rabbit was avoided (because rabbit ChrA was used earlier). Thus, even if the ChrA antibody was still present it would not be expressed due to lack of appropriate secondary antibody. Finally, somatostatin (rat), insulin (guinea pig), and glucagon (mouse) were used to re-stain the tissue after stripping.

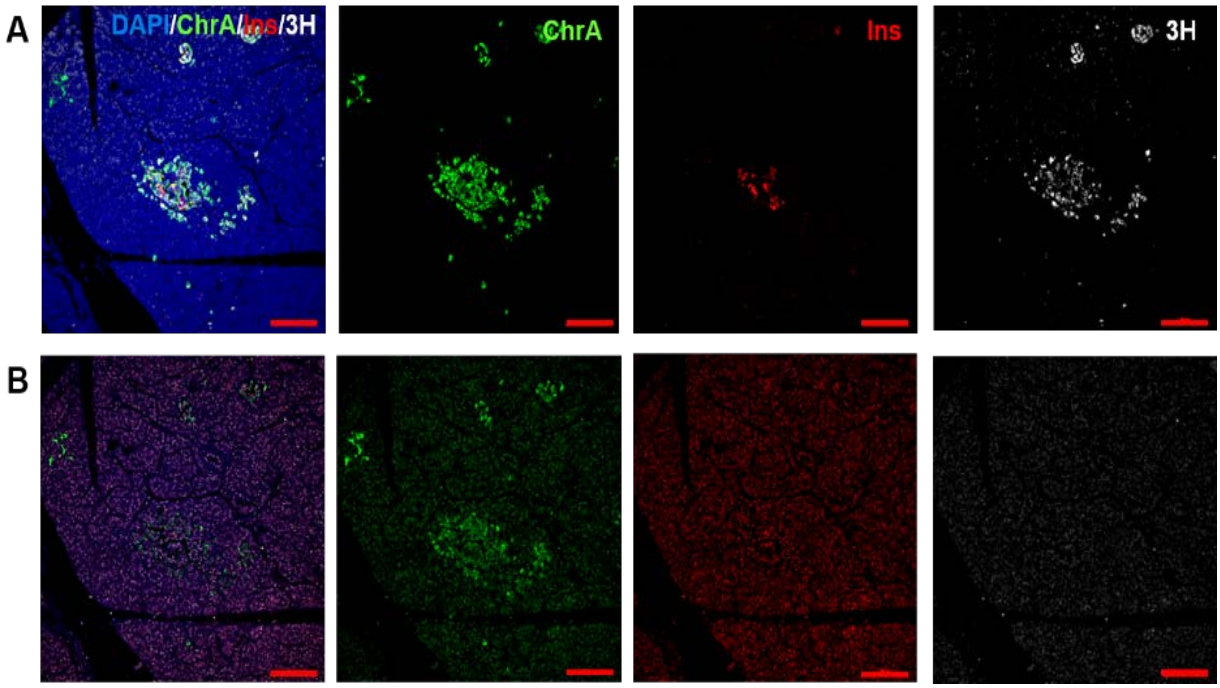


*Figure 3.18: Problems with antibody stripping*

*Representative images of IF staining of pancreatic tissue taken at 10x magnification on Nikon confocal A1R microscope, pre-stripping (A) and post-stripping (B). B still shows ChrA expression after antibody stripping and re-staining with secondary antibody, while clear removal of insulin and hormone cocktail was achieved. Scale bars represent 100 µm.*

Figure 3.18 highlights the problems associated with antibody stripping protocol. ChrA antibody was still detectable post-stripping. Higher than normal exposure parameters were used while imaging on Nikon confocal microscope to ensure complete (or lack of) loss of expression. Changing parameters like stripping times led to loss of tissue from section. As ChrA antibody was raised in rabbit, use of rabbit

antibodies (post-stripping) was avoided to make the ChrA expression undetectable. Thus, somatostatin (rat), insulin (guinea pig), and glucagon (mouse) were used to re-stain the tissue after stripping (Figure 3.19B). This led to clear expression of insulin, glucagon and somatostatin in the islets. Islets were further analysed to evaluate expression of each hormone.

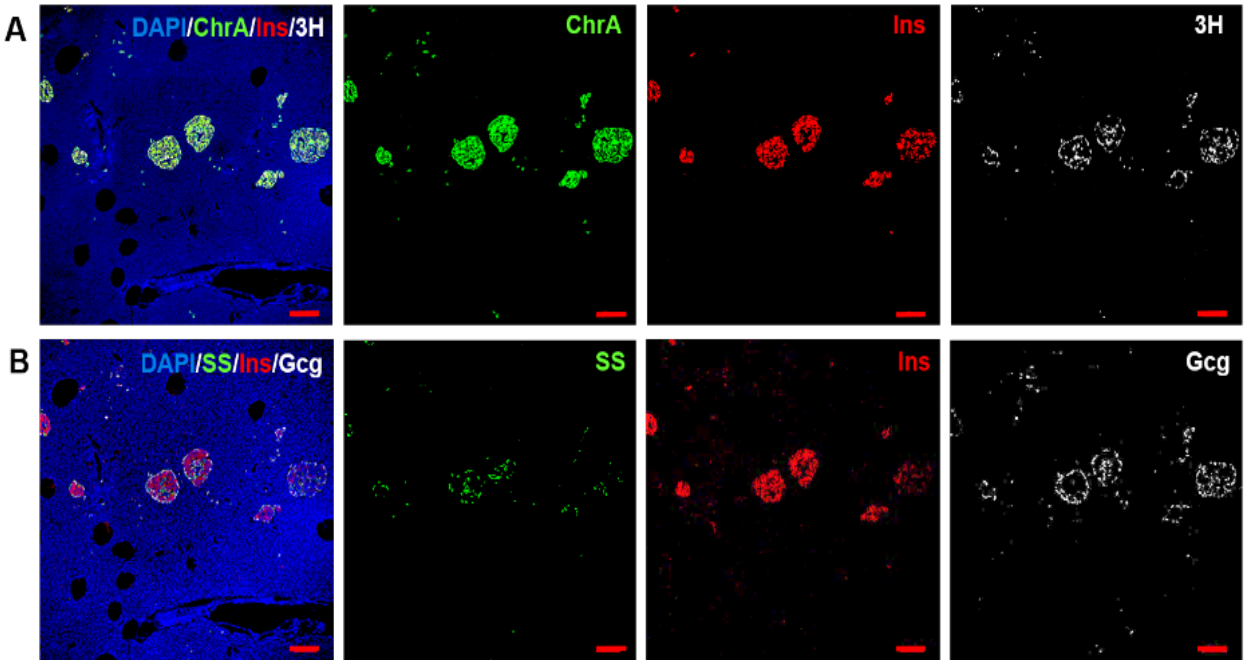


*Figure 3.19: Representative images of pancreatic tissue sections before and after antibody stripping and re-staining*

*Representative images of IF staining of pancreatic tissue taken at 10x magnification on Nikon confocal A1R microscope, pre-stripping (A) and post-stripping (B). (A) Islets showing expression of ChrA, insulin (Ins) and non-β-hormone cocktail (3H). (B) The tissue was then stripped to remove antibodies, and then re-stained with somatostatin (SS), insulin (Ins), and glucagon (Gcg). Scale bars represent 100 µm.*

### 3.3.19 Changes in number of insulin$^+$ cells after antibody stripping and re-staining of tissues

Antibody stripping was not performed on all the donors, but only on the Cohort 1 donors that contained maximum number of polyhormonal cells. Two donors each from non-diabetic (PM333 and 12142), short-duration T1DM (E396 and SC57), mid-duration T1DM (E560 and E386) and one donor from long-duration T1DM (SC100) were selected for antibody stripping and re-staining of tissue sections. As the only common parameter between the original and post-stripping and re-staining of tissue was insulin, number of insulin$^+$ cells were determined and compared to determine the effectiveness of the protocol and the reliability of data. 50 islets were analysed on each

tissue section and mean number of insulin$^+$ cells was determined. Figure 3.20 below indicates the changes in number of insulin$^+$ cells before and after stripping.

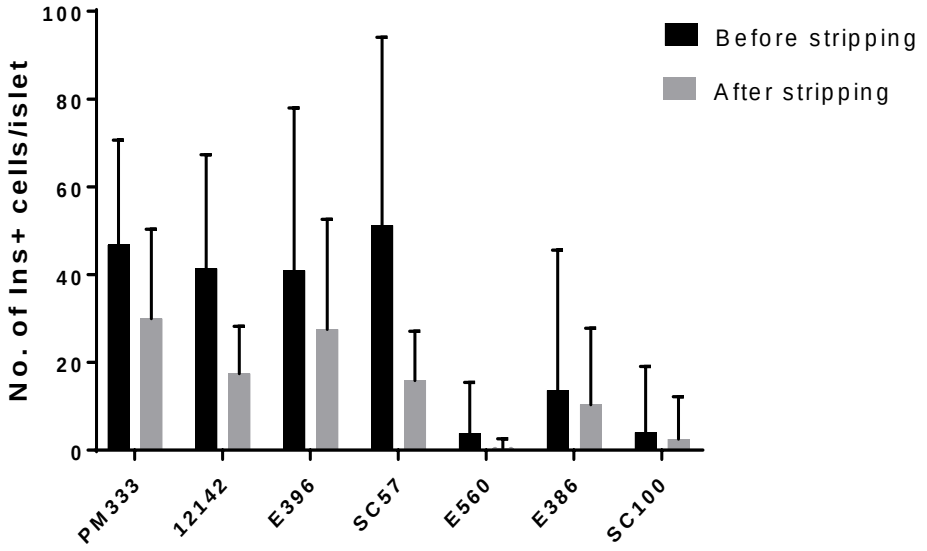


*Figure 3.20: Changes in number of insulin$^+$ cells before and after antibody stripping and re-staining of tissue sections*

*Number of insulin$^+$ cells was significantly ($p<0.05$) reduced in each donor after stripping and re-staining compared to before. Bars represent mean ± SD.*

Assessment of insulin hormone expression pre- and post-stripping was performed in 50 islets. Significant reductions in number of insulin$^+$ cells (Figure 3.20) were observed as well as visual loss of tissue was evident, indicating loss of tissue integrity following the stripping protocol. This could also be because the same islets were not assessed before and after stripping and re-staining. However, as insulin was consistently decreased in all donors, further analysis was still carried out to estimate patterns of hormone expression in the islets, which is described below.

### 3.3.20 Post-stripping changes in islet hormone expression and number of bi-hormonal cells

Islet hormone expression was evaluated in 50 islets and a mean number of insulin, somatostatin, and glucagon-expressing cells were determined for each donor (Figure 3.21A). Also, to determine co-expression of insulin$^+$ cells with glucagon and/or somatostatin, 'bi-hormonal' cells expressing insulin and somatostatin (SS-Ins) and insulin and glucagon (Gcg-Ins) were quantified for each donor (Figure 3.21B).

As can be seen in Figure 3.21A, analysis of individual hormone expression in islets revealed an expected decrease in insulin, with a simultaneous increase in

glucagon in T1DM compared to controls, based on the disease duration. Levels of somatostatin did not change significantly across the different groups. As each group only entails 1-2 donors, statistical analysis on this data were not possible. Also, cells co-expressing insulin and glucagon and insulin and somatostatin were both found in non-diabetic and diabetic pancreas (Figure 3.21B), with higher number of cells co-expressing insulin and glucagon indicating higher possibility of fate switches between α- and β-cells.

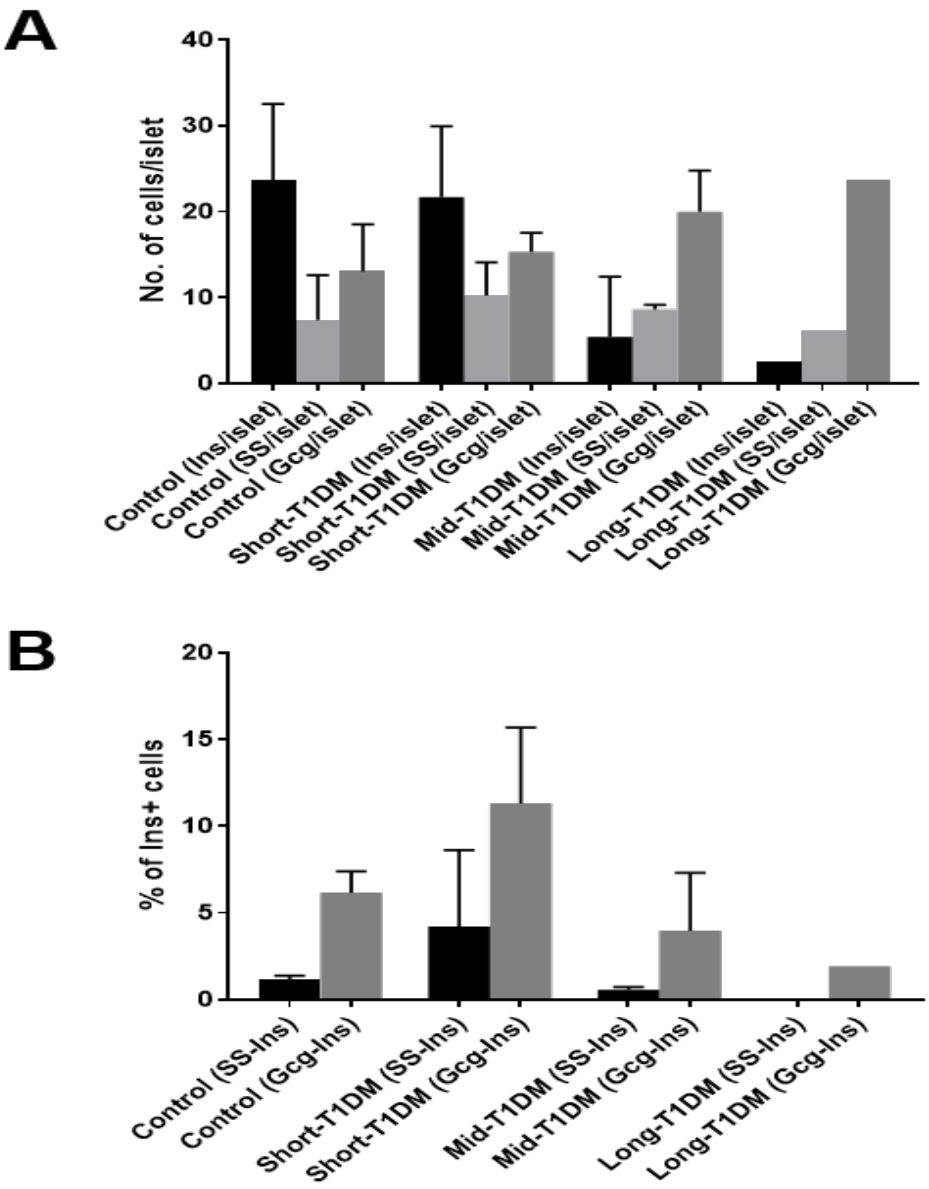


*Figure 3.21: Post-stripping changes in islet hormone expression and number of bi-hormonal cells.*

*Insulin, glucagon and somatostatin hormone expression in the non-diabetic (n = 2) and diabetic donors indicating reduction of insulin and increase in glucagon with increasing duration of disease from short (n = 2) to mid (n = 2) to long (n = 1). (B) Cells co-expressing insulin and glucagon (Gcg-Ins) and insulin and somatostatin (SS-Ins) were*

*both found in non-diabetic and diabetic donors. Insulin$^+$ cells co-expressing glucagon were more frequent than insulin$^+$ cell co-expressing somatostatin in all the groups. Bars represent mean ± SD.*

## 3.4 Discussion

The main question addressed by this study was the possible existence of alternate mechanisms, other than apoptosis, for the loss of β-cells in human T1DM. The hypothesis was that β-cell trans-/de- differentiation is an intermediate response to the inflammatory stress in T1DM preceding β-cell death. To test this, human pancreatic tissue from deceased patients with T1DM was used with thoroughly optimized staining protocol and analytical techniques. The key findings of the study were as follows:

1. Presence of residual insulin-producing β-cells even after long-standing T1DM in Cohort 1 (patients diagnosed over the age of 13 years).
2. Endocrine (ChrA) cell number in islets of T1DM pancreas is comparable to age-matched control donors, across both cohorts.
3. Reduced β-cell number is associated with an increased number of cells expressing non-β-cell hormones in islets of patients with T1DM.
4. Polyhormonal and 'hormone-empty' cells were identified in patients with T1DM, which may represent β-cell trans- and de-differentiation events respectively.

Firstly, an important observation of this study, as is also seen in previous studies (Baiu et al., 2011, Leete et al., 2016), is the presence of residual β-cells in the islets of T1DM patients, both those diagnosed after 13 years (Cohort 1) and those diagnosed before 7 years of age (Cohort 2). While the loss of ICIs was more pronounced in Cohort 2 (>80 %), change in the number of ICIs in Cohort 1 was associated with the disease duration. Cases that were recently diagnosed with T1DM (short-duration) in Cohort 1

had over 50 % of islets still showing insulin expression compared to around 18 % in T1DM Cohort 2, indicating that extent of β-cell loss is less in Cohort 1. This finding contradicts the widely-accepted belief that symptoms of T1DM, generally, exist when β-cell destruction is over 90 % (Atkinson et al., 2014), suggesting a role for β-cell dysfunction in disease presentation. This conclusion is supported by a study assessing the function of islets isolated from donors with recently diagnosed T1DM (Krogvold et al., 2015). Here, following *ex vivo* culture for 3-6 days, the glucose stimulated insulin response significantly improved, indicating that β-cell function can be restored, at least partially, following removal from the stressors of the T1DM environment.

Historically, β-cell loss in T1DM has been attributed to apoptosis. However, studies have found that the rate of apoptosis is fairly modest compared with the extent of β-cell loss (Butler et al., 2007). A limitation of such a type of study involving human tissue sections is that examination of certain phenomena like apoptosis (presence of apoptotic cells) can be difficult due to tissue heterogeneity and also macrophagic removal of apoptotic cells (Willcox et al., 2009). Thus, assessment of β-cell death in this type of study is challenging. While there was a clear loss of β-cells, a characteristic finding of the current study was that the number of endocrine cells (total ChrA+ cells) in islets examined from patients with T1DM was comparable to age matched controls in both cohorts. If β-cell apoptosis was a significant contributor to insufficient insulin secretion in T1DM, the number of $ChrA^+$ cells would be expected to decrease. Strikingly, this was later explained by the increase in number of other non-β endocrine cells in T1DM compared to non-diabetic controls. This was also observed in one of the studies by Butler group on T1DM (Md Moin et al., 2017). As non-β endocrine cells were marked using a cocktail of glucagon, somatostatin, and PP antibodies, it was not possible to determine from our initial analysis which hormone-producing cells were increased in islets of patients in T1DM. However, stripping and re-staining of the same

tissue section revealed an increase in glucagon expression in T1DM compared to non-diabetic, control donors. Literature suggests an increase in both glucagon-producing α-cells (Brissova et al., 2018) and somatostatin-producing δ-cells in T1DM (Piran et al., 2014b). Gepts et al. (1978) also found an increase in number of α-cells in both, recent-onset and long-standing cases of T1DM (Gepts and De Mey, 1978). Moreover, there is an increasing amount of evidence implicating hyperglucagonaemia, contributing to increased glucose levels in both T1DM and T2DM (Brown et al., 2008, Cryer, 2012, Brissova et al., 2018) . A study describing time-dependent alterations in insulin and glucagon secretions in recent-onset to two years of diagnosis of T1DM patients (Brown et al., 2008) observed 37 % increase in glucagon secretion in response to mixed-meal. On the other hand, while Brissova et al. (2018) reported a 2-fold increase in glucagon in T1DM islets, they observed an impaired glucagon secretion in response to hypoglycaemia (Brissova et al., 2018). They also observed abnormal expression of β-cell genes like Nkx6.1 in T1DM α-cells, but no expression of α-cell regulators like ARX and MAFB, indicating altered genetic profiles of T1DM α-cells. Together, these results indicate that loss of β-cells and an increase in the non-β-cells (especially α-cells) in the pancreatic islets, both contribute to the development and progression of T1DM with aberrant glucagon responses (glucose stimulated secretion and absences of hypoglycaemia induced secretion) suggestive of potential β-cell-like stimulus-secretion coupling.

The observed loss of β-cells followed by compensatory increase in non-β-cells points towards alternative mechanisms of β-cell dysfunction, may also suggest that not all β-cells die as a result of the immune insult. Based on recent studies, lots of theories have been proposed regarding the fate of β-cells post-immune attack. Recent research by Talchai et al. (2012) suggests that β-cell loss in human (type 2) diabetes may be a result of conversion of β-cell to other endocrine cells like α, δ, or PP cell (trans-

differentiation) or due to degranulation of β-cells leading to a more 'progenitor-like' state (de-differentiation) (Talchai et al., 2012). Data involving such mechanisms in T1DM is rather limited and hence, the scope of this study was to determine if such mechanisms exist in human T1DM pancreata.

A study by Piran et al. (2014), reported that autoimmune attack in T1DM is associated with islet cell trans-differentiation (Piran et al., 2014b). Trans-differentiation is the fate conversion of islet endocrine cells for e.g. β-cells to α-cells or δ-cells or vice versa (Kim and Lee, 2016a). Piran et al. (2014) found an increased number of glucagon-producing α-cells in recent-onset T1DM pancreas, while a marked increase in somatostain-producing δ-cells in a patient with T1DM for 44 years (long-duration) (Piran et al., 2014b). They also reported conversion of α-cells to β-cells and β-cells to δ-cells in caerulein-induced mouse model that mimic T1DM autoimmunity in humans. This conversion was associated with presence of intermediate phenotypes of cells co-expressing hormones i.e. insulin and glucagon co-expression and insulin and somatostatin co-expression. Thus, we set out to look for such transitional (trans-differentiation) phenotypes in T1DM pancreata.

In light of this, analysis of IF staining to assess co-expression of insulin with other non-β islet hormones (polyhormonal cells) in T1DM tissue was performed. Co-expression of insulin and non-β-cell hormones was 2-fold higher ($p<0.05$) in $ChrA^+$ cells in T1DM compared to controls in Cohort 1. In contrast to this, polyhormonal cells were significantly lower in the islets of T1DM donors compared to non-diabetic in Cohort 2. Presence of polyhormonal cells correlated with the presence of residual β-cells in T1DM. Thus, short-duration T1DM donors in Cohort 1 had highest expression of polyhormonal cells, followed by mid-duration and long-duration donors. This could also be the reason for the negligible presence of these cells in Cohort 2 T1DM donors as the extent of insulin loss (compared to controls) was more dramatic within this

group. It is difficult to examine function and origin of these cells in this study that assesses only one slice of a small section of pancreatic tissue. Nevertheless, stripping and re-staining of tissue (even if not perfect), revealed co-expression of insulin with glucagon and somatostatin both in control and T1DM pancreata, with a higher number of cells co-expressing insulin and glucagon. These cells may represent a β- to non-β-cell conversion or even vice versa. However, we found a significant increase in presence of non-β-cells in T1DM islets which may suggest that a β- to non-β-cell conversion is more likely in this situation. A similar finding has been reported in a human study involving T2DM where β-cell transcription factors were found in glucagon- and somatostatin-expressing cells (Cinti et al., 2015). Also, a study by Brereton et al. (2014) has reported similar findings with a decrease in β-cells and an increase in α-cells, without changes in islet cell turnover in response to hyperglycaemia (Brereton et al., 2014). They also observed 'bi-hormonal' cells co-expressing insulin and glucagon, with expression of β-cell genes like Pdx1 and glucose transporter 2 (Glut2), as well as α-cell transcription factor MafB.

Studies have also been reporting the presence of dedifferentiated cells in the form of $ChrA^{+}$/$hormone^{-}$ (CPHN) cells in T1DM (Md Moin et al., 2016, Md Moin et al., 2017) and T2DM (Talchai et al., 2012, Cinti et al., 2016). These hormone-empty endocrine cells represent loss of cell identity (cell failure) and are thought to be associated with a progenitor like phenotype (Accili et al., 2016). The present study explored the presence of such CPHN cells in T1DM pancreata to determine evidence for β-cell de-differentiation in T1DM.

A very small number of CPHN cells in T1DM donors in both cohorts and also some in younger, non-diabetic donors was observed in this study. Such small numbers of these cells may suggest the possibility that these might be ghrelin-producing cells that represent less than 1 % of islet cells. While this may be true to some extent (at

least in control donors), it is unlikely as no such cells were found in normal, non-diabetic pancreas in Cohort 1. The CPHN cells were less frequent in the islets, but were more evident as single, scattered cells in the exocrine pancreas. Similar observation of CPHN cells, more in the form of scattered single or cluster of endocrine cells than the islets, was found in a study by the Butler group (Md Moin et al., 2016, Md Moin et al., 2017) who also reported that the pattern of distribution of these cells was similar to one in neonatal pancreas suggesting possibility of attempted β-cell regeneration. Pro-inflammatory cytokines (like IL-1β, TNF-α and IL-6) have been shown to cause β-cell de-differentiation and dysfunction *in vitro* cultures of mouse and human islets (Nordmann et al., 2017). Moreover, a study demonstrated that viral infection of human islets leads to loss of insulin production, but not β-cell apoptosis, suggesting a loss of β-cell identity and dysfunction of β-cells. They reported a decrease in β-cell genes and an increase in progenitor marker SOX9 within the islets (Oshima et al., 2018). Thus, these cells could either indicate loss of β-cell identity or could be representative of β-cell regeneration as a response to immune attack. It is challenging to make specific conclusions about these cells as they are observed in such low numbers within the islets.

Thus, this study, with its own strengths and limitations, gives circumstantial evidence of alternate or additional mechanisms involved in the pathology or progression of T1DM.

The strength of the study lies in the use of rare, human pancreatic tissue samples which enables better understanding of the human disease. Quantification data from such a large number of samples also gives good basis for statistical analysis to make meaningful conclusions on islet hormone expression profiles. Moreover, the study of two different cohorts with T1DM led to the appreciation of differences between the donors with younger and older age at onset of diabetes.

However, due to limited availability of tissue sections from these rare donors; and difficulties with the stripping protocol, it was not possible to do further studies to characterize which type of endocrine cells increase in T1DM i.e, glucagon-, somatostatin-, or PP- producing cell. Further studies should use individual hormone staining to address this issue and also to more definitely determine the co-expression of hormones. Also, tissue heterogeneity is a factor that limits meaningful analysis in studies of this nature. This study is limited by the use of a single section of tissue derived from a single region of pancreas, which may or may not be representative of the whole pancreas. The area of pancreas (head, body, or tail) where the tissue is derived from dictates islet distribution within that region. A study indicated that there was 2-fold higher density of islets in the tail region compared to the head and body in normal pancreas (Wang et al., 2013). Moreover, patterns of insulitis differ in different regions of pancreas in T1DM. Most of the islets do not show any signs of insulitis, and certain lobes can be absolutely unaffected by the process (Gepts, 1965). In this study, the region of pancreas where the tissue section was taken is unknown, which may bear an impact on islet distribution and possibly, hormone expression.

Also, β-cell identity and function is associated with regulatory transcription factors like Nkx6.1, Pdx1, MafA, and Glut2. Loss of these factors is implicated in the studies describing loss of β-cell identity (Spijker et al., 2015, Remedi and Emfinger, 2016, Brereton et al., 2016). Staining for these specific markers will give better insight into loss of function and/or identity of β-cells. A recent study by Atkinson and colleagues (Wasserfall et al., 2017) indicated the presence of high levels of proinsulin (precursor to insulin) and INS mRNA in parallel to low levels of insulin and C-peptide in T1DM patients. They further added that the insulin gene is silenced in most of the T1DM pancreata. Persistence of proinsulin with lower levels of insulin and absence of C-peptide indicates that proinsulin is not converted to insulin due to lack of proinsulin

to insulin processing machinery (Wasserfall et al., 2017). Thus, differential proinsulin and insulin staining can benefit our knowledge of β-cell function in T1DM.

Nevertheless, the results from these studies provide novel quantitative analysis of differences in islet hormone expression profiles in non-diabetic and T1DM pancreata. They also demonstrate evidence of transitional ‘endocrine’ phenotypes that may be an alternate or additional mechanism, in parallel with β-cell death, resulting in β-cell dysfunction. Even if a small population of such transitional endocrine cells exist in patients with T1DM, it can lead to hope for future re-differentiation therapy targets. The end goal will be to find solutions and treatments to prevent loss of β-cells in T1DM and/or reverse trans-/de-differentiation of other endocrine cells or progenitor-like cells to regenerate functional β-cells.

# Chapter 4: Validation of automated image analysis method for high throughput quantification of islet cell phenotypes

## 4.1 Introduction

### 4.1.1 Heterogeneity of pancreatic islets in normal and diseased states

Islets of Langerhans are clusters of cells that make up the endocrine compartment of the pancreas central to glucose homeostasis (Szablewski, 2014). They largely consist of four major secretory cell types: insulin-producing β-cells, glucagon-producing α-cells, somatostatin-producing δ-cells, and PP-producing PP cells (Cabrera et al., 2006). Another hormone-producing cell types, ε-cells produce ghrelin are also occasionally found in the pancreatic islets.

Under normal conditions, β-cells make up about 65 – 80 % of islets and the rest is occupied by many non-β-cells (α, δ, or PP) (Brissova et al., 2005). In diabetes, however, these proportions of β- and non-β-cells in the islets are shown to be disturbed (Brereton et al., 2014, Cinti et al., 2016, Butler et al., 2016, Moin et al., 2018). Studies indicate loss of β-cells due to stressors like hyperglycaemia and inflammation, with a rise in the population of non-β-cells (specifically α- and δ-cells) in T1DM and T2DM (Chung et al., 2010, Piran et al., 2014a, Talchai et al., 2012, Morgan and Richardson, 2016, Nordmann et al., 2017). This brings about a change in the cytoarchitecture of the islets leading to disturbances in underlying endocrine and paracrine interactions (Castiello and Tabrizian, 2018). Moreover, a lot of studies demonstrating changes in islet hormone expression have used rat or mouse pancreas that differs in cellular organization and structure of islets compared to human pancreas (Brissova et al., 2005, Chen et al., 2013). Thus, quantification of such changes in human pancreas is essential to understand the underlying pathology of clinical diabetes development.

Also, islets are believed to differ between different pancreatic lobes; for example, the PP-rich lobe in the head of the pancreas shows different islet composition to the other islets with reduced α- and β-cell mass (Wang et al., 2013). Moreover, certain parts of pancreas (head and body) have denser distribution of islets than the other (tail) (Morgan and Richardson, 2018). Further, in T1DM, the disease is shown to present in a lobular manner in which certain lobes are unaffected by autoimmunity, while some show extensive or total β-cell loss (Morgan and Richardson, 2016). Hence, it is

essential that islets from different parts of the pancreas are studied in order to make conclusions representative of the whole pancreas.

Thus, microscopic analysis of histopathological changes in islets associated with diabetes is central to understanding the development and progression of disease.

### 4.1.2 Manual quantification / analysis and associated problems

Many advances in medicine can be attributed to the science of medical pathology which enables the use of microscopy for studying animal and/or human tissues to characterize and understand the tissue morphology and patterns of protein expression in normal and diseased states. Since its first description in 1942 (Coons et al., 1941, Coons, 1942), the technique of IHC has continued to evolve. Use of fluorescent-labelled antibodies for detection of antigen-of-interest, called as immunofluorescence (IF) staining, is now a common method to study biological tissue samples in research (Duraiyan et al., 2012).

Quantification has gained considerable importance in medicinal pathology in order to estimate levels of protein expression *in situ*, measurements of relevant tissue area, and also fluorophore intensity determination (Mansfield, 2014). Manually acquiring images of interest on appropriate microscope, followed by manual analysis using relevant software like ImageJ, Volocity, Nikon, Leica, etc. has been the preferred gold standard method of choice for this purpose due to its adaptability and cost-effectiveness. While this technique provides accuracy in determining exact cell phenotypes, it is tedious and impractical for a large number of samples and is challenged by slow throughput rate (Chen et al., 2013), subjective analysis and inter-user variations due to human errors (Cadena-Herrera et al., 2015). Also, thoroughly optimized IHC protocols can lead to visually noisy images, thereby limiting consistent and unbiased interpretation (Chen et al., 2013). Hence, there is need for a more sophisticated method of imaging and analysis that can address these issues.

### 4.1.3 Need for automated image analysis

The rise of digital imaging and automated computational methods for analysis has enabled high-throughput analysis of a larger number of samples, while also providing a low cost option due to reduction in manned work hours and reducing subjective interpretations associated with manual analysis (Cadena-Herrera et al., 2015). However, quantification of hormone expression in islets is limited by lack of

sophisticated software which can provide precise and automated imaging and quantification of multiple cell types associated with the islet cluster (Chen et al., 2013).

### 4.1.4 Aims

This study aimed to validate the effectiveness of an automated method of image analysis using PerkinElmer Vectra 3.0 Automated Quantitative Pathology Imaging system (Vectra) and inForm® software. The Vectra machine helps in automated multi-spectral image (MSI) acquisition of the desired region-of-interest, which could then be analysed using a customized, pre-defined algorithm in inForm® software. The use of this automated image analysis system provides combination of multi-colour staining and MSI along with cellular and/or tissue segmentation thereby, enabling quantification and analysis of each individual cell for many proteins/markers of interest (Mansfield, 2014).

In Chapter 3, quantification of individual hormone expression by way of counting insulin$^+$, and hormone-cocktail$^+$ cells, along with total endocrine cell count determined by ChrA$^+$ cells was performed in 50 islets from each of the non-diabetic and T1DM donors. For the studies in Chapter 4, the same donors were used for automated image analysis and cell counting using the approach described above. The same phenotypes that were assessed on manual analysis were set on the inForm® software and phenotype-training was performed by the same analyst (myself) so as to avoid any subjective variabilities.

### 4.1.5 Specific objectives

1. To determine validity of an automated method of imaging by Vectra 3.0 and analysis by inForm® software for high throughput analysis of islet cell phenotypes.
2. To compare data obtained from manual cell counting and automated cell counting using statistical tools - t-test and Bland Altman plots.

## 4.2 Study design

IF staining was performed on pancreatic tissue samples obtained from non-diabetic and T1DM donors for ChrA, insulin, and hormone cocktail (glucagon, somatostatin, and PP). Manual counting using Nikon Elements AR software to determine number of cells expressing each marker was performed on 50 islets (where possible) that were imaged on confocal microscope. The slides were then scanned using the Vectra 3.0 machine to obtain whole-scan images of each tissue section

(Figure 2.1). Then with the help of Phenochart™ software, an equivalent number of islet-containing regions were marked to get magnified (20x) MSI images of each region (Figure 2.2). Further, on inForm® software, the islets in each of the MSI images (Figure 2.3) were marked for analysis and the cells were segmented using pre-determined parameters (Figure 2.4). Cells were then assigned the appropriate phenotype (Figure 2.5), and the software was trained until an acceptable level of automated phenotyping was achieved. The data were processed using Matlab software to quantify number of cells expressing each phenotype. Results were compared to those obtained from manual cell counting analysis as is described below. Student's paired t-test was used to analyse the difference between methods i.e. no significance ($p>0.05$) meant closer agreement between the manual and automated methods. Bland-Altman plots were also used for method comparison.

## 4.3 Results

### 4.3.1 Number of islets counted by manual and automated assessment

As was the case in Chapter 3, approximately 50 islets were imaged and analysed as per the described protocol. However, for automated analysis, islets were picked and imaged by an independent, blinded Vectra user. While (almost) 50 islets were analysed for some donors, there was a large difference in the number of islets counted on other donors. Table 4.1 and Table 4.2 below shows the number of islets counted by each method.

Table 4.1 and Table 4.2 highlight the difference in number of islets counted by the two methods in each donor in both cohorts. In Cohort 1, the mean number of islets counted manually was 47.7 ± 7.03 (mean ± SD), while that by Vectra was 57.8 ± 57.9. In Cohort 2, the mean number of islets counted manually was 50.5 ± 1.1, while that by Vectra was 179.5 ± 119.4. Table 4.3 demonstrates the difference in the number of islets counted per donor group and highlights if they are significant based on Student's paired t-test. The number of islets counted per donor group was significantly different in the T1DM Cohort 1 where less than 50 islets were counted by automated method; as well as non-diabetic donors in Cohort 2 where over 4-times more than 50 islets were counted on each donor by the automated method.

*Table 4.1: Number of islets analysed by manual and automated counting in Cohort 1*

| Donor ID | Tissue type | No. of islets counted | |
|---|---|---|---|
| | | Manual | Automated |
| 65/71 | Non-diabetic | 50 | 212 |
| 191/67 | Non-diabetic | 50 | 116 |
| 12142 | Non-diabetic | 50 | 45 |
| PM146 | Non-diabetic | 50 | 67 |
| PM333 | Non-diabetic | 49 | 65 |
| PM447 | Non-diabetic | 50 | 231 |
| E168 | T1DM | 49 | 31 |
| E396 | T1DM | 47 | 38 |
| E431 | T1DM | 50 | 35 |
| SC57 | T1DM | 50 | 28 |
| SC76 | T1DM | 49 | 48 |
| E260 | T1DM | 45 | 19 |
| E385 | T1DM | 47 | 19 |
| E386 | T1DM | 50 | 64 |
| E556 | T1DM | 17 | 16 |
| E560 | T1DM | 49 | 46 |
| E557 | T1DM | 48 | 25 |
| SC100 | T1DM | 50 | 33 |
| SC107 | T1DM | 50 | 26 |
| SC109 | T1DM | 46 | 23 |
| SC112 | T1DM | 50 | 20 |
| SC116 | T1DM | 52 | 64 |

*Table 4.2: Number of islets analysed by manual and automated counting in Cohort 2*

| Donor ID | Tissue type | No. of islets counted | |
|---|---|---|---|
| | | Manual | Automated |
| 240/90 | Non-diabetic | 50 | 361 |
| 242/89 | Non-diabetic | 49 | 288 |
| 2189 | Non-diabetic | 50 | 213 |
| 27491 | Non-diabetic | 50 | 272 |
| E254 | T1DM | 50 | 108 |
| E308 | T1DM | 51 | 65 |
| SC41 | T1DM | 52 | 46 |
| SC115 | T1DM | 52 | 83 |

*Table 4.3: Difference in number of islets counted by the two methods*

| Donor group | Number of islets counted (mean±SD) | | Significance (t-test) |
|---|---|---|---|
| | Manual | Automated | |
| Non-diabetic, cohort 1 | 49.83 ± 0.41 | 122.7 ± 80.27 | No (p>0.05) |
| T1DM, cohort 1 | 46.81 ± 8.15 | 33.44 ± 15.13 | Yes (p<0.01) |
| Non-diabetic, cohort 2 | 49.75 ± 0.5 | 283.5 ± 60.91 | Yes (p<0.01) |
| T1DM, cohort 2 | 51.25 ± 0.96 | 75.5 ± 26.41 | No (p>0.05) |

### 4.3.2 Quantification of total number of cells per islet in T1DM and non-diabetic donors by manual and automated methods

Total number of cells per islet were assessed by combining the numbers of insulin$^+$ (alone), hormone cocktail$^+$ (alone) and polyhormonal cells obtained from manual and automated counting. Figure 4.1 describes the comparison of total number of cells per islet between manual and automated counting.

Figure 4.1 shows the total number of cells per islet in control and T1DM donors analysed by manual and automated methods. There was no significant difference in the number of cells per islet counted by the two methods in non-diabetic and T1DM donors of Cohort 2 (Figure 4.1B) and non-diabetic donors of Cohort 1 (Figure 4.1A). However, a significant difference was observed in the number of cells per islet of T1DM donors of Cohort 1 between the two methods. Even still, the number of cells per islet counted by both methods suggest maintenance of number of endocrine cells per islet between non-diabetic and T1DM donors in both cohorts.

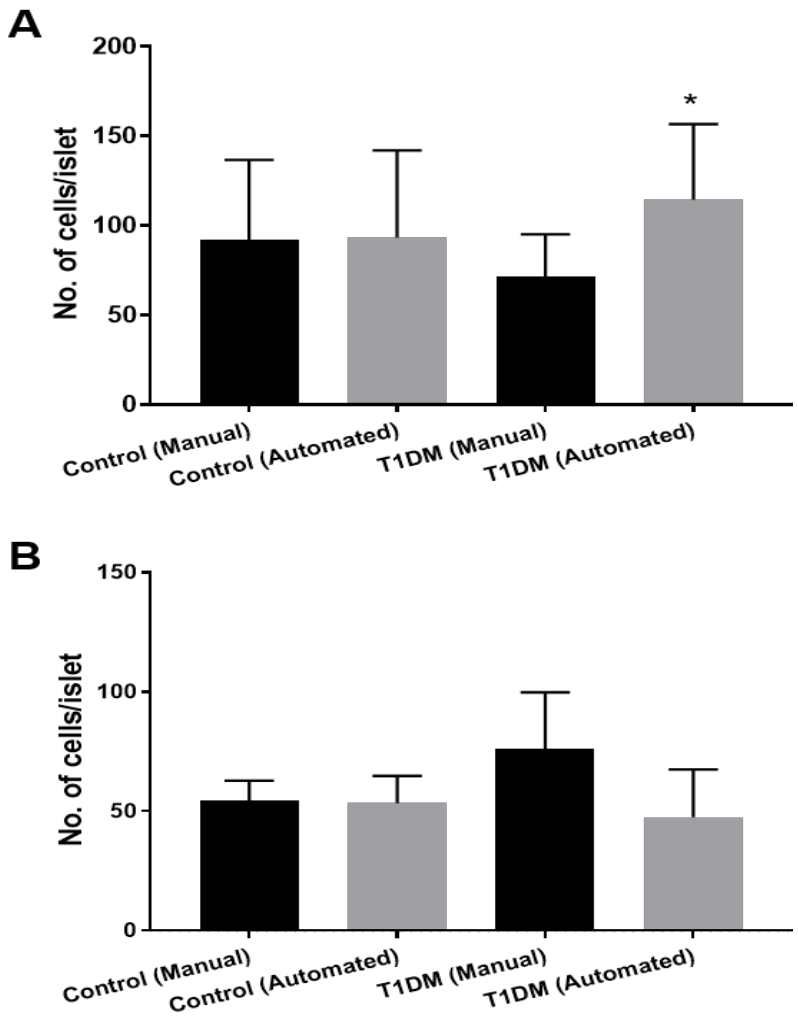


*Figure 4.1: Quantification of total number of cells per islet by manual and automated methods*

*Quantification of mean total number of cells per islet achieved by combining numbers of insulin$^+$, hormone cocktail$^+$ and polyhormonal cells. Data represent mean ± SD. (A) Cohort 1: No change in total islet cell number was observed between automated and manual counting in the control, non-diabetic donors from Cohort 1, but a significant increase in the T1DM donors between manual and automated counting. (B) Cohort 2: No significant change in the number of cells per islet quantified by the 2 methods was observed in non-diabetic and T1DM donors. (*$p<0.05$ compared to manual, Student's unpaired t-test)*

### 4.3.3 Assessment of hormone expression profiles in T1DM and non-diabetic pancreata by manual and automated methods

To assess hormone expression profiles in non-diabetic and T1DM islets, number of insulin$^+$ and hormone cocktail$^+$ (3H) cells were determined by manual and automated counting. The results obtained from manual and automated counting are indicated in Figure 4.2.

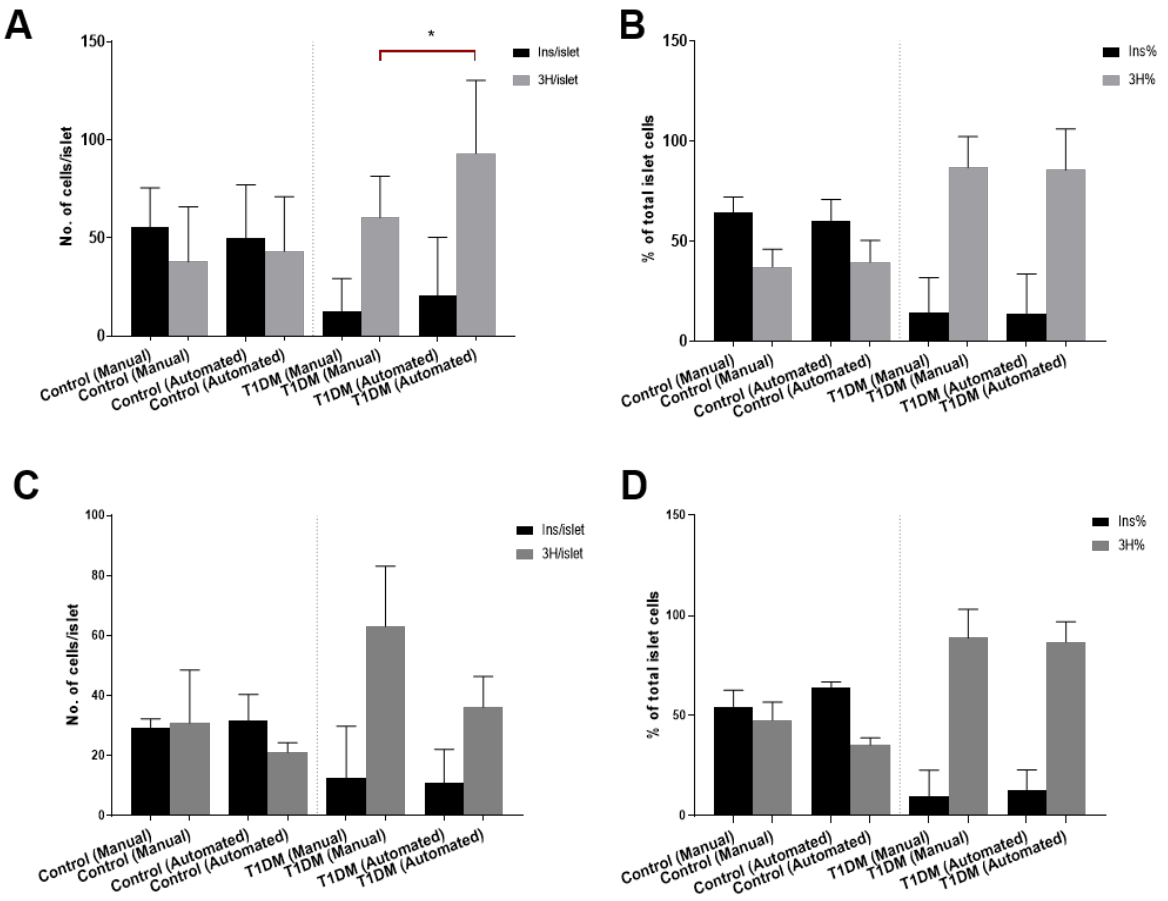


*Figure 4.2: Assessment of islet hormone expression profiles by manual and automated methods*

*Changes in islet hormone expression profiles in T1DM and non-diabetic donors by manual and automated counting in Cohort 1 and 2. Data represent mean ± SD. (A) In Cohort 1, no change in number of insulin$^+$ and hormone cocktail$^+$ cells was observed in non-diabetic pancreas, but a significant increase in number of hormone cocktail$^+$ cells in T1DM cohort was noted. (B) When analysed as a percentage of total islet cells, hormone expression profiles were not different between manual and automated counting in Cohort 1. (C) No significant change was observed in number of insulin$^+$ and hormone-cocktail$^+$ cells in either categories of Cohort 2 donors by manual and automated analysis. (D) When analysed as a percentage of total islet cells, hormone expression profiles were not different between manual and Vectra counting in Cohort 2. (*p<0.05 compared to corresponding manual, Student's paired t-test).*

Islet hormone expression was assessed by quantification of cells expressing insulin and non-β-hormones and is described in Figure 4.2 above. No significant change in islet hormone expression profiles were observed by manual and automated assessment in non-diabetic donors of Cohort 1 (Figure 4.2A) and non-diabetic and T1DM donors of Cohort 2 (Figure 4.2B). A significant change ($p<0.05$) in non-β-cell hormone expression was observed in the T1DM donors of Cohort 1 between manual (60.5 ± 21) and automated counting (93.1 ± 37.2) (denoted as number of cells per islet). This could be because the number of islets counted in this group varied

significantly between manual and automated methods and less than 50 islets were counted by automated assessment. However, when expressed as percentage of total number of cells per islet, the percentage of insulin$^+$ and non-β-hormone$^+$ cells were similar between manual and automated methods. This indicates that even if there are differences in the actual cell counts, the automated method can give accurate results for the percent hormone expression in islets.

### 4.3.4 Quantification of polyhormonal cells in T1DM and non-diabetic pancreata by manual and automated methods

Polyhormonal cells were determined by the assessment of cells co-expressing insulin and hormone cocktail. Figure 4.3 describes the number of polyhormonal cells in the non-diabetic and T1DM donors across the two cohorts by the two methods.

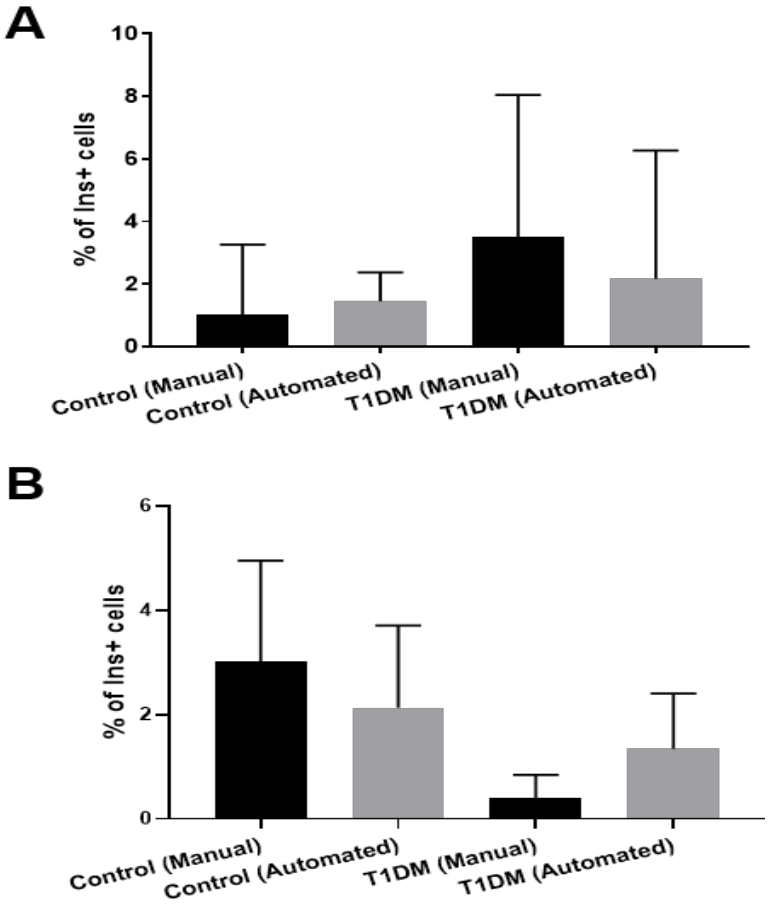


*Figure 4.3: Quantification of polyhormonal cells per islet by manual and automated methods*

*Assessment of polyhormonal cells per islet was done by measuring the number of insulin$^+$ cells co-expressing non-β-cell hormones. Data represent mean ± SD. (A-B) Bar graph showing percentage of insulin$^+$ cells co-expressing non-β-cell hormones in*

*Cohort 1 (A) and Cohort 2 (B). No significant change was observed in the number of polyhormonal cells determined by manual and automated methods in both cohorts.*

Figure 4.3 describes the assessment of polyhormonal cells in non-diabetic and T1DM donors in Cohorts 1 and 2 done by manual and automated assessment. The mean percentage of insulin$^+$ cells co-expressing non-β-cell hormones was found to be comparable in non-diabetic and T1DM groups. Also, polyhormonal cells were higher in T1DM Cohort 1 compared to controls in Cohort 1 and lower in T1DM compared to controls in Cohort 2. This pattern was similarly observed in both manual and automated assessments.

### 4.3.5 Statistical comparison of manual and automated methods

#### *4.3.5.1 Paired t-tests*

Paired t-tests were performed on all analytical parameters in non-diabetic and T1DM groups in both Cohorts to determine p-values to show if the data obtained from automated assessment were significantly different those obtained from manual assessment or not. Paired Student's t-tests were performed using Graphpad Prism software and the results are described in Table 4.4 and Table 4.5 below.

*Table 4.4: Paired t-test on data obtained by manual and automated assessment of Cohort 1 donors*

| Analysis parameter | Manual v Automated (Paired t-test) | |
|---|---|---|
| | Non-diabetic | T1DM |
| Total number of cells per islet | p>0.99 | p=0.006* |
| Number of insulin$^+$cells | p=0.69 | p=0.054 |
| Number of non-β-cells | p=0.68 | p=0.0017* |
| Polyhormonal cells | p=0.44 | p=0.054 |

*Table 4.5: Paired t-test on data obtained by manual and automated assessment of Cohort 2 donors*

| Analysis parameter | Manual v Automated (Paired t-test) | |
|---|---|---|
| | Non-diabetic | T1DM |
| Total number of cells per islet | p=0.88 | p=0.25 |
| Number of insulin$^+$cells | p=0.63 | p>0.99 |
| Number of non-β-cells | p=0.63 | p=0.63 |
| Polyhormonal cells | p>0.99 | p>0.99 |

Table 4.4 and Table 4.5 highlight the extent of differences in data obtained from manual and automated assessment. Table 4.4 shows the results of paired t-tests comparing data from non-diabetic and T1DM donors in Cohort 1. There was no significant difference ($p>0.05$) in the analysis of non-diabetic donors of Cohort 1 between the two methods. However, there were significant differences in the data obtained for T1DM donors of Cohort 1 by the two methods ($p<0.05$). The total number of cells per islet and number of non-β-cells differed significantly ($p<0.01$), while the number of insulin$^+$ and polyhormonal cells was not found to be significantly different. Table 4.5 shows the results of paired t-tests comparing data from non-diabetic and T1DM donors in Cohort 2. Data obtained from manual and automated assessment of non-diabetic and T1DM donors of Cohort 2 were not significantly different between the two methods.

*4.3.5.2 Bland-Altman plots*

Bland-Altman analysis was used to compare quantitative data obtained by two different methods. In this, limits of agreement are determined on the basis of mean and standard deviation of the difference between the two values. On the plot, the X-axis represents the mean of the two counts, while the Y-axis represents the difference between the mean of each donor by each method (manual minus automated). A value of zero indicates total agreement between the counts i.e. they are the same. As per Bland-Altman recommendation, if 95 % of the points lie between the limits of agreement, then the two methods provide comparable results (Giavarina, 2015).

Bland-Altman plots provide a good means of visual depiction to compare results obtained from two different methods. As per Bland-Altman recommendation, if 95 % of points fall within the positive and negative limits of agreement (calculated as 1.96 times of SD), then the data from the two methods are in good agreement and the methods provide comparable results. Bland-Altman plot analysis was performed for each of the parameters (total number of cells per islet, insulin$^+$ cells, non-β-cells, and polyhormonal cells) for non-diabetic and T1DM donors separately across both cohorts. Figure 4.4 shows Bland-Altman plots for data obtained on Cohort 1 donors. In the Figure, except plots B and F, all the plots show good agreement between manual and automated assessment as all the points lie between the limits of agreement. B and F only have 87.5 % points within the acceptable limits of agreement and therefore, shows significant difference between the data obtained from manual and automated assessment. This is also corroborated by significant p-values ($p<0.05$) obtained from

paired t-tests for these two parameters (Table 4.4). Figure 4.5 shows Bland-Altman plots for data obtained on Cohort 2 donors. All the plots from Cohort 2 data show good agreement between manual and automated assessment as all the points lie within the acceptable limits of agreement. Results from paired t-tests also confirm this finding as no analytical parameters are significantly different ($p>0.05$) between the two methods (Table 4.5).

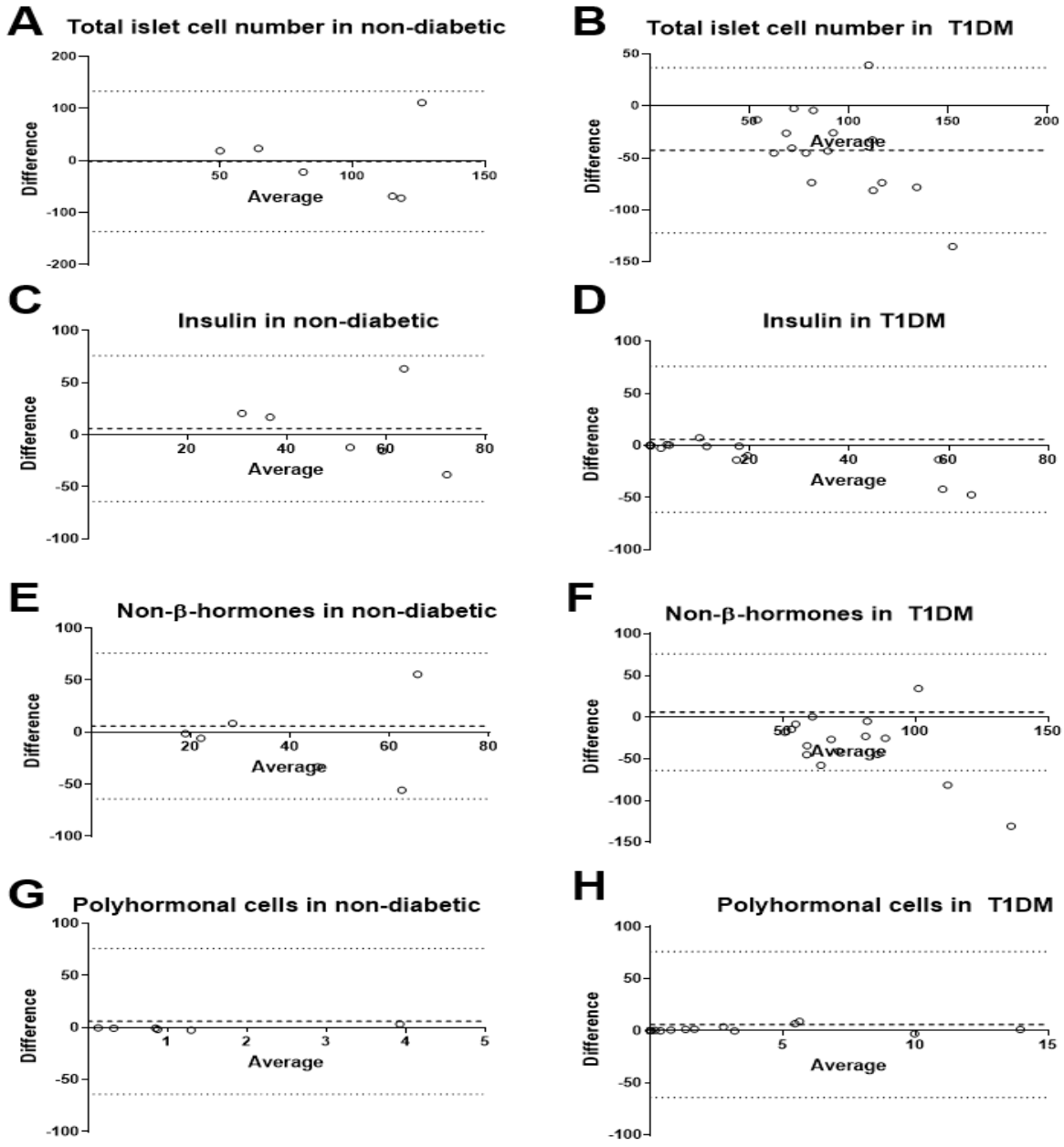


*Figure 4.4: Bland-Atman plots for method comparison of Cohort 1 data*

*Bland-Altman plots were used to compare results obtained for each parameter by manual and automated analysis of Cohort 1 donors. (A-H) Bland-Altman plots showing difference vs average between manual and automated assessment of total islet cell number in non-diabetic (A) and T1DM (B) donors, insulin in non-diabetic (C) and T1DM*

*(D) donors, non-β-cell hormones in non-diabetic (E) and T1DM (F) donors, and polyhormonal cells in non-diabetic (G) and T1DM (F) donors. Dotted lines represent limits of agreement (± 1.96 SD). Dashed lines represent bias between the two data sets. Most plots showed good agreement between manual and automated counts (A, C-E, G-H), while plots B & F had values outside the acceptable limits of agreement.*

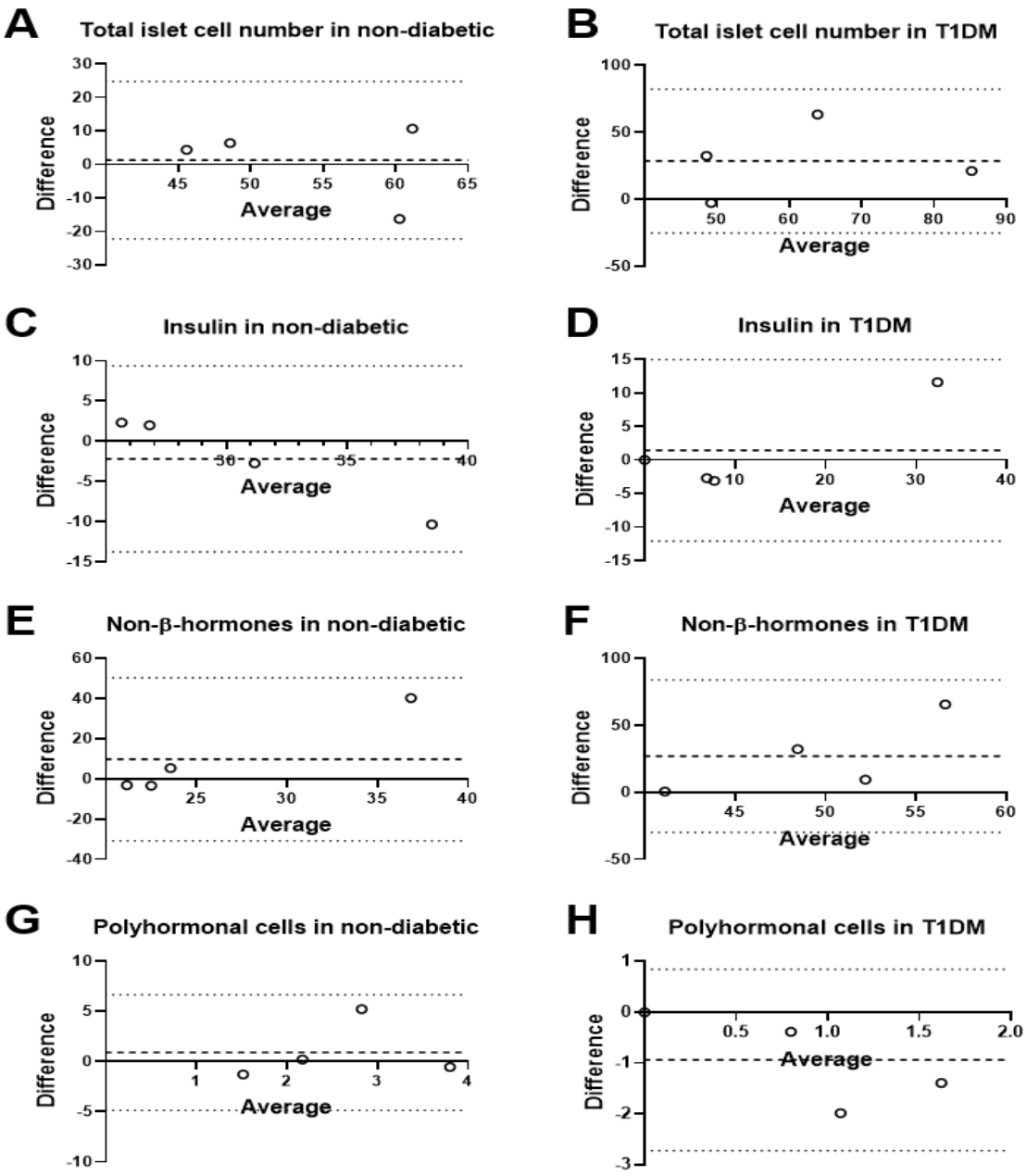


*Figure 4.5: Bland-Altman plots for method comparison of Cohort 2 data*

*Bland-Altman plots were used to compare results obtained for each parameter by manual and automated analysis of Cohort 2 donors. (A-H) Bland-Altman plots showing difference vs average between manual and automated assessment of total islet cell number in non-diabetic (A) and T1DM (B) donors, insulin in non-diabetic (C) and T1DM (D) donors, non-β-cell hormones in non-diabetic (E) and T1DM (F) donors, and polyhormonal cells in non-diabetic (G) and T1DM (F) donors. Dotted lines represent limits of agreement (± 1.96 SD). Dashed lines represent bias between the two data sets. All the plots showed good agreement between manual and automated counts as all the values lie within the acceptable limits of agreement.*

## 4.4 Discussion

The main goal of the study was to identify and establish a protocol for automated imaging and cell quantification that would give comparable results to the gold standard manual assessment. Automated assessment would be time-efficient and less tedious and also eliminate subjective difference that could arise in manual analysis. This study was conducted to compare results obtained for islet cell quantification from non-diabetic and T1DM pancreas by manual and automated analysis. The major findings were as follows:

1. Differences in number of islets counted can influence results obtained; larger number of islets counted gives better accuracy and more comparable results to manual analysis.

2. While the exact cell number may differ between the two methods (mostly non-significant), an accurate estimate of overall hormone expression patterns can be obtained by automated analysis.

3. Paired t-tests and Bland-Altman plots suggest good agreement between results obtained from manual and automated assessment.

The main advantage of using automated tools for quantification by this protocol was that the time needed for analysis was reduced by nearly 50 % of what is needed for manual assessment. Moreover, larger number (or all) of islets on the tissue section can be counted by this method without extra investment of time. Thus, it provides a way of high throughput analysis with avoidable human error.

A general observation of the study was the number of islets (sample size) analysed by each method had a large impact on the results obtained. The mean number of islets counted by the two methods differed significantly (Table 4.3). These differences could be the reason for varied results between groups, where when the difference was significant, it produced larger (significant) difference in cell number quantified by the two measurements (Figure 4.1A). However, interestingly, if the number of islets counted by automated assessment was higher than manual method, there was no significant difference in the results obtained by two methods (Figure 4.1B). That means, automated assessment needs over 50 islets to be analysed to provide more comparable results to manual measurement. In instances where less than 50 islets (like in T1DM Cohort 1) were analysed, results failed to match the ones

from manual counting. Higher number of islets counted provide more accurate results and will be representative of the whole tissue.

The exact cell counts for total number of cells per islet as well as individual hormone expression differed greatly between the two methods. This could be because the same islets were not counted by the two methods. Also, another reason, as discussed above, can be the number of islets analysed by each method.

Bland-Altman plots are a statistical tool to compare results obtained from two different methods. For most comparisons (Figure 4.4 and Figure 4.5) all the points lie between the limits of agreement suggesting good agreement between the results obtained from the two methods. Paired t-tests also confirm this observation as most p-values were not significant. The results from automated assessment followed conclusions from manual method, indicating the suitability of automated analysis for such a study.

Although automated analysis enabled faster measurements, a few issues were encountered in the process of automated analysis. The level of accuracy of the software to differentiate between different cell types depends on the extent of training of phenotypes carried out by the user. This could be highly extensive or minimal depending on the individual user which could bear an impact on the results. Over-training can lead to an increase in time for analysis, while under-training can result into improper identification of cell phenotypes. Hence, it should be ensured that sufficient (not over or under) training of assigned phenotypes is completed by the analyst, depending on the project. However, even after this problem is addressed, another issue is the quality of tissue and staining. Not all types of staining are compatible with this method of automated analysis. Moreover, the quality of staining at different parts of the section can be varied which may lead to discrepancies in differentiating between the cells. It was also impossible to get the whole-scan of some sections due to problems in setting up the Vectra 3.0 machine to the desired plane in the section. To overcome all these issues, sections should be cut and stained according to the standards acceptable by the Vectra 3.0 machine. Thus, appropriate ‘in-house’ validation is required for the use of this method for different biological studies.

Another problem that was faced during automated assessment was the quantification of cell types that were low in number in the tissue i.e. polyhormonal cells and CPHN cells. Presence and number of polyhormonal and CPHN cells varied

between the non-diabetic and diabetic tissues and hence, it was harder to assess these phenotypes. The algorithm used for analysis must be standard throughout the whole study and each cell type has to be available (minimum 5) for phenotype training in each section. Because this was not the case with CPHN cells, this category was deleted from automated analysis. Also, polyhormonal cells, because they express both insulin and non-β hormones, were difficult to differentiate by automation. Hence, most polyhormonal cells were identified by manual selection only. Occasionally, software also has difficulty in segmenting cells that are touching or overlap each other. All these factors can affect the results greatly and must be controlled for if this method needs to be used on a routine basis.

While manual analysis remains the gold standard for cell quantification studies due to the accuracy of results, the approach validated here for ‘semi-automated’ analysis may provide a reliable method for cell quantification as it gives comparable results to manual assessment with the elimination of user bias. Estimates of islet hormone expression profiles can be made accurately by this method of automated assessment. However, rarer types of cells in the pancreas can be hard to quantify by this method. If all the above-mentioned problems are controlled for, this method could be used as a faster tool for high throughput, quantitative assessment of a large number of tissue samples.

# Chapter 5: Evaluation of CFTR expression in normal human pancreas and assessment of endocrine cell constitution in CF/CFRD pancreas

## 5.1 Introduction

### 5.1.1 CFTR: the problem underlying CF and CFRD

CF is a genetic disorder caused by mutations in the CFTR gene leading to impairment in CFTR channel function (Elborn, 2016). The CFTR channel in epithelial cells regulates chloride and bicarbonate transport across the cell membrane, thereby contributing to normal fluid secretion by epithelial cells (Yoon, 2017). Mutations in this channel due to abnormal CFTR result in viscous mucus secretions leading to obstruction of various organ systems, mainly, the airways, gastrointestinal tract and the pancreas (Gibson-Corley et al., 2016a). With the advent of new therapies that have led to enhanced life expectancy in CF patients, the incidence of age-related diabetes (CFRD) has increased affecting about 20 % of adolescents and nearly 50 % of young adults (Bogdani et al., 2017). Moreover, CFRD worsens CF disease and associated complications leading to poor treatment outcomes and increased mortality rate (Marshall et al., 2005).

### 5.1.2 Characteristics of CFRD

The clinical characteristics of CFRD differ from T1DM and T2DM (Kelsey et al., 2019). The type of CFTR mutations, often, dictate the severity of disease, and patients with F508del mutation are the most susceptible to developing CFRD (Koivula et al., 2016). CFRD leads to impairment of first phase insulin response, following meal ingestion (Koivula et al., 2016), resulting in failure of glucose regulation characterized by normal fasting glucose and post-meal hyperglycaemia (Bridges et al., 2018). Pancreatic exocrine insufficiency is a hallmark of CF occurring in about 85 % of patients (Singh and Schwarzenberg, 2017), and CFRD is only seen in this patient group (Litvin and Nwachukwu, 2016). CFRD is characterized by changes in islet structure and function secondary to exocrine fibrosis and insufficiency (Cory et al., 2018), leading to an age-dependent decline in insulin secretion as well as number of β-cells in the islets (Bridges et al., 2018). Abnormal glucose tolerance is present earlier in life in CF patients (possibly from birth) as is demonstrated in CF ferrets (Olivier et

al., 2012), pigs (Uc et al., 2015) and young children aged between 3-months and 5-years (Yi et al., 2016a).

### 5.1.3 Histopathology of pancreas in CFRD

Although, pulmonary pathology associated with CF is the largest cause of morbidity and mortality in patients, the pancreas remains one of the earliest organs affected by CFTR mutation (Gibson-Corley et al., 2016a). CFTR is highly expressed in the epithelial cells of the pancreatic ducts which facilitate the transport of $Cl^-$ and $HCO^{3-}$ secretions into the ducts (Wilschanski and Novak, 2013). These bicarbonate-rich secretions play an important role in neutralizing the gastric acid and providing optimal pH and solubility for digestive enzymes secreted by the pancreatic acinar cells (Park and Lee, 2012). Faulty CFTR leads to altered composition of ductal secretions characterized by low pH, reduction in volume secreted, and exaggerated protein content (Durie and Forstner, 1989, Wilschanski and Novak, 2013, Gibson-Corley et al., 2016a), which in turn disturbs the zymogen (precursor to enzymes) secretions leading to pancreatic duct obstruction (Gibson-Corley et al., 2016). This is followed by the early signs of pancreatic changes seen in CF that include obstruction of small ducts and acinar tissue due to aggregation of protein and digestive enzymes. This, soon, progresses to plugging of acini and dilation of ducts leading to disruption of intact ductal epithelial layer. As a result of this, inflammation ensues causing exocrine fibrosis and fatty infiltration until there is total replacement of exocrine tissue with fat, the end-stage pancreatic pathology in CF (Gibson-Corley et al., 2016a). The endocrine compartment of the pancreas i.e. islets, are usually spared (although in an altered state) in spite of extensive exocrine fibrosis (Norris et al., 2019).

### 5.1.4 Endocrine pathology in CF

Insulin deficiency is considered the primary driver of CFRD (Barrio, 2015). Even though CF is associated with near-absolute loss of pancreatic acini, islets seem ‘spared’ in the pool of fibrotic or lipoatrophic exocrine tissue. But the morphology and functionality of these spared islets is questioned in the literature.

Examination of post mortem human CF pancreas demonstrates a reduction in number of islets (Lohr et al., 1989) and approximately 50 % decline in number of β-cells (Bogdani et al., 2017). Moreover, β-cell area is reduced in CF patients (without CFRD) by 11 to 52 % (Norris et al., 2019). Ferret models of CF have also shown decline of β-cells at birth (Olivier et al., 2012). These β-cells further decline progressively due to inflammation and exocrine fibrosis (Bridges et al., 2018). Also, overall islet

architecture is severely disrupted due to presence of fibrosis, immune infiltrate and amyloid deposits (Iannucci et al., 1984, Couce et al., 1996). Lohr et al. (1989) also reported that β-cell loss was associated with an increase in non-β-cells in the islets. An increase in the number of α-cells (Bogdani et al., 2017, Hart et al., 2018, Hull et al., 2018), and δ-cells (Lohr et al., 1989, Bogdani et al., 2017) has been observed in CF patients. This indicates changes in islet constitution that may suggest a disturbance in islet cell interactions thereby causing β-cell dysfunction.

As discussed earlier, impaired first-phase insulin response along with abnormal glucose tolerance are some of the earliest defects observed in CF (Sun et al., 2017, Norris et al., 2019), even when the structural damage to endocrine and exocrine pancreas is not apparent (Sun et al., 2017). β-cell function is diminished in CF patients compared to normal (Sheikh et al., 2017, Nyirjesy et al., 2018). Proinsulin secretion is also higher in CF patients with impaired glucose response thereby, suggesting early defects in β-cell function (Sheikh et al., 2017, Nyirjesy et al., 2018). Moreover, almost 50 % of the β-cell mass is retained in CFRD patients, which is insufficient to cause diabetes (Sun et al., 2017). Altogether this indicates that exocrine fibrosis is not the only driver β-cell loss in CF with a possibility of existence of β-cell dysfunction due to inflammatory stress and fatty infiltration (Litvin and Nwachukwu, 2016).

### 5.1.5 Potential causes of CFRD

Whilst there is evidence for β-cell dysfunction in the development of CFRD (Cano Megias et al., 2015), the mechanism by which the CFTR channel influences insulin secretion remains debated. Currently, three major hypotheses have been proposed (Sun et al., 2017):

1. Intrinsic CFTR-dependent pathways of insulin secretion

Various studies, mainly in animal models of CF, have indicated a direct role of CFTR in regulation of insulin secretion from pancreatic β-cells (Litvin and Nwachukwu, 2016). These studies have put across different views to explain the role of CFTR in the normal functioning of the endocrine pancreas and the mechanisms by which CFTR mutations may lead to the development of CFRD (Sun et al., 2017). The first study to demonstrate presence of CFTR protein and mRNA in rat pancreatic islet cells was done by Boom et al. (2007). They reported presence of CFTR mRNA in isolated rat islets by polymerase chain reaction (PCR), and by application of flow cytometry found that CFTR was highly expressed in non-β islet cells compared to β-cells. They further

confirmed CFTR protein expression in non-β islet cells by immunoblotting techniques, with IHC demonstrating localization of CFTR in α-cells of rat islets (Boom et al., 2007). Another study in 2009 reported that islets from CFTR-deficient mice are more susceptible to injury, from a low dose of streptozotocin, compared to controls and resulted into impaired glucose secretion. Moreover, as CFTR-deficient mice failed to develop exocrine fibrosis and scarring, they concluded that exocrine pathology could not be the only mechanism leading to CFRD in CF patients (Stalvey and Flotte, 2009).

Since then, expression of CFTR in various cultured β-cell lines and isolated murine islets have been reported, with impaired glucose-stimulated insulin secretion in response to faulty CFTR (Edlund et al., 2014, Guo et al., 2014, Ntimbane et al., 2016). Edlund et al. also demonstrated CFTR expression in human β-cells from isolated islets by way of small CFTR current detected using whole cell patch clamp studies. Edlund et al. showed a novel function of CFTR channel in islet β-cells as regulator of insulin secretion and exocytosis. They have also demonstrated role of CFTR in regulation of Anoctamin 1 (Ano1) which facilitates insulin release from β-cells (Edlund et al., 2014). In another study, they also demonstrated presence of CFTR channels in human and rodent α-cells and indicated that faulty CFTR leads to disturbances in glucagon secretion (Edlund et al., 2017). Guo et al. (2014) also reported similar conclusions demonstrating absence of membrane depolarization in mouse β-cells in presence of CFTR channel inhibitors like CFTRinh-172 and glyH-101.

Further, a pilot study consisting of five patients (with and without CFRD) by Bellin et al. studied effects of a drug, ivacaftor which corrects CFTR function in people with G551D mutation. In this study, they demonstrated improved insulin secretion in CF patients following ivacaftor therapy, suggesting a direct role of CFTR in insulin secretion in human subjects (Bellin et al., 2013). A recent study by Kelly et al. reported similar finding of improved of insulin secretion following four months of ivacaftor therapy (Kelly et al., 2019).

However, in contrast to this, CFTR mRNA and protein was found to be undetectable in ferrets as well as human pancreas (Sun et al., 2017, Hart et al., 2018). Sun et al. reported absence of CFTR mRNA in ferret β-cells (by ISH) indicating the role of pro-inflammatory mediators affecting islet function via paracrine mechanisms (Sun et al., 2017). A study by Hart et al., in human CF pancreas, found absence of CFTR protein in β-cells by immunostaining (Hart et al., 2018). Further, RNA sequencing of β-cells (mouse and human) showed extremely low level expression of

CFTR mRNA in about 5 % of β-cells (Blodgett et al., 2015, Hart et al., 2018). CFTR is thought to affect insulin secretion by disrupting β-cell electrophysiology (Norris et al., 2019). Hence, it is important to robustly determine if (and the levels) CFTR is expressed within β-cells or not.

2. Pancreas-extrinsic CFTR defects

Animal and human studies involving different CF models indicate the role of pancreas-extrinsic factors like gastrointestinal abnormalities leading to impaired incretin secretion, as a possible mediator of CFRD (Kelly and Moran, 2013). Under normal physiology, incretin hormones, GIP and GLP-1, are released from the small intestine in response to meal ingestion and play a key role in glucose-stimulated insulin release from pancreatic β-cells (Kim and Egan, 2008). The levels of these hormones are shown to be disturbed in some CF studies. A study by Sheikh et al. reported low levels of GLP-1 and GIP in response to a mixed meal test, especially in CF patients that have pancreatic insufficiency indicating that this may contribute to postprandial hyperglycaemia in CF (Sheikh et al., 2017). Also, Hillman et al. demonstrated low levels of active GLP-1 in patients with CF and CFRD (Hillman et al., 2012). In a study of 5 CF patients with pancreatic insufficiency, it was noted that CF patients suffer from rapid gastric emptying, impaired GLP-1 and GIP secretion, and postprandial glucose disturbances compared to healthy subjects (Kuo et al., 2011). However, some studies did not observe any changes in these gut hormone levels in CF compared to control subjects (Lanng et al., 1993, Anzeneder et al., 2011). Nevertheless, Perano et al. (2014) demonstrated improvement in postprandial hyperglycaemia by enhancing incretin secretion is possible by a strict pancreatic enzyme supplementation regimen, suggesting a role of incretins in hyperglycaemia underlying CFRD.

3. Remodelling of islets following loss of exocrine tissue due to inflammation

Pancreatic duct obstruction, an established pathology in CF, leads to fibrosis of the exocrine tissue and consequent fat deposition leading to stress and inflammation in the pancreatic environment, also bringing about a change in islet architecture. This, in turn, leads to faulty insulin secretion and β-cell dysfunction (Kelly and Moran 2013, Barrio 2015). Studies in human pancreas have reported presence of immune infiltrate composed of CD8+ and CD4+ T-cells in the islets of CF patients (Bogdani et al., 2017, Hart et al., 2018). Moreover, Bogdani et al. found 50 % reduction in β-cell numbers in CF children (<4-years of age) compared to control pancreas, and an even exaggerated

decrease in adult CFRD pancreata, with reduced islet density and presence of amyloid deposits in the CFRD islets (Bogdani et al., 2017). These changes are even observed in CFTR-knockout ferrets (Sun et al., 2017). At birth, CF ferrets demonstrate abnormal glucose tolerance and impaired first phase insulin secretion response, even in the absence of obvious structural damage to the endocrine and/or exocrine pancreas (Sun et al., 2017). However, in the first three months post-gestation, remodelling of both pancreatic compartments occurs as a result of persistent inflammation. In the first two months after birth, inflammation is higher and leads to a decline in β-cell mass, with consequent hyperglycaemia. In the third month, the inflammatory signal subsides, and hyperglycaemia improves with an increase in β-cell mass and expression of other islet hormones. This indicates that although inflammation mediated changes in β-cells occur in early stages of life (in ferrets), these changes are improved following remodelling of islets leading to recovery of normal glucose regulation (Yi et al., 2016b). Such a phase of normoglycaemia, following early life glucose regulation abnormalities, has also been observed in human CF patients (children under 6 years of age) (Yi et al., 2016a), pointing towards a dysfunctional β-cell phenotype capable of restoring normal insulin secretion in absence of stressors.

### 5.1.6 Aims

While there is existing literature to support all the above hypotheses, the contribution (if any) of each to β-cell failure and progression to CFRD remains relatively unknown. Here, through assessment of a number of pancreatic tissue samples from donors with and without CF/CFRD, the aim was to determine the potential for a direct, intrinsic effect of CFTR on insulin secretion, and whether mutations in this channel causes alterations in islet structure and endocrine cell constitution in human CF/CFRD.

CFTR localisation and expression within human pancreas was determined using novel and highly sensitive approaches. CFTR mRNA detection via ISH technique called as RNAscope® and protein expression via IF staining in human tissue was carried out to answer the critical question, whether CFTR affects insulin secretion by β-cell intrinsic or β-cell extrinsic pathways.

Further, a range of pancreatic tissue sections obtained from CF deceased patients, with or without CFRD, were studied and examined to characterize any histopathological changes in and around islets. IF staining, using a similar protocol as optimised in the T1DM study (Chapter 3) and subsequent quantitative analysis, were

also used to assess changes in islet hormone expression profiles along with evaluation of transitional endocrine phenotypes.

### 5.1.7 Specific objectives

1. To evaluate CFTR protein and mRNA expression in β-cells and any other endocrine cells in the islets of normal (control), non-diabetic pancreata.
2. To assess histopathological changes that occur in CF pancreata with or without CFRD compared to control pancreata.
3. To determine islet endocrine cell number in CF islets and age-matched controls.
4. To assess differences in islet hormone expression profiles between CF and age-matched controls.
5. To evaluate evidence of transitional endocrine phenotypes in CF pancreata with or without CFRD.

## 5.2 Study design

Control (normal) pancreatic tissue sections from Newcastle Tissue Biobank were studied to check for localisation and expression of CFTR protein and mRNA by IF staining and RNAscope® respectively.

Mercuric chloride-fixed, CF and CFRD pancreatic tissue sections, along with age-matched controls, from the EADB were studied to assess changes in histopathology to identify any abnormalities in the islets or the exocrine compartment. Further, IF staining, as performed previously in T1DM study, was carried out to quantify differences in hormone expression profiles between normal and diseased islets.

## 5.3 Results

### 5.3.1 Tissue sampling for evaluation of CFTR expression in donors with no known pancreatic pathology

Due to the ambiguity in literature concerning whether CFTR is expressed within human β-cells and/or islet endocrine cells under normal conditions (Edlund et al., 2014, Hart et al., 2018, Norris et al., 2019), the first aim of the study was to understand localisation and expression of CFTR within normal human pancreatic sections. Thus, pancreatic sections from ten donors between the age of 23 and 71 years were obtained from the Newcastle Tissue Biobank and are presented in Table 5.1. Donors were selected across a range of body mass index (BMI), between normal and obese (21-30), as obesity has been shown to be a risk factor for pancreatic diseases (Kim and Han, 2012).

*Table 5.1: Information on donors with no known pancreatic pathology*

| Donor ID | Age (years) | Sex | BMI |
|---|---|---|---|
| LDIS156 | 23 | M | 21.13 |
| LDIS072 | 24 | F | 25 |
| LDIS159 | 32 | M | 27.7 |
| LDIS158 | 39 | F | 29.98 |
| LDIS155 | 48 | F | 23 |
| LDIS152 | 49 | M | 25 |
| LDIS194 | 59 | M | 26.77 |
| LDIS204 | 57 | F | 26.89 |
| LDIS161 | 63 | F | 24 |
| LDIS174 | 71 | M | 26.73 |

### 5.3.2 Optimization of CFTR antibody for IF staining

Conventional anti-CFTR antibodies that can detect CFTR in a cell line that over-expresses CFTR protein, are often not sensitive enough to detect it in human tissues. Moreover, certain epitopes detected by these antibodies becomes inaccessible (undetectable) after routine tissue processing and fixation (Claass et al., 2000)

The Cystic Fibrosis Foundation (University of North Carolina, USA) generates and supplies various monoclonal antibodies for research purposes. CFTR 596 is one of the monoclonal antibodies provided by the foundation that is shown to be suitable for use in Western blotting, flow cytometry and IHC by IF staining (van Meegen et al., 2013).

The CFTR 596 primary antibody along with either ChrA, insulin, or keratin 7 (KRT7) was used for IF staining of pancreatic sections. ChrA was used as a marker for all endocrine cells in the islets, while insulin served as β-cell marker specifically. The epithelial cell marker, KRT7, was used to identify ductal compartments in the pancreas (Jimenez et al., 1999).

The CFTR 596 antibody needed optimization to determine optimal conditions for IF staining (Figure 5.1). Regular antibody diluents like 0.05 % foetal bovine serum (FBS) in PBS or DAKO antibody diluent failed to give good staining results due to presence of background staining. Thus, solution B was used to dilute the antibody to

address this issue. CFTR antibody was used at 1:2000 concentration of the stock because this demonstrated optimal signal with least noise. Another study by Chin et al. has used the same concentration of CFTR 596 antibody for IHC (Chin et al., 2017).

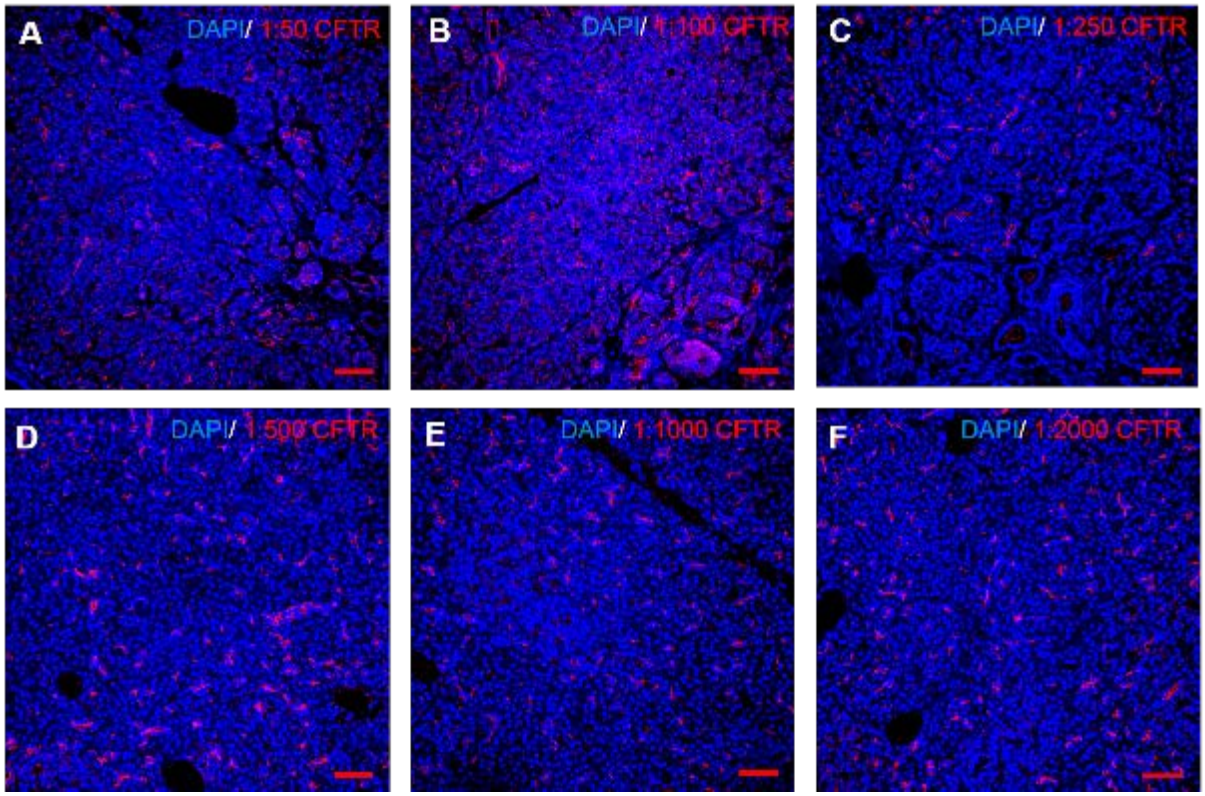


*Figure 5.1: Optimization of anti-CFTR primary antibody in normal pancreas.*

*Anti-CFTR (red) primary antibody optimization done by testing at six different dilutions of stock. Scale bars represent 100 µm. Tested dilutions were 1:50 (A), 1:100 (B), 1:250 (C), 1:500 (D), 1:1000 (E) and 1:2000 (F).*

### 5.3.3 Determination of CFTR antibody specificity

Ferret models to study CF are gaining increasing interest because of their close resemblance to human lung anatomy and cell biology (Sun et al., 2010). In light of this, Professor John Engelhardt and his colleagues at the University of Iowa have developed various CFTR knockout ferret models that mimic human CF and CFRD pathology. In a study conducted by Sun et al. (2010), they reported the generation of a CFTR-null (knockout) neonatal ferret model that demonstrates mild pancreatic pathology at birth and at the age of 1-month, develops progressive pancreatic inflammation and exocrine atrophy (Olivier et al., 2012). Hence, pancreatic tissue sections from these CFTR-knockout and wild-type ferrets (kindly provided by Prof John Engelhardt) were used to test antigen specificity of CFTR 596 antibody.

For this, IF staining with CFTR 596 and insulin was performed on ferret pancreatic tissue sections and representative images are shown in Figure 5.2.

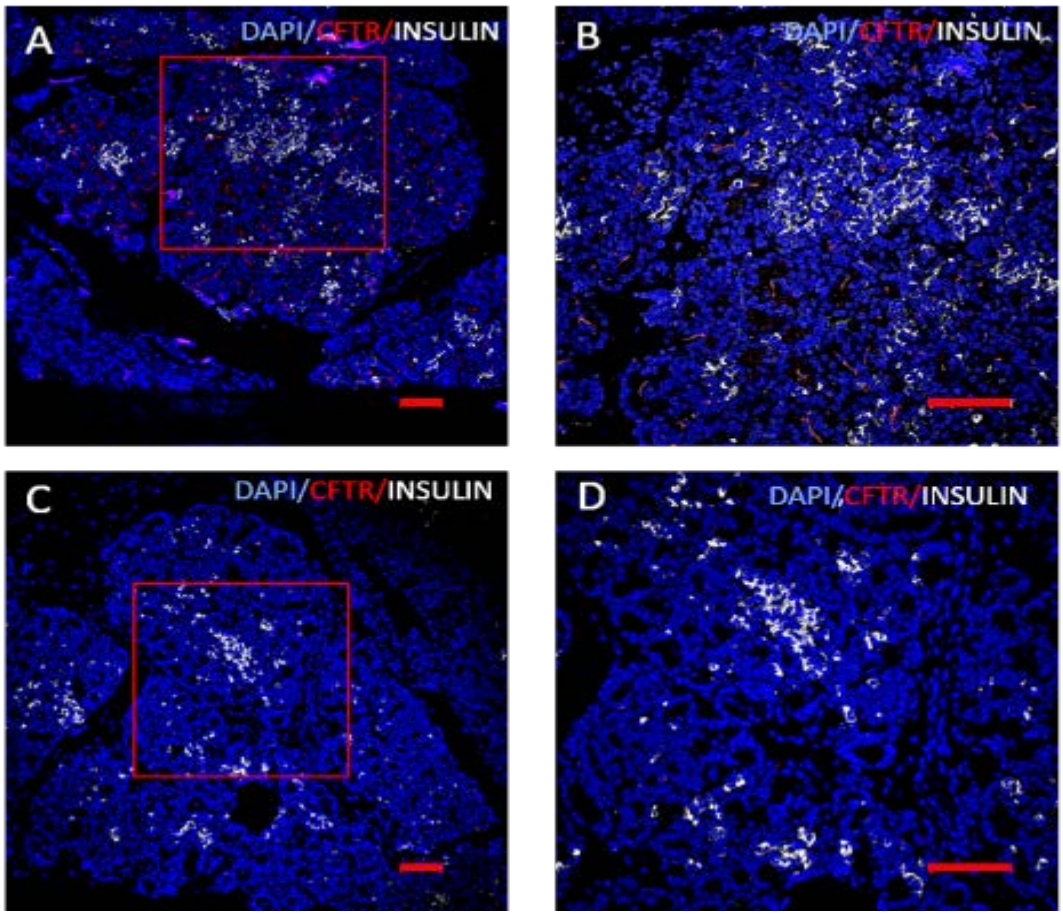


*Figure 5.2: Determination of CFTR 596 antibody specificity.*

*Representative IF staining for CFTR (red) and insulin (white) in wild-type (A-B) and CFTR knockout (C-D) ferret pancreas. The antibody was specific to CFTR channel as no expression was detected in CFTR-null ferret tissue. Boxes represent magnified regions (B & D). Scale bars represent 100 µm.*

Expression of CFTR 596 was only observed in wild-type ferret pancreas and not in CFTR knockout ferret pancreas. This shows that the antibody used is highly specific to CFTR protein and does not bind to non-specific targets. Hence, CFTR 596 antibody was used for further studies.

### 5.3.4 Evaluation of CFTR RNA expression in donors without known pancreatic pathology

As discussed above, due to the ambiguity in literature concerning whether CFTR is expressed within human β-cells, the first aim of the study was to understand localisation and expression of CFTR within normal human pancreatic sections. To this

end, we undertook ISH studies (RNAscope®) to determine the expression of CFTR RNA in a range of pancreas sections from donors with no known pancreatic disease pancreas. This experiment was performed by Claire Jones in the Pathology node of Royal Victoria Infirmary, Newcastle (RVI) and was supervised by Dr Michael White who kindly provided these data for inclusion in this thesis. Pancreatic tissue sections from ten donors with no known pancreatic pathology were used for CFTR ISH. Tissue slides were scanned in Leica side scanner and images were acquired at 10x and 20x magnification (

Figure 5.3).

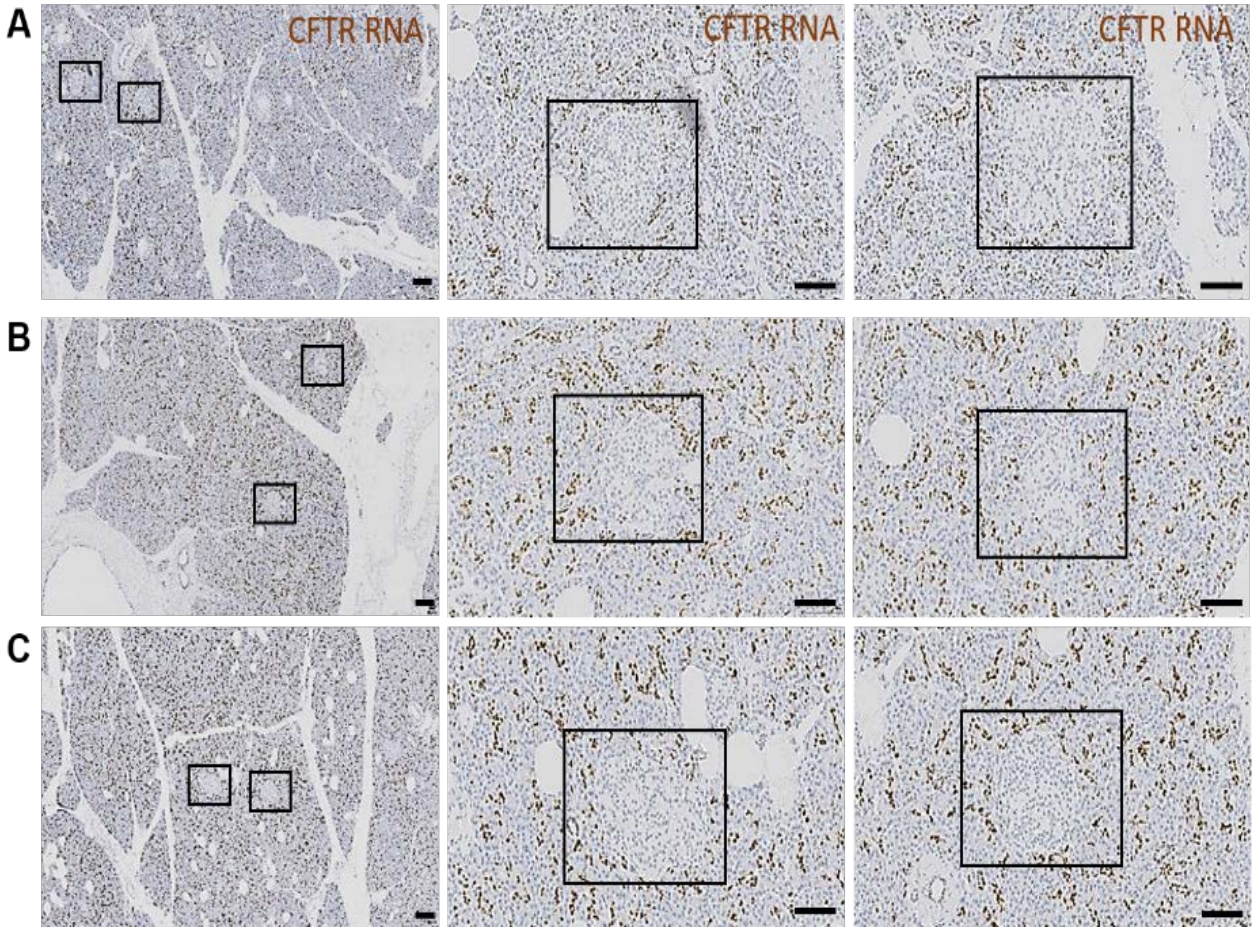


*Figure 5.3: CFTR RNA expression in human pancreas*

*Representative CFTR ISH (brown) images in pancreatic tissue from three separate donors (A-C). Pancreatic tissue section from donors LDIS072 (A), LDIS174 (B), and LDIS152 showing CFTR RNA expression in exocrine pancreas, and no expression in apparent islet regions. Rectangles represent magnified regions. Scale bars represent 100 µm.*

As can be seen in Figure 5.3, apparent 'islet-like' regions showed no expression of CFTR RNA. To confirm these regions are islets and to check for any localisation of

CFTR RNA within β-cells or any endocrine cells, CFTR ISH combined with IHC staining for either insulin or ChrA was performed.

### 5.3.5 Evaluation of CFTR RNA and protein expression in β-cells

To understand the role of CFTR in β-cell dysfunction and consequent CFRD, it is crucial to assess the expression of CFTR within the pancreas which will help in determining if CFTR impacts β-cell function via islet-intrinsic or islet-extrinsic pathways.

Hence, expression of CFTR RNA by ISH and CFTR protein by IF staining, along with insulin (β-cell marker) was assessed. To evaluate CFTR RNA expression within β-cells, CFTR ISH combined with IHC staining for insulin was performed (Figure 5.4). To assess CFTR protein expression, IF staining with insulin (Ins) and CFTR was undertaken along with ductal cell marker KRT7 (Figure 5.5) to check localization of CFTR within insulin$^+$ β-cells and KRT7$^+$ ductal cells.

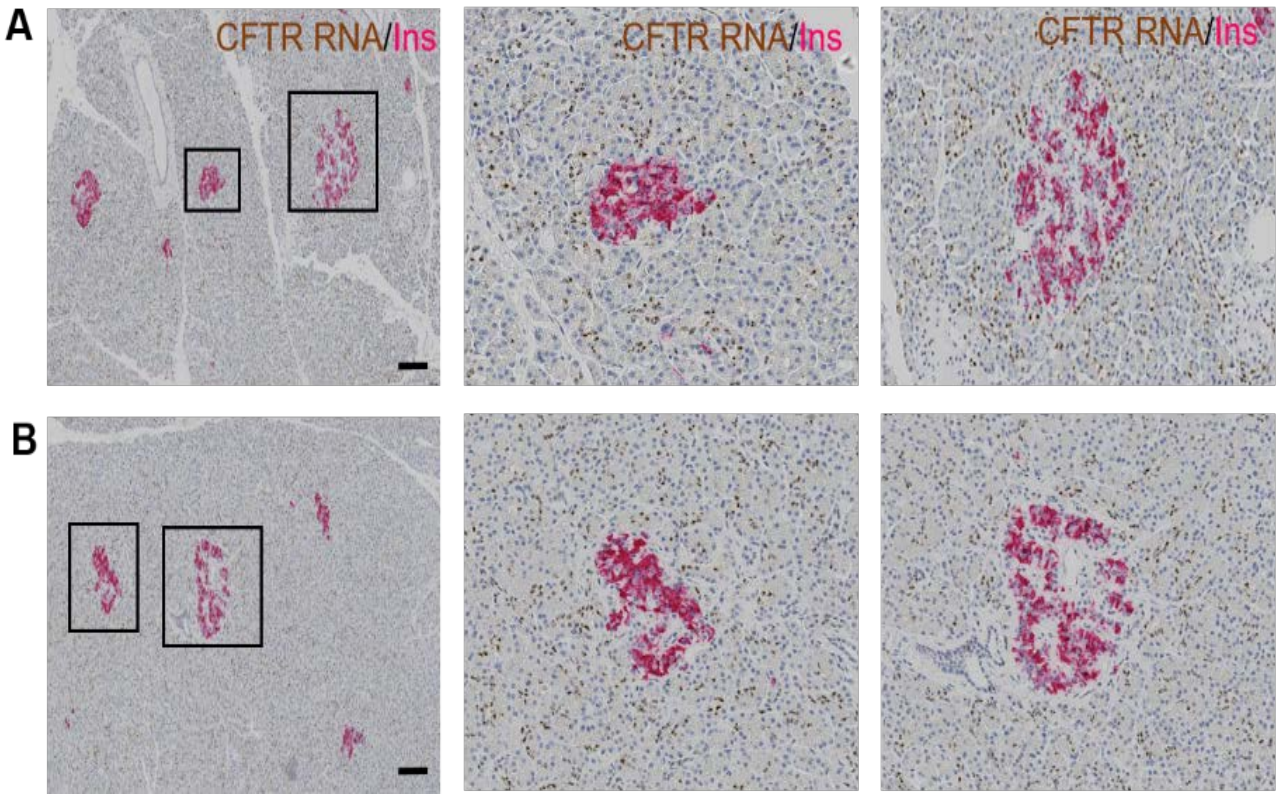


*Figure 5.4: CFTR RNA expression in β-cells*

*Representative CFTR ISH (brown) with insulin IHC images in pancreatic tissue from two separate donors (A-B). Pancreatic tissue section from donors LDIS072 (A), and LDIS155 (B) showing CFTR RNA expression in exocrine pancreas, and no expression in insulin$^+$ regions (pink). Rectangles represent magnified regions. Scale bars represent 100 µm.*

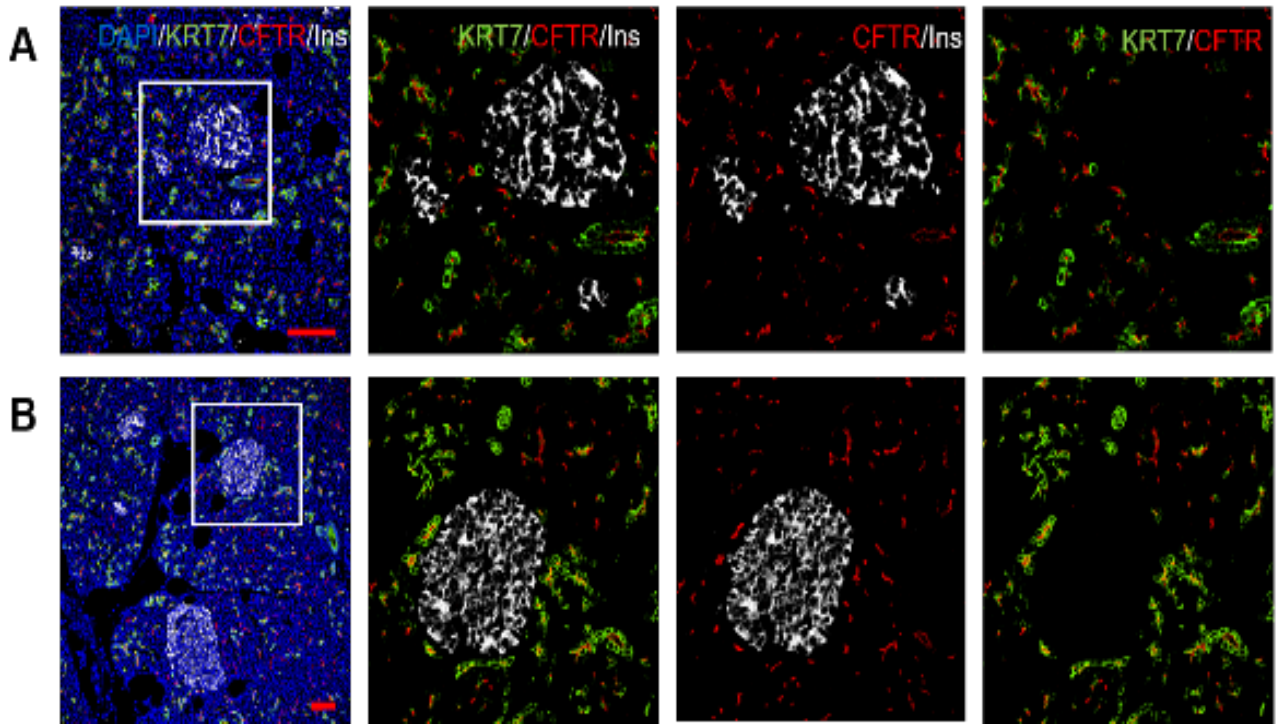


*Figure 5.5: CFTR protein expression in β-cells*

*Representative IF staining for CFTR (red), insulin (white) and KRT7 (green) in pancreatic tissue from two donors (A-B). Pancreatic tissue section from donors LDIS156 (A), and LDIS194 (B) showing CFTR expression in KRT7$^+$ cells, and no expression in insulin$^+$ cells. Rectangles represent magnified regions. Scale bars represent 100 µm.*

As is seen in Figure 5.4 and Figure 5.5, CFTR RNA and protein were absent in human β-cells and mainly localized in exocrine pancreas.

### 5.3.6 Evaluation of CFTR RNA and protein expression in all endocrine cells (ChrA$^+$) of islets

As CFTR was not found to be expressed in β-cells, the next question to address was if any islet endocrine cells expressed CFTR. Hence, expression of CFTR RNA by ISH and CFTR protein by IF staining, along with ChrA (endocrine cell marker) was assessed. To evaluate CFTR RNA expression within islet endocrine cells, CFTR ISH combined with IHC staining for ChrA was performed (Figure 5.6). Further, IF staining with ChrA and CFTR (Figure 5.7) was performed to check localization of CFTR within islet endocrine cells.

Figure 5.6 and Figure 5.7 indicated that CFTR was not expressed in any islet endocrine cells and is confined to the exocrine compartment in the human pancreas.

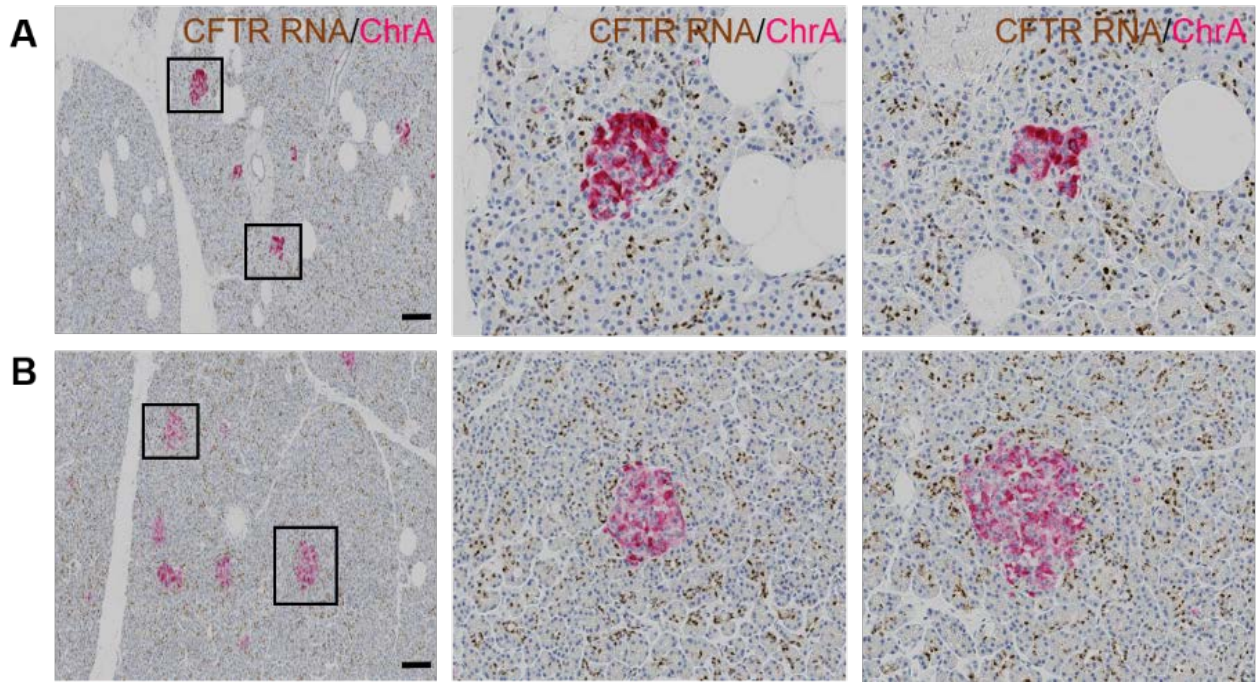


*Figure 5.6: CFTR RNA expression in islet endocrine cells*

*Representative CFTR ISH (brown) with ChrA IHC images in pancreatic tissue from two separate donors (A-B). Pancreatic tissue section from donors LDIS072 (A), and LDIS152 (B) showing CFTR RNA expression in exocrine pancreas, and no expression in ChrA$^+$ regions (pink). Rectangles represent magnified regions. Scale bars represent 100 µm.*

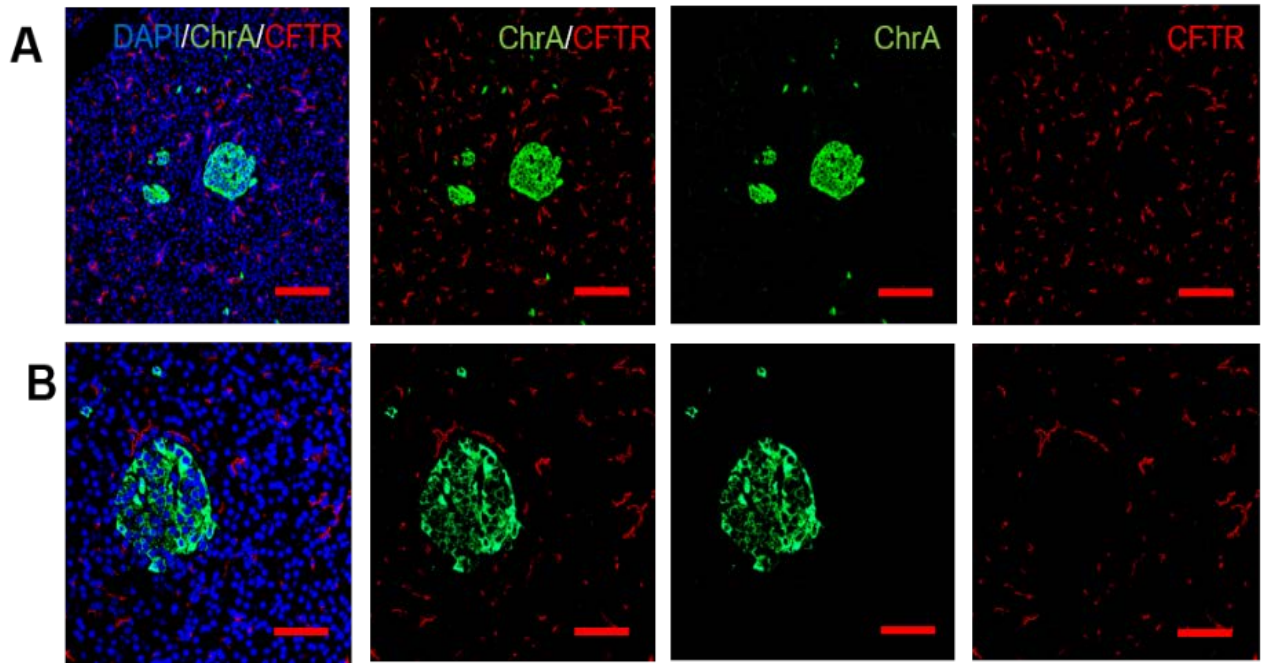


*Figure 5.7: CFTR protein expression in islet endocrine cells*

*Representative IF staining for CFTR (red), and ChrA (green) in pancreatic tissue from two donors (A-B). Pancreatic tissue section from donors LDIS161 (A), and LDIS156 (B) showing CFTR expression in exocrine pancreas, and no expression in ChrA$^+$ cells. Scale bars represent 100 µm.*

From the above results, it is evident that CFTR, protein or RNA, was not generally expressed in β-cells or other islet endocrine cell types, thereby, suggesting that CFTR may affect β-cell function by islet-extrinsic, exocrine pathways.

To address the next aims of the study, assessment of CF and CFRD pancreata was carried out by histopathological evaluation and IF staining to understand pancreatic disease development and associated exocrine and endocrine changes in CF.

### 5.3.7 Tissue sampling and donor characteristics

Pancreatic tissue sections were obtained from the UK EADB. Appropriate ethical clearance from University of Exeter and Newcastle University was obtained for the use and transport of the tissues.

Pancreatic tissue sections from nine CF and two CFRD deceased donors between the ages of 1-month to 19-years and six age-matched control, non-diabetic (no known pancreatic pathology) pancreatic donors were acquired for the study. Table 5.2 provides information on the CF donors and their non-diabetic controls.

*Table 5.2: Donor information on CF, CFRD and control cases*

| Group | Donor ID | Age |
|---|---|---|
| Non-diabetic controls | 12422 | 3 weeks |
| | 8582 | 1 year |
| | 8503 | 2 years |
| | 8618 | 5 years |
| | 8579 | 7 years |
| | 12142 | 17 years |
| CF | 11094 | 4 weeks |
| | 10763 | 2 years |
| | 10966 | 2 years |
| | 10842 | 3 years |
| | 10563 | 4 years |
| | 10920 | 4 years |
| | 11316 | 7 years |
| | 11328 | 14 years |
| | 211/71 | 19 years |
| CFRD | 10423 | 12 years |
| | 10386 | 14 years |

### 5.3.8 Histological evaluation of CF and CFRD tissue by H & E staining

Analysis of CF and CFRD tissue morphology was performed on sections obtained from mercuric chloride fixed, paraffin embedded tissue through employment of H & E staining. As can be seen in Figure 5.8, histological features differed between donors. All these features were evaluated by an experienced consultant pathologist Dr Dina Tiniakos at RVI (Newcastle, UK) on the basis of a scoring system adapted from a historic study characterizing changes in CF pancreas (Lohr et al., 1989). The results are presented in Table 5.3.

As described in Figure 5.8, varying extents of islet and acinar tissue damage (acinar atrophy) was observed across the CF donors. Moreover, severe fibrosis and lipid infiltration (lipomatous atrophy) was prevalent in some cases. Duct dilation was a more striking feature in some donors with presence of very large, dilated ducts, while some other donors showing extensive duct loss. Islets embedded in a pool of fatty tissue looked spared from the extensive fibrotic attack.

Histopathological scoring on one control, eight CF and two CFRD donors (Table 5.3) was conducted on the basis of eight clinical features described below:

1. Pattern: The pattern of exocrine damage i.e. fibrotic (presence of scarred acinar tissue showing fibrosis) or lipoatrophic (replacement of acinar cells by fatty adipose tissue).
2. Inflammatory score: Extent of inflammatory cells observed in the pancreas.
3. Acinar atrophy: Extent of loss of acinar tissue.
4. Acinar and/or ductal lumen dilation: Extent of duct dilation and loss of surrounding acini.
5. Duct loss: Extent of loss of defined ductal epithelium.
6. Insular atrophy: Extent of fibrotic damage observed in islets, accompanied by changes in shape and/or structure of islets.
7. Exocrine pancreas fibrosis: Extent of fibrotic damage to exocrine (acini) tissue.
8. Ductuloinsular complexes: Presence of ‘islet-like’ structures attached to the ducts that seem to be budding from the ducts; also known as nesidioblastosis (Soejima and Landing, 1986, Goossens et al., 1989, Kaczirek and Niederle, 2004)

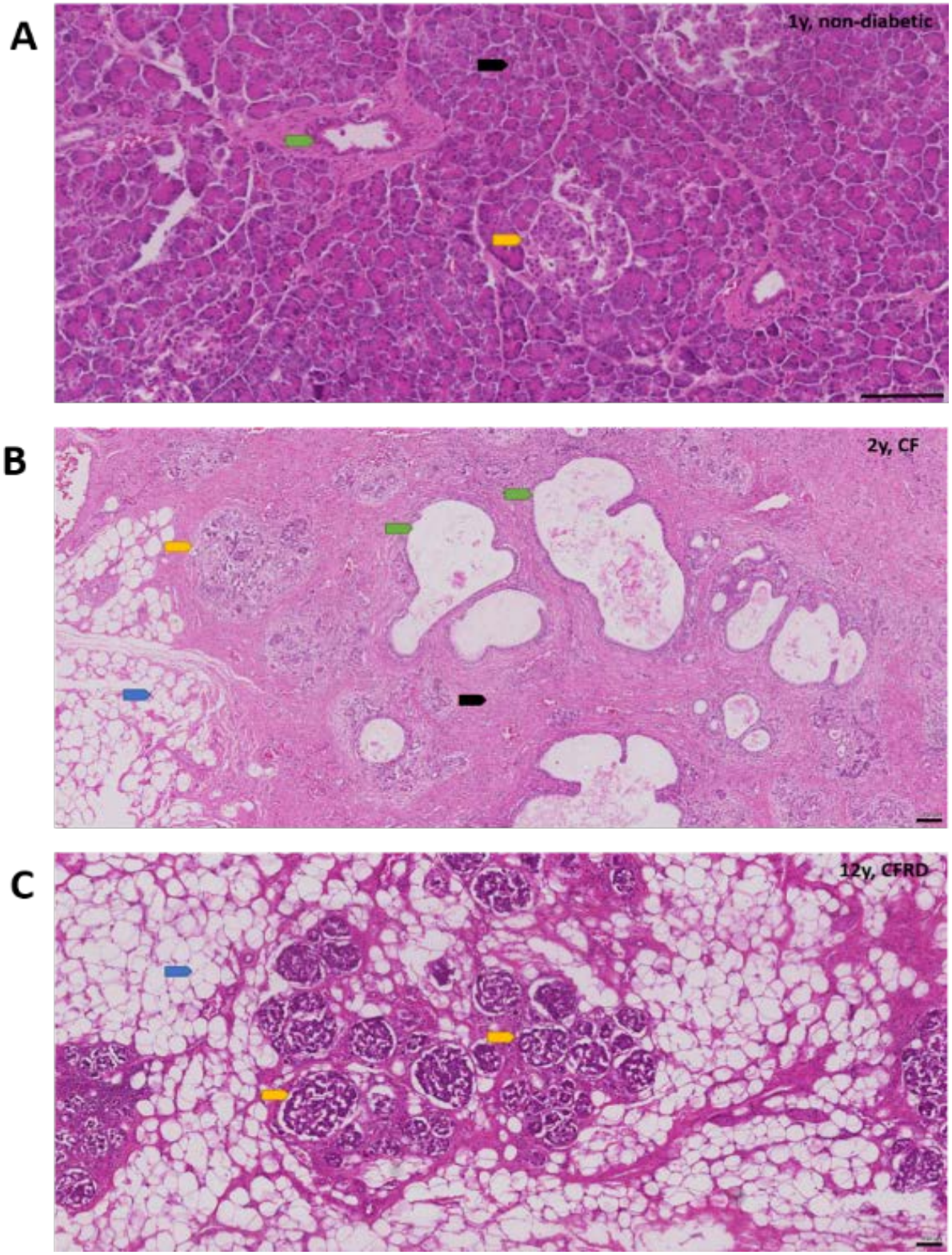


*Figure 5.8: Representative H & E images of control, CF, and CFRD donors*

*Representative H & E images of normal (A), CF (B), and CFRD (C) pancreas. (A) 1-year old non-diabetic pancreas (8582) showing intact acinar tissue (black arrow), islet (yellow) and duct (green). (B) 2-year old CF pancreas showing dilated ducts (green), and inflamed islets (yellow). Black arrow represent exocrine fibrosis, and encroaching fatty tissue is indicated in blue. (C) 12-year old CFRD pancreas showing spared islets (yellow) in a bed of lipoatrophic exocrine tissue (blue). Scale bars represent 100 µm.*

*Table 5.3: Histopathological scoring chart of control, CF and CFRD donors*

| Donor ID | Pattern | Inflammatory score | Acinar atrophy | Acinar and/or ductal lumen dilation | Duct loss | Insular atrophy | Exocrine pancreas fibrosis | Ductuloinsular complexes |
|---|---|---|---|---|---|---|---|---|
| 8582 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 10763 | 1 | 2 | 3 | 2 | 0 | 0 | 3 | 0 |
| 10966 | 1 | 1 | 2 | 2 | 1 | 2 | 2 | 0 |
| 10842 | 1 | 1 | 3 | 2 | 0 | 1 | 3 | 0 |
| 10920 | 1 | 1 | 2 | 1 | 1 | 1 | 2 | 0 |
| 10563 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 |
| 11316 | 2 | 2 | 3 | 1 | 2 | 1 | 1 | 1 |
| 10423 | 2 | 3 | 3 | 1 | 2 | 1 | 0 | 0 |
| 11328 | 1 | 1 | 3 | 3 | 1 | 2 | 3 | 1 |
| 10386 | 1 | 1 | 3 | 3 | 1 | 2 | 3 | 1 |
| 211/71 | 2 | 1 | 3 | 2 | 2 | 1 | 1 | 1 |
| **Scale** | 0=normal 1=fibrotic 2=lipoatro -phic | 0=none 1=mild 2=moderate 3=severe | 0=none 1= <1/3 acini lost 2= 1/3-2/3 acini lost 3= >2/3 acini lost | 0=none 1= mild 2= moderate 3= severe | 0=none 1=focal 2=exten -sive | 0=none 1= mild 2= moderate 3= severe | 0=none 1= mild 2=moderate 3= severe | 0=none 1= focal 2=extensive |

The control donor with no known pancreatic pathology (8582) showed normal pancreas histology with no evidence of the above-mentioned features. Among the CF and CFRD donors, the extent of acinar, islet and ductal damage differed greatly independent of age or exocrine fibrosis. Three (11316, 10423, and 211/71) out of ten CF donors exhibited lipoatrophic pattern of exocrine damage while the others had fibrotic damage. Surprisingly, even after total replacement of acinar cells with adipose tissue, islets of these three donors showed mild damage. Moreover, even the islet damage in the two CFRD donors was not extreme with one (10386) showing moderate insular atrophy while the other (10423) showing mild lesions. Over 65 % of acini were lost in five CF and two CFRD donors, showing aggressive pancreatic disease in these donors.

### 5.3.9 Evidence of budding in CF

Assessment of pancreas histology on H & E stained sections highlighted the presence of ‘islet-like’ group of cells that appeared to be budding from the epithelium of dilated ducts in some CF pancreata (Figure 5.9).

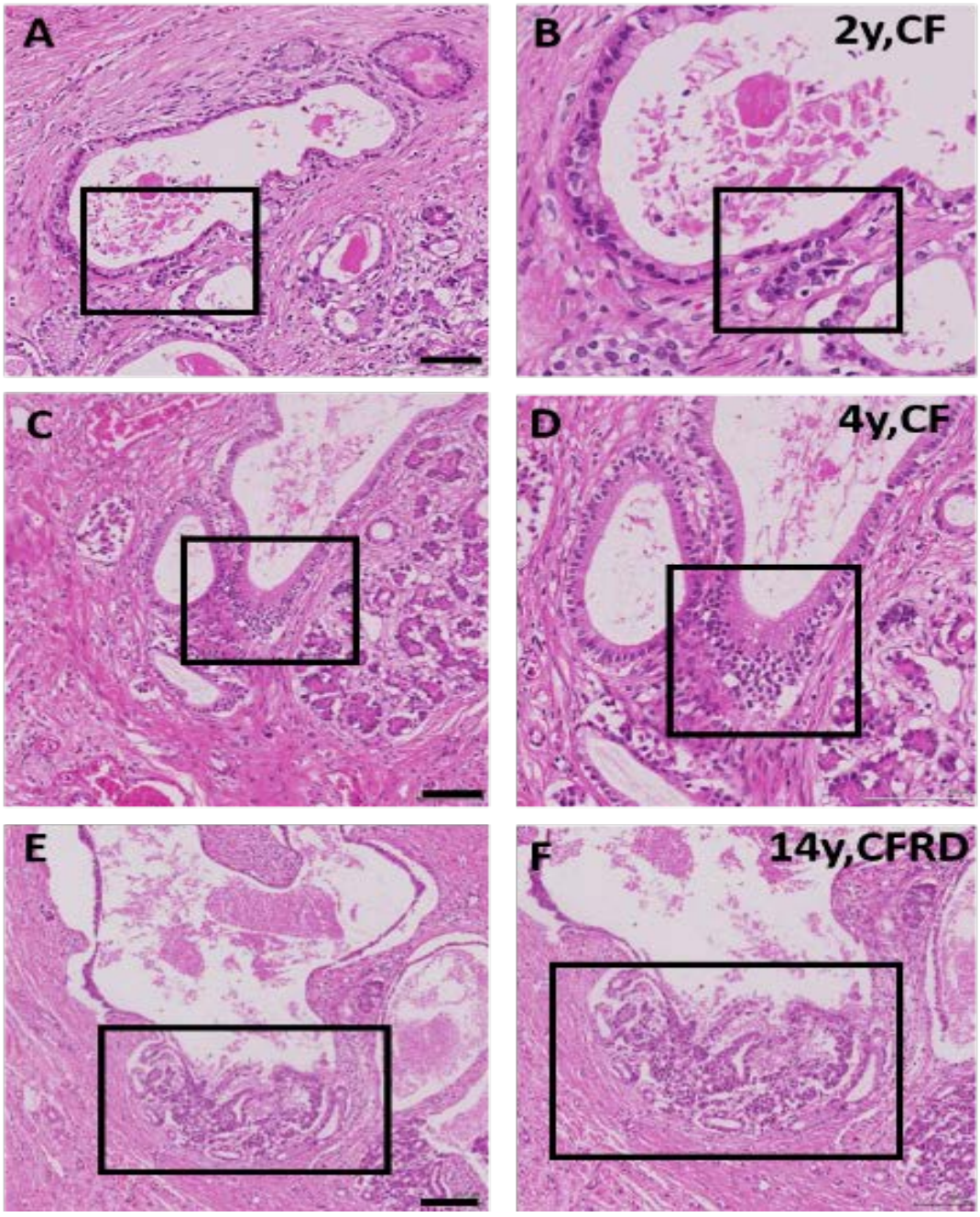


*Figure 5.9: Representative H & E images showing budding of ducts*

*Representative H & E images showing budding of 'islet-like' structures in CF (A-D) and CFRD (C-F) pancreas from three different donors. Pancreatic tissue section from donors 10966 (A-B), 10563 (C-D), and 10386 (E-F) showing dilated ducts and groups of cells budding from ducts (box). B, D, E represent magnified regions of A, C, E respectively. Scale bars represent 100 µm.*

As can be seen in Figure 5.9, there was evidence of budding of 'islet-like' structures in some CF and CFRD pancreata. These 'budding' structures are called 'ductulo-insular complexes' and are thought to represent new endocrine cell formation originating from multipotent ductal epithelial cells (Kim et al., 1992).

For the assessment and quantification of endocrine (and exocrine) changes in the normal, CF and CFRD pancreas, IF staining (on serial sections) with ChrA, insulin and hormone cocktail (3H) consisting of glucagon, somatostatin and PP antibodies was performed. The results are described below.

### 5.3.10 IF staining indicating hormone expression in and around ducts

Firstly, to confirm if the cells observed in the ductal lining (Figure 5.9) are of endocrine origin, qualitative assessment of IF staining with ChrA, insulin and hormone cocktail (3H) was performed on CF, CFRD and normal pancreatic sections.

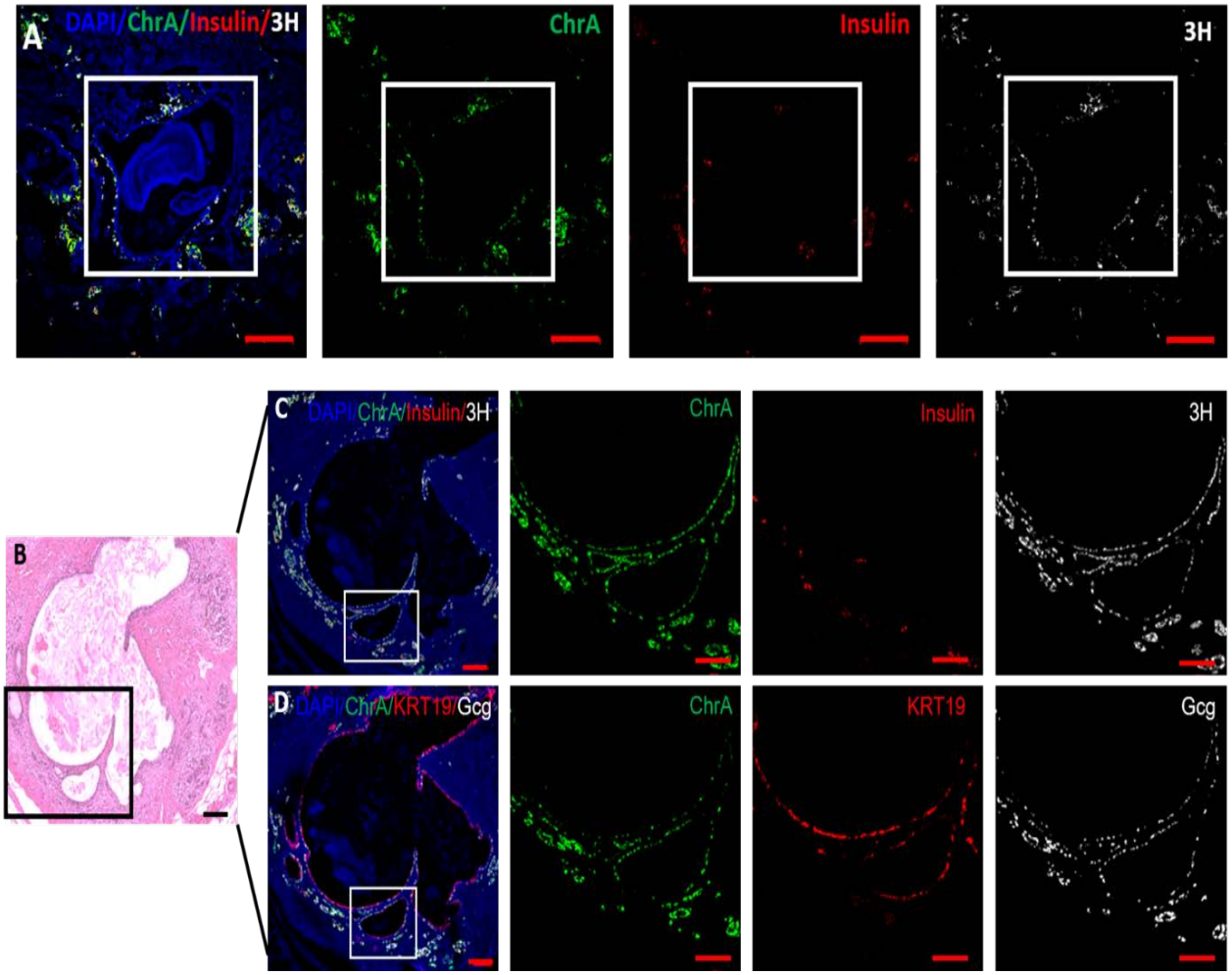


*Figure 5.10: Representative IF images of hormone expression around ducts in CF donors.*

*(A) Representative IF image showing hormone expression around a duct in the 4-year old CF pancreas (10563). (B) H & E image from donor 10966 showing hormone like cells around ducts (indicated in box). (C) IF staining on serial section showed ChrA$^+$ cells lining the ducts, with majority of them co-expressing non-β-hormones (3H). (D) Further staining on serial section showed presence of glucagon$^+$ cells around the ducts. Scale bars represent 100 µm.*

As shown in Figure 5.10, ChrA$^+$ cells lining the ducts were found to be mostly hormone cocktail$^+$ (i.e. of non-β origin). Based on a study showing ductal derived β-cell neogenesis through an α-cell intermediate (Ben-Othman et al., 2017), it was hypothesized that these non-β-cells in the ducts could be glucagon-expressing cells. Thus, further staining on serial sections with ChrA, keratin 19 (KRT19- to confirm ductal regions), and glucagon was performed. It was found that most ChrA$^+$ cells in the ducts were glucagon$^+$. Occasional glucagon$^+$ cells co-expressed ductal cell marker KRT19, indicating that these cells might be originating from ducts.

Further assessment of endocrine changes in the islets of CF, CFRD and normal pancreas was performed and is described below.

### 5.3.11 Assessment of islet size and endocrine area

Histopathological examination of CF and CFRD tissue revealed presence of larger-than-normal islets in diseased conditions compared to age-matched controls with no known pancreatic pathology. However, on IF staining on serial sections, the spread of ChrA$^+$ endocrine cells within these large islets seemed modest to the islet size. Hence, we sought to assess islet and respective endocrine area within each donor. To do this, twenty islets were assessed on five normal, and nine CF (including two CFRD) donors. Islet and endocrine area was determined using Nikon Elements AR software. Islet endocrine cell area was measured by automatic thresholding of ChrA-stained region. Total islet area was measured by automated contouring of the whole islet structure which included ChrA-stained area as well as unstained regions (like intraislet capillaries) (Kilimnik et al., 2012). Table 5.4 and Figure 5.11 below describe information for the same. The islets were classified as below for the purposes of analysis:

| **Mean islet area** | **Terminology** |
|---|---|
| <5 mm$^2$ | Small islets |
| 5-10 mm$^2$ | Medium islets |
| >10 mm$^2$ | Large islets |

*Table 5.4: Quantification of islet and endocrine area*

| Donor ID | Group | Mean islet area ($mm^2$) | Mean endocrine area ($mm^2$) | Endocrine/ islet area (%) |
|---|---|---|---|---|
| 12422 | Control | 6.3 | 3.7 | 61.2 |
| 8503 | Control | 8.7 | 5.0 | 58.6 |
| 8618 | Control | 4.9 | 3.5 | 69.8 |
| 8579 | Control | 5.7 | 4.3 | 78.3 |
| 12142 | Control | 5.7 | 4.4 | 76.9 |
| 10763 | CF | 8.1 | 3.8 | 50.1 |
| 10966 | CF | 4.8 | 2.9 | 61.7 |
| 10842 | CF | 4.5 | 2.3 | 49.6 |
| 10563 | CF | 9.9 | 5.1 | 59.6 |
| 10920 | CF | 11.2 | 5.6 | 50.8 |
| 11316 | CF | 8.5 | 4.3 | 54.1 |
| 10423 | CFRD | 11.9 | 6.6 | 56.1 |
| 10386 | CFRD | 14.5 | 8.1 | 62.8 |
| 211/71 | CF | 27.9 | 14.6 | 50.7 |

As is evident from Figure 5.11, there were dramatic differences in the islet size between control and CF pancreata. The mean islet area (Figure 5.11A) in CF (11.3 $mm^2$) was nearly two-fold higher than control (6.3 $mm^2$) donors. However, the ratio of endocrine area ($ChrA^+$) to the whole islet size significantly decreased in CF (55 %) compared to control (69 %) donors (Figure 5.11B). This may be associated with loss of endocrine cells from the islet core or can suggest presence of fibrous tissue in the islet structure thereby, increasing islet size. Also, it can indicate that islet aggregation in fibrous or lipoatrophic tissue leads to union of two or more islets. Moreover, an age-dependent increase in the size of islets was found in CF pancreas, while the islet size remained unchanged in control donors (Figure 5.11C).

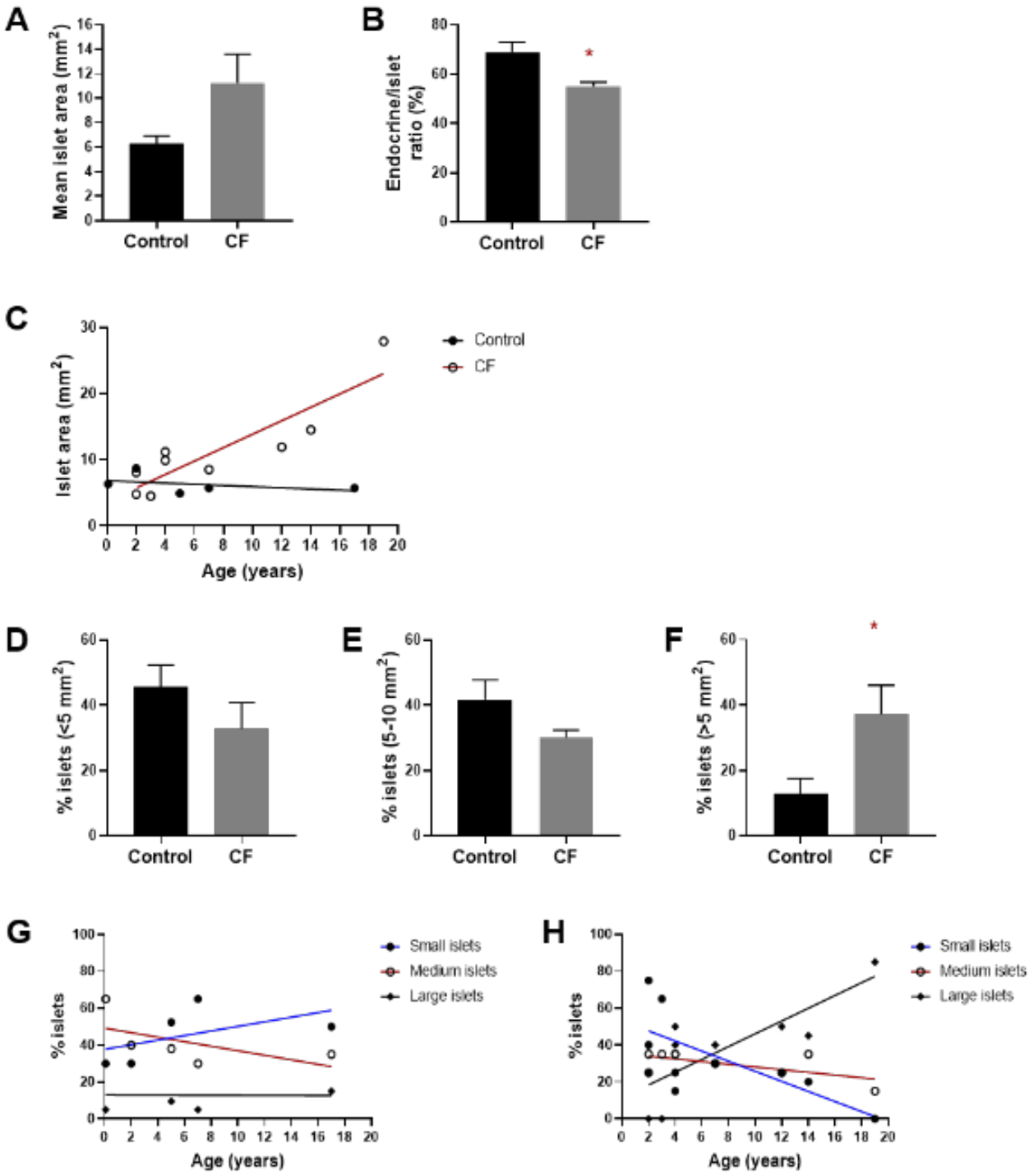


*Figure 5.11: Assessment of changes in islet and endocrine area in control and CF pancreas*

*The mean islet area increased in CF compared to controls (A), but the percentage ratio of endocrine area to islet area was found to decrease significantly in CF (B). (C) Correlation plot of age and islet area revealed that while size/area of islets remained constant in controls, there was an age-dependent increase in the area of islets in CF. The number of small islets (D) and medium islets (E) was found to decline in CF, while the number of large islets (F) increased significantly compared to controls. (G) Correlation plot revealed age-dependent changes in the number of small, medium and large islets in control (G) and CF (H) donors. Bars represent mean ± SEM ($p<0.05$, Student's t-test).*

Islets were then classified as small (<5 $mm^2$), medium (5-10 $mm^2$) and large (>10 $mm^2$) depending on the islet area, It was found that while the percentage of small (Figure 5.11D) and medium (Figure 5.11E) islets decreased in CF compared to controls, the percentage of large islets (Figure 5.11F) increased nearly three-times in CF (37 %) compared to controls (13 %). Also, these changes were found to be age-dependent (Figure 5.11H). In control pancreas (Figure 5.11G), however, presence of large islets was between 10-15 % and did not change with age.

### 5.3.12 Selection of donors for the assessment of changes in islet hormone expression in CF and control pancreas

Most of the tissue samples used for this study were fixed in mercuric chloride (a fixation reagent no longer used). Due to this, the quality of IF staining obtained was not standard in all tissue sections and thus, not ideal for quantitative assessment. Nevertheless, seven samples were selected for quantitative analysis to get an estimate of changes in hormone expression profiles. Of these seven, two each of CF and CFRD donors were chosen because they showed extensive fibrosis, large islets, and ductal pathology characteristic of CF pancreas, and three age-matched controls were selected (Table 5.5). As before, the tissue was stained by IF for ChrA, insulin and hormone cocktail (glucagon, somatostatin and PP). The tissue section was imaged using confocal microscopy and hormone expression in islets, single scattered cells, ducts and ductuloinsular complexes was evaluated using manual counting.

*Table 5.5: Donors selected for quantification of hormone expression*

| Group | Donor ID | Age (years) |
|---|---|---|
| Controls | 8503 | 2 |
| | 2189 | 4 |
| | 12142 | 17 |
| CF | 10966 | 2 |
| | 10563 | 4 |
| CFRD | 10423 | 12 |
| | 10386 | 14 |

### 5.3.13 Quantification of endocrine cell number in the islets

To determine the extent of loss of endocrine cells from islet core, the number of ChrA$^+$ cells was determined by manual counting. Fifty images, containing islets and ducts, from each donor were captured using Nikon confocal microscope. To determine changes in islet cell number, ChrA$^+$ cells were counted in the islets in the seven donors and a mean number of ChrA$^+$ cells per islet was obtained. The number of islets counted on each donor is described in Table 5.6 below. Figure 5.12 indicates the changes in number of ChrA+ cells per islet in CF donors compared to controls.

*Table 5.6: Number of islets counted in each donor*

| Group | Donor ID | No. of islets counted |
|---|---|---|
| CF | 10966 | 94 |
| CF | 10563 | 141 |
| CFRD | 10423 | 92 |
| CFRD | 10386 | 127 |
| Control | 8503 | 50 |
| Control | 2189 | 50 |
| Control | 12142 | 49 |

The mean number of islets counted between CF (133.5 ± 24.4) and non-CF controls (49.7 ± 0.6) differed largely. Figure 5.12C indicates that there is a trend (insignificant) towards decrease in ChrA+ endocrine cells in CF (45.35 ± 6.8) compared to controls (55.58 ± 2.02). While this could be due to differences in number of islets counted, this could also indicate loss of some islet cells secondary to fibrotic damage of the tissue.

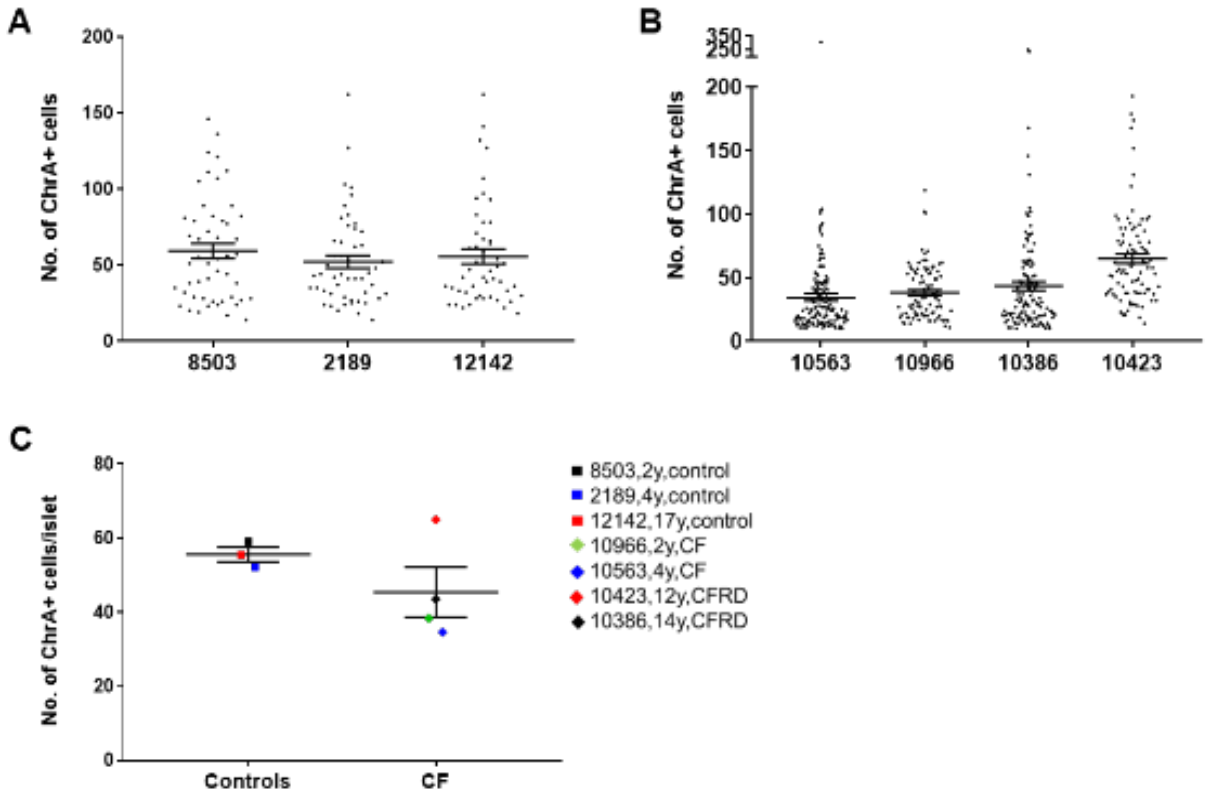


*Figure 5.12: Maintenance of ChrA⁺ endocrine cell number in CF compared to controls.*

*Quantification of endocrine cells (ChrA⁺) per islet in normal and CF donors. Data represent mean ± SEM. (A-B) Scatter dot plot of the number of ChrA⁺ cells in individual islets in control (A), and CF donors (B). (C) Dot-plot of the mean number of ChrA⁺ cells per islet in each control (n = 3) and CF (n = 4) donor (denoted as separate dots), showing a slight decrease in the number of ChrA⁺ cells in CF islets compared to controls.*

### 5.3.14 Assessment of islet hormone expression

To assess hormone expression profiles in non-CF and CF islets, number of insulin⁺ and hormone cocktail⁺ (3H) cells were determined by manual counting. The results obtained are indicated in Figure 5.13.

Figure 5.13 describes the changes in hormone expression in normal and diseased states. A significant decrease in insulin-expressing β-cells was evident in CF donors (17.08 ± 1.35) compared to the controls (30.4 ± 2.86). No changes in expression of other non-β hormones leads to the possibility that the β-cells are more affected by the fibrosis and exocrine insufficiency in CF pathology.

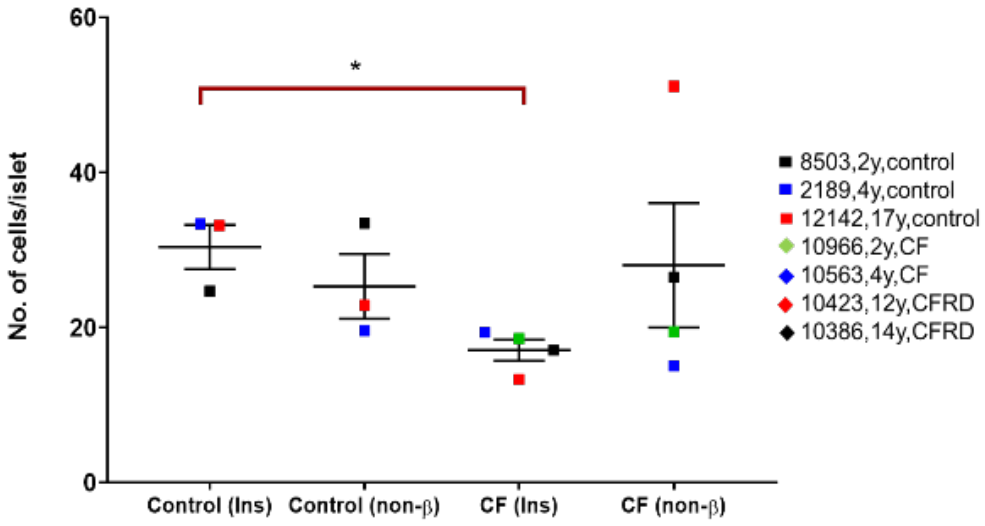


*Figure 5.13: Changes in islet hormone expression profiles in CF and control donors.*

*Quantification of hormone expression demonstrates a significant decrease ($p<0.05$) in insulin expression in the CF donors when compared to the controls. In parallel, no change in expression of non-β-cell hormones was observed in the CF donors compared to controls. Data represents mean ± SEM.*

### 5.3.15 Assessment of CPHN cells in CF and non-CF islets

Recently, a study by Cory et al. (2018) reported presence of CPHN cells in CF pancreata suggesting attempted endocrine cell regeneration. Thus, prompted determination of the presence of CPHN cells in the islets of CF and control donors. To do this, cells expressing ChrA but no insulin, or other non-β-hormones (glucagon, somatostatin and PP) were evaluated.

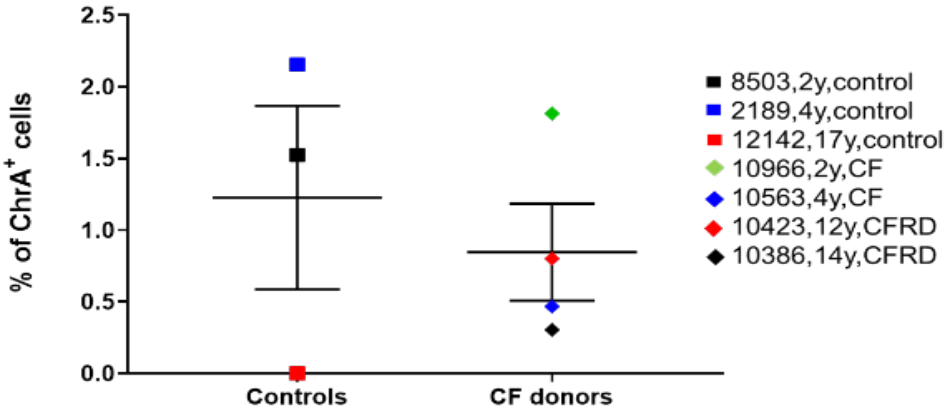


*Figure 5.14: Presence of CPHN cells in islets of control and CF donors.*

*The percentage of hormone-empty ChrA⁺ cells in the islet was greater in control than in CF donors. Data represents mean ± SEM.*

Figure 5.13 indicates no significant difference between the number of hormone-empty endocrine cells in the islets of control donors (1.227 ± 0.64) compared to CF (0.85 ± 0.34). This is in contrast to the observation by the Butler group (Cory et al., 2018) in which they found a higher number of CPHN cells in CF islets, rather than control subjects.

### 5.3.16 Assessment of hormone expression in single, scattered cells

A lot of scattered, single cells, or small cell clusters (maximum three cells) in exocrine pancreas, were found to be $ChrA^+$ (i.e. of endocrine nature). While this phenomenon was observed in normal pancreas too, it was much more pronounced in CF donors. A total of 1582 $ChrA^+$ single cells across the four donors were quantified to determine their phenotypic identity. The Table 5.7 below shows the number of single cells counted in each donor, and the hormone expression within these cells is indicated in Figure 5.15.

*Table 5.7: Number of scattered single cells counted in four CF donors*

| Donor ID | Number of cells counted |
|---|---|
| 10563 | 661 |
| 10966 | 552 |
| 10386 | 202 |
| 10423 | 167 |

Many single, scattered endocrine cells in the exocrine pancreas were observed in CF donors. The hormone expression within these cells was evaluated and is described above. Figure 5.15 shows that mostly, scattered single cells express non-β-hormones. The expression of non-β-hormones varied from about 45 % to 99 %, depending on the severity of exocrine fibrosis (Table 5.3). As the extent of exocrine fibrosis increased, the number of β-cells and CPHN cells decreased. 10423 did not show exocrine fibrosis but had a lipoatrophic pattern of exocrine damage (full replacement of acini with fatty tissue). This could be a reason for absolute loss of β-cells and CPHN cells from the exocrine tissue. CPHN cells in the form of single scattered units in the exocrine pancreas are believed to be representative of endocrine cell regeneration as is seen in foetal and infant pancreata (Moin et al., 2018). Presence

of nearly 5 to 25 % of CPHN cells in CF pancreata may indicate attempted endocrine cell regeneration triggered by exocrine fibrosis.

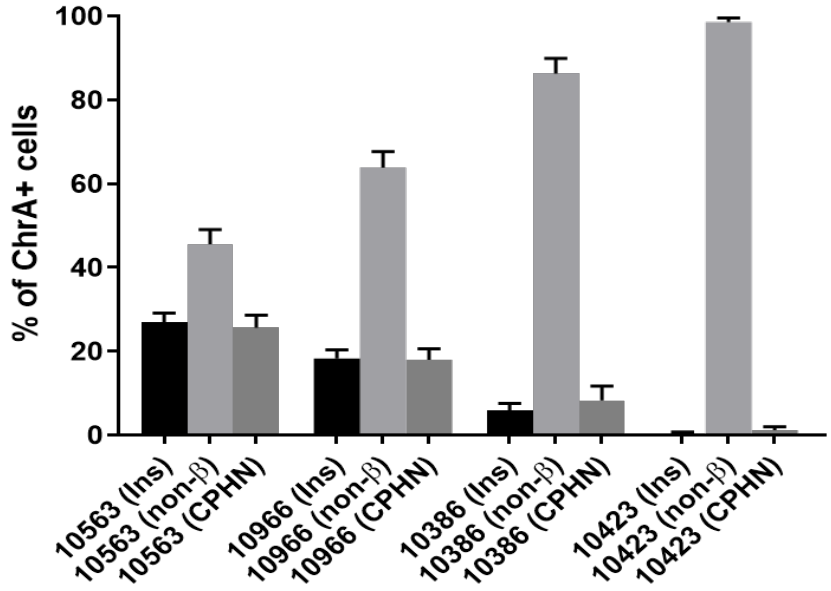


*Figure 5.15: Quantification of hormone expression in scattered single cells/small clusters in the four CF donors*

*Most scattered endocrine single cells or in clusters were non-β-cells, while the percentage of insulin-expressing and CPHN cells was almost similar in all four donors.*

### 5.3.17 Assessment of hormone expression in the ducts

While there was the presence of occasional single ChrA$^+$ cells in ducts of normal pancreas, these cells were found to be densely lining the ductal lumen in three out of four CF donors. The number of ducts counted in each donor is depicted in Table 5.8. Pancreatic section from donor 10423 showed extensive duct loss and no hormone expression around existing ducts. The hormone expression within these cells was evaluated and is depicted below in Figure 5.16.

*Table 5.8: Number of ducts counted in four CF donors*

| Donor ID | Number of ducts counted |
|---|---|
| 10563 | 7 |
| 10966 | 21 |
| 10386 | 45 |
| 10423 | 0 |

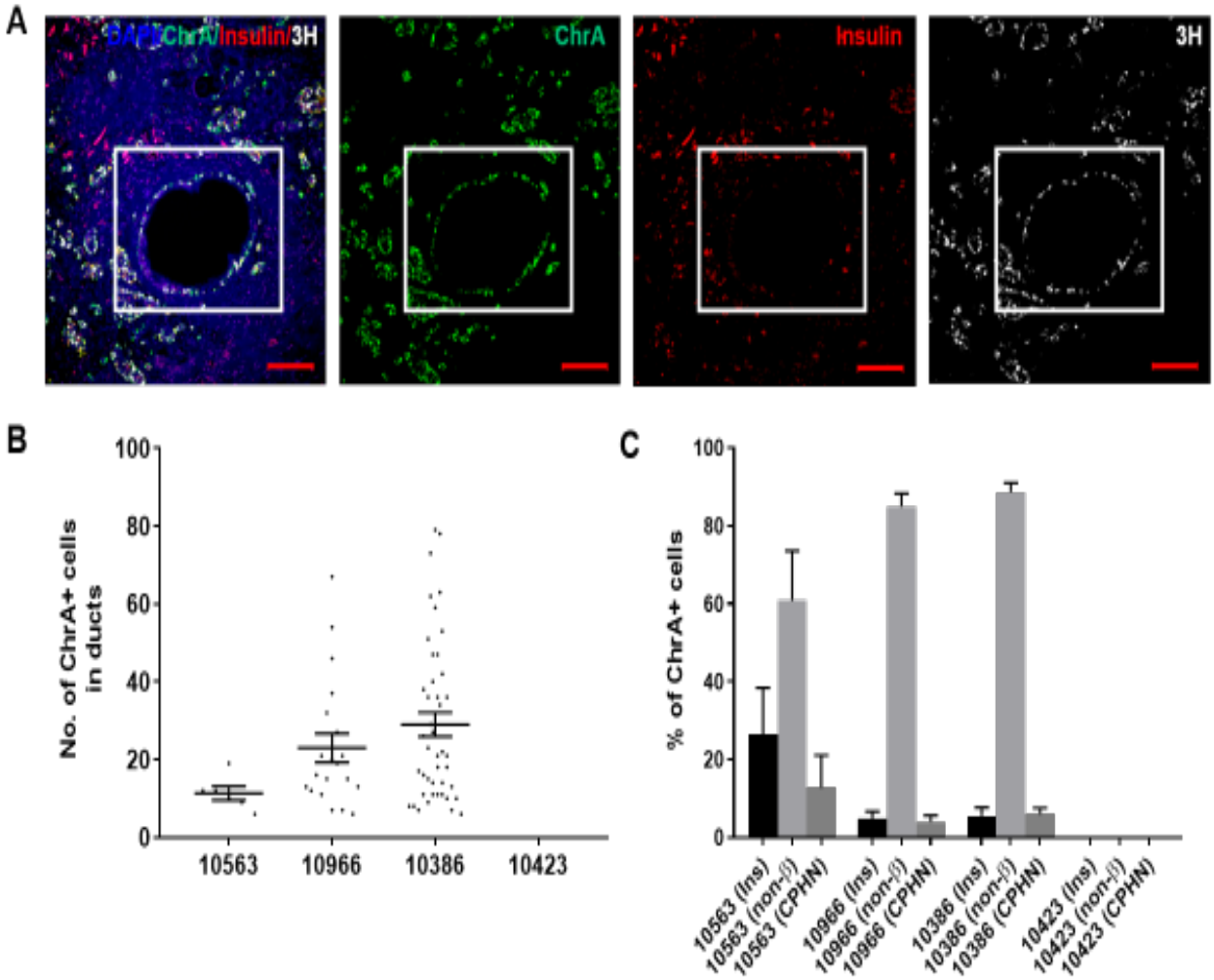


*Figure 5.16: Assessment of hormone expression in CF ducts*

*(A) Representative image from IF staining of 2-year old CF (10966) pancreas showing ChrA⁺ cells lining ducts (green), which are mostly of non-β phenotype (white). Box indicates ductal region. Scale bars represent 100 µm. (B) Scatter dot-plot showing number of ChrA⁺ cells in each, individual ducts. (C) Quantification of hormone expression in CF ducts reveals presence of higher percentage of non-β cells compared to β- or CPHN cells. Data represents mean ± SEM.*

Figure 5.16 shows the evaluation of hormone expression in the ducts. The number of ducts showing hormone-expressing endocrine cells correlated directly with the extent of fibrosis (i.e. donors with milder fibrosis had lesser number of hormone-expressing ducts). Also, this hormone expression was only found in donors displaying a fibrotic pattern of exocrine damage, and not lipoatrophic exocrine destruction. As is evident from Figure 5.16A and Figure 5.16C, most of the ChrA⁺ cells lining the ducts were of non-β-origin i.e. either glucagon, somatostatin or PP cell. Also, CF pancreatic donor with milder exocrine fibrosis (10563) showed presence of nearly 25 % of insulin⁺ cells and over 10 % of CPHN cells, while the number of β-cells and CPHN cells declined with increasing fibrosis.

### 5.3.18 Assessment of hormone expression in ductuloinsular complexes in CF pancreata

'Islet-like' groups of cells were found to be extending from the ChrA$^+$ ductal lining in CF donors termed as 'ductuloinsular complexes'. Such ductuloinsular complexes were found in three out of four CF donors. The number of ductuloinsular complexes counted in each donor is described in Table 5.9. Pancreatic section from donor 10423 showed extensive duct loss and no hormone expression around ducts, hence, no ductuloinsular complexes. Analysis of hormone expression within these structures was performed and is described in Figure 5.17 below.

*Table 5.9: Number of ductuloinsular complexes counted in four CF donors*

| Donor ID | Number of ductuloinsular complex counted |
|---|---|
| 10563 | 8 |
| 10966 | 5 |
| 10386 | 9 |
| 10423 | 0 |

In line with no hormone expression in ducts of donor 10423, no ductuloinsular complexes were found either. Figure 5.17 demonstrates the hormone expression in ductuloinsular complexes. Ductuloinsular complexes, like the ducts, were mainly composed of non-β-cells. But a difference was the percentage of insulin$^+$ cells was 15-40 % in the ductuloinsular complexes, which was higher than the ducts. CPHN cells were rarely observed in the ductuloinsular structures.

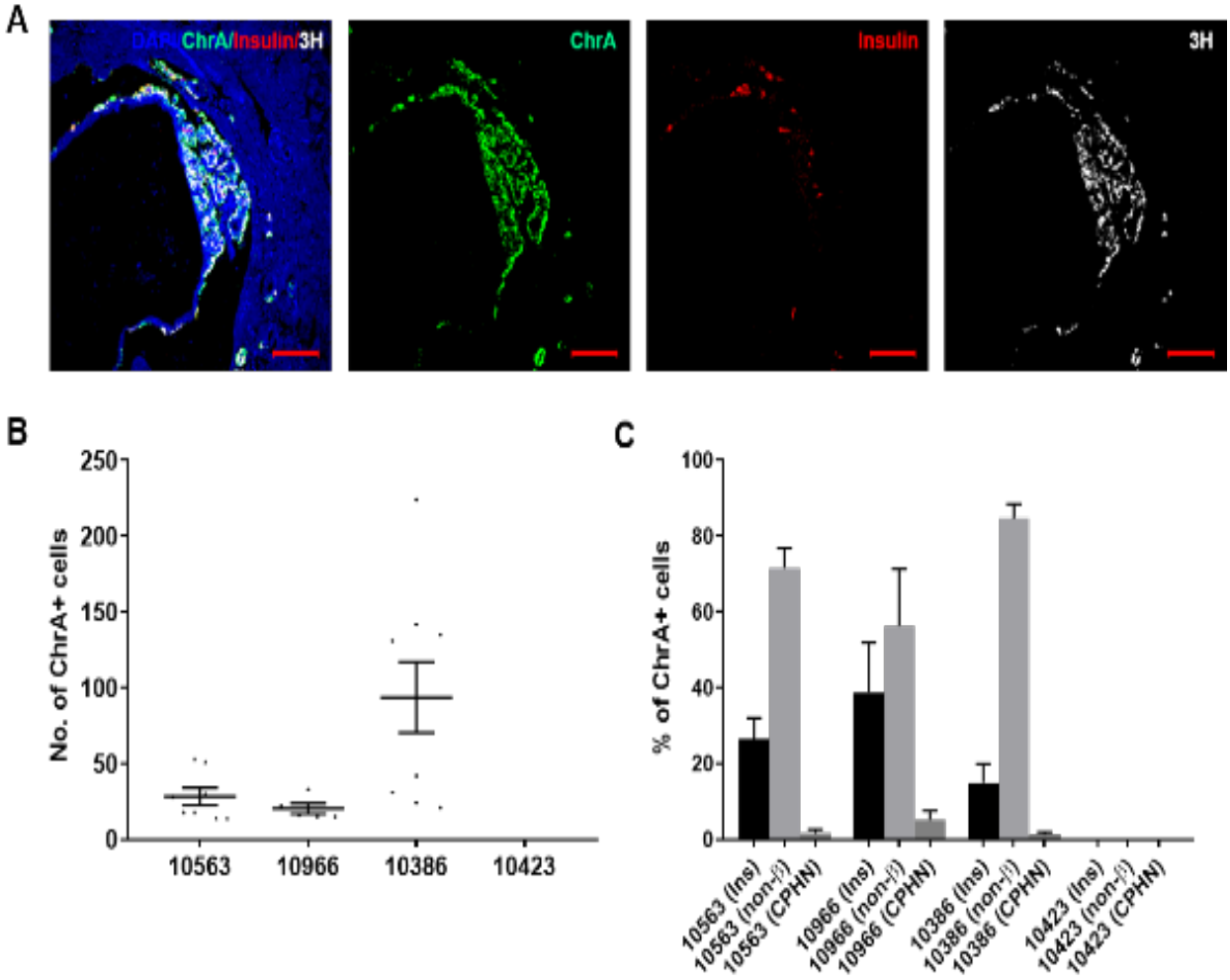


*Figure 5.17: Assessment of hormone expression in ductuloinsular complexes*

*Representative IF images of ductuloinsular hormone expression from 14-year old CFRD (10386) pancreas. Scale bars represent 100 µm. (B) Scatter dot-plot showing number of ChrA⁺ cells per ductuloinsular complex in CF donors. (C) Evaluation of hormone expression in ductuloinsular complexes in the CF donors indicates that percentage of β-cells decrease with advancing age. Data represents mean ± SEM.*

## 5.4 Discussion

The focus of the study was to characterize CFTR expression in human pancreas with no known pancreatic pathology and assess histopathological and hormone expression changes in CF and CFRD pancreas compared to age-matched controls.

The major findings of the study were as follows:

1) CFTR protein and mRNA is not expressed in islet β-cells or any other ChrA⁺ endocrine cells.

2) The histopathology of CF pancreas with or without CFRD is very different from normal pancreas due to presence of acinar tissue damage, fibrosis and interstitial fatty tissue.
3) Presence of ductal associated endocrine cells, mostly glucagon-expressing, provides evidence for islet neogenesis/regeneration by duct cells.

Firstly, absence of detectable CFTR mRNA in β-cells (Figure 5.4) or any other islet endocrine ($ChrA^+$) cells (Figure 5.6) by ISH in normal pancreas with no known pancreatic pathology was observed. CFTR mRNA was localised in the exocrine pancreas (Figure 5.3). A study by Sun et al. (2017) also reported similar finding with absence of CFTR mRNA in endocrine cells of isolated human islets, and co-localization of CFTR with $KRT7^+$ cells. These data are consistent with data obtained from three other studies in which single cells RNA sequencing of β-cells revealed very low levels of CFTR in a very small population of β-cells (less than 5 %) (Blodgett et al., 2015, Segerstolpe et al., 2016, Hart et al., 2018).

In the present study, there was an absence of CFTR protein expression in β-cells (Figure 5.5) or any other islet endocrine ($ChrA^+$) cells (Figure 5.7) by IHC. This is in line with the observations of Hart et al. (2018), in which they failed to detect CFTR protein expression in β-cells by immunostaining of human pancreas. As also observed by Sun et al. (2017), CFTR was mainly found to be localized in $KRT7^+$ cells In this study, CFTR protein expression was assessed using CFTR 596 antibody which has been demonstrated to detect CFTR by IF staining (van Meegen et al., 2013). Because conventional CFTR antibodies have been identified to have problems with non-specific binding (van Meegen et al., 2013), thorough optimization (Figure 5.1) was completed before use, and antibody specificity (Figure 5.2) was confirmed in wild-type and CFTR-null ferret pancreatic sections. Wild-type ferret pancreatic tissue section showed presence of CFTR expression, while CFTR-null ferret pancreatic tissue section did not show CFTR expression.

However, our observation of absence of CFTR is in contrast to the studies that have been reporting CFTR expression in single human β-cells following IF staining (Edlund et al., 2014). The CFTR antibody (MATG-1061) used in that study was different to ours, and also showed lack of (expected) CFTR expression in ductal cells, highlighting technical difficulties with use of MATG-1061 antibody (Edlund et al., 2017). Our data, along with existing literature, would strongly suggest that CFTR RNA or protein may not be expressed in sufficient quantities in islet cells, to contribute to β-cell

function through cell-intrinsic mechanisms. Thus, CFTR may cause β-cell dysfunction and subsequent CFRD development, by β-cell-extrinsic, paracrine mechanisms.

Assessment of CF and CFRD pancreas revealed key histopathological changes associated with the pancreatic disease in CF. Pancreatic tissue sections from CF donors between the age of 1-month and 19-years were examined by H & E staining and were scored on the basis of histopathological lesions (Table 5.3). The pathology and disease severity between all CF donors varied greatly and seemed to be independent of age although there was no information on CF genotype (severity) of disease in these donors. Fibrotic and lipoatrophic pattern of exocrine damage, along with presence of varying levels of inflammatory cells and acinar damage was observed in CF donors. Cystic dilation of ducts was also observed, along with ductuloinsular complexes (nesidioblastosis) were also found in CF pancreas. Another observation noted in CF pancreas was that the islets looked relatively spared, and clustered closely in together, in fibrotic or fatty exocrine tissue (Figure 5.8C). These histopathological features have also been observed by other studies involving human CF pancreas (Iannucci et al., 1984, Lohr et al., 1989, Bogdani et al., 2017, Hart et al., 2018) and highlight the degree of exocrine damage in CF pancreatic disease.

Islets in CF appeared bigger in size than those in control pancreas. Thus, islet and endocrine area was evaluated in CF/CFRD and control pancreata. The islet area was found to be increased in CF compared to controls. However, the ratio of endocrine area to the islet area was significantly decreased in CF. Moreover, the number of large islets (>10 $mm^2$) increased significantly in CF, while the number of small and medium islets decreased with age. This may suggest that smaller islets coalesce to form larger islets. Such a phenomenon is also observed in aging CF ferrets (Rotti et al., 2018), indicating that extensive islet remodelling may be occurring secondary to exocrine fibrosis. Further quantification of islet hormone expression also revealed characteristic endocrine changes in CF. The number of $ChrA^+$ endocrine cells per islet decreased in CF, compared to normal islets. Moreover, quantification of hormone expression changes in the islets reported about 50 % reduction in insulin and no changes in the levels of non-β-cell hormones. While the loss of insulin in CF islets is consistent with other studies (Bogdani et al., 2017), studies have been reporting an increase in expression of non-β-hormone cells (Lohr et al., 1989, Bogdani et al., 2017, Hart et al., 2018).

Altogether these results indicate extensive pancreatic (exocrine and endocrine) remodelling due to CF pathology, as demonstrated by other human and ferret studies too (Hart et al., 2018, Rotti et al., 2018). Loss of β-cells only from the islet niche points towards the vulnerability of β-cells to the fibrotic damage of exocrine pancreas. This raises the question as to how exocrine insufficiency and ductal damage influence islets, particularly insulin function. Is this due to disruption in the 'islet-acinar axis' defining relationship between acinar and endocrine tissue or due to the ductal impact on β-cell function? (Bertelli and Bendayan, 2005). Fibrotic damage of acinar cells is shown to disrupt vascularization and intra-pancreatic secretion of insulin (Partha Pratim Chakraborty, 2015). This could result in hypoxia which is proven to affect β-cell function (Gerber and Rutter, 2017). Another way by which CF exocrine pathology can affect β-cells is lipotoxicity due to fat replacement of acinar cells (Lee et al., 1994). Moreover, presence of inflammation has also been shown to affect islet loss and β-cell function in CF (Hart et al., 2018).

While the ability of ductal cells to regenerate and give rise to new islets is long established in animal models of pancreatic diseases, this study observed the presence of ductal associated endocrine cells in human CF pancreas (Figure 5.16), even noting budding-like phenomenon of endocrine cells from pancreatic ducts on H & E stained sections (Figure 5.9). A study by Lohr et al. (1989) also observed such budding of endocrine cells from the ductal epithelium of patients with CF and CFRD. This phenomenon is shown to exist in patients with fibrotic pancreas and not lipoatrophic lesions (Lohr et al., 1989), as was also observed in our study.

Numerous *in vitro* and *in vivo* studies have looked into the possibility and subsequent pathways of differentiation of ductal cells to mature islet cells (Wang et al., 1995, Bonner-Weir et al., 2000, Inada et al., 2008, Xu et al., 2008, Reichert and Rustgi, 2011). A study using pancreatic duct ligation in rats have reported differentiation of ductal cells to hormone-producing islet cells (Wang et al., 1995). Moreover, human ductal tissue can be cultured to produce insulin$^{+}$ cells using strictly controlled parameters *in vitro* (Bonner-Weir et al., 2000). Another study by Xu et al. (2008) demonstrates presences of β-cell progenitors in the ducts of mouse pancreas that when injured, can be stimulated to proliferate into all islet cell types. Inada et al. (2008) reported pancreatic ductal-specific human carbonic anhydrase II (CAII) expressing cells within the pancreas act as cell progenitors with the ability to differentiate into both islets and acinar cells normally after birth or post injury induced by pancreatic duct

ligation. These studies collectively indicate that a subpopulation of ductal cells and/or endocrine cells lining the ductal epithelium can give rise to islet cells in adults (Reichert and Rustgi, 2011).

While conclusions from animal studies cannot be directly extrapolated to humans, the data obtained from this study provides similar evidence of endocrine neogenesis from the pancreatic ducts in human CF and CFRD as is evident by the presence of $ChrA^+$ and CPHN cells around ducts. Most of the endocrine cells found in ducts were $glucagon^+$, with the presence of $insulin^+$ cells mostly confined to the ductuloinsular complexes. A recent study by Ben-Othman et al. (2017) demonstrated role of γ-aminobutyric acid (GABA) in β-cell neogenesis via ductal-derived α-cell intermediate. The observance of large number of $glucagon^+$ cells around ducts may suggest a possible route for restoration of β-cells from α-cells, as demonstrated in this study.

More studies focusing on lineage tracing of these ‘abnormal’ endocrine cells are required to thoroughly explain the presence of these cells around the ducts. However, occasional $ChrA^+$ cells within ductal regions were found to be expressing KRT19 indicating ductal origin. On this basis it was hypothesized that fibrotic insult of exocrine pancreas triggers, ductal-derives endocrine cell regeneration via α-cell generation primarily. As these α-cells move away from the ducts, into ductuloinsular complexes, they can give rise to new β-cells. However, ongoing fibrosis inhibits maturation or survival of these β-cells, leading to an overall decline in β-cells in CF that can lead to CFRD.

While the present study highlights CFTR expression in human pancreas and key pathologies underlying CF pancreatic disease, it also has some limitations. Firstly, all the conclusions are drawn from observations of IHC in one section of pancreatic tissue from each donor which may not be representative of the histopathology of the whole tissue. Also, although CFTR protein and mRNA were both found to be absent in human pancreas, the techniques used are not sensitive enough to detect very low level expression of CFTR, which has been observed in other studies (Edlund et al., 2014). Moreover, quantification of changes in hormone expression was not conducted on all the donors, and is therefore, limited, by statistical comparisons between the groups. Also, the cohort size is not sufficient to get an accurate evaluation of the disease development and progression.

This study while answering questions about CFRD pathophysiology, also raises a few new ones. Absence of CFTR from islet endocrine cells demands further explanation as to how CFTR impacts β-cell function via exocrine-endocrine cross talk. Future work should incorporate cell culture models showing the influence of exocrine secretions on β-cell following CFTR mutation. Lineage tracing studies should also be carried out in human cell models to outline the origin and fate of these ‘regenerating’ endocrine cells. Further work to explore how ductal cell plasticity can be harnessed to generate a potential source of new, functional human β-cells is also essential.

# Chapter 6: General Discussion

The pancreas is an organ that serves the critical function of regulating macronutrient digestion, energy metabolism and homeostasis, by the production and release of various digestive enzymes and pancreatic hormones (Röder et al., 2016). The exocrine compartment of the pancreas is largely made up of the acinar cells that release digestive enzymes including amylase, trypsinogen, and pancreatic lipase into the pancreatic ducts (Jennings et al., 2015). The endocrine function of pancreas in regulation of glucose homeostasis is performed by clusters of highly-specialized cells, the islets of Langerhans (Jennings et al., 2015). β-cells in the islets produce and secrete the hormone, insulin, which lowers blood glucose levels in response to meal ingestion (Röder et al., 2016). Defects or damage to these β-cells can lead to diabetes mellitus which is classically characterized by abnormal glucose regulation and hyperglycaemia. Diabetes presents itself in various forms including T1DM, T2DM, and gestational diabetes, and can also occur secondary to diseases of the pancreas.

This thesis explored the changes in islet endocrine cell constitution associated with T1DM and CF. T1DM is associated with immune-mediated damage of β-cells leading to insulin deficiency, which requires life-long insulin therapy (Donath and Halban, 2004). On the other hand, CF is a multi-organ disorder occurring due to mutated CFTR gene (Barrio, 2015) leading to thickened mucus secretions that obstruct the airways and small ducts in the body. As a result of this, pancreatic ducts are obstructed causing inflammation and fibrosis of exocrine pancreas, and subsequent pancreatic insufficiency. With the advancement of medical therapies and improvement of life expectancy in CF patients, over-time, this pancreatic exocrine insufficiency may lead to CFRD, which is now the largest co-morbidity in CF. Even in the absence of CFRD, exocrine pancreatic insufficient CF patients suffer from abnormal glucose tolerance early on in life.

The global incidence of diabetes has increased substantially in the last few decades. Not only is the number of people affected by this disease increasing at a concerning rate, the age of disease onset has decreased now affecting many young children (Fradkin and Rodgers, 2013). Diabetes development can be slow taking years to present. It is now, generally, well-accepted that the associated β-cell damage precedes years before diabetes onset or diagnosis and may well even start *in utero*

and or in early postnatal life. Thus, study of this disease across the life span is important to understand the early alterations in pancreatic environment as well as age-related changes associated with diabetes pathogenesis. While animal models give meaningful insights into disease development and progression, these findings are often hard to extrapolate to humans. Thus, study of the human pancreas, normal and diseased, is essential to fully describe diabetes pathology in relation to the circulating changes in insulin and glucose levels associated with it. Even after the advancement in procedures for safe tissue procurement, availability of human pancreatic tissue fit for study of this organ in normal and/or diseased states has been challenging. A reason for this is the inaccessibility of pancreas in living individuals because of risk of pancreatitis associated with biopsy procedures. Hence, most knowledge of diabetes comes from *in vitro* models of disease, and *in vivo* studies in animals.

Thus, the aim of this project was to assess and quantify islet endocrine cell constitution changes in T1DM and CF human pancreas compared to control, non-diabetic, and non-CF organs. While the major focus was assessment of changes in the islet niche, some very interesting exocrine alterations (in CF) were also observed. In light of recent research on β-cell dysfunction in T2DM, another important question addressed by this thesis was the assessment of any alterations in β-cell phenotypes indicating β-cell plasticity / dedifferentiation / regeneration associated with pancreatic pathology in T1DM, CF and CFRD.

## 6.1 Characterising histopathological changes in pancreatic islets of two clinically distinct cohorts of T1DM compared to non-diabetic

β-cell dysfunction, as a cause of insulin deficiency in diabetes, is a well-accepted phenomenon in T2DM. Improvement / remission of hyperglycaemia following weight loss in T2DM patients, drew attention to a subset of dysfunctional β-cells whose function can be restored by removal of stressors such as glucotoxicity and hyperglycaemia. This (potentially reversible) loss of β-cell function is associated with phenotypic shifts driven by mechanisms of de-differentiation and trans-differentiation which are characterized by loss of mature (differentiated) identity of β-cells and conversion to a more progenitor like state (de-differentiation) and/or direct conversion to other endocrine cell types (trans-differentiation).

Studies, in T1DM pancreata, have indicated that β-cell dysfunction may be the driver of hyperglycaemia in T1DM patients at least at diagnosis (Pugliese et al., 2014) and that this dysfunction may be present at least 5-years before diagnosis (Evans-

Molina et al., 2018). The presence / absence of de-differentiation and trans-differentiation as a driver of β-cell dysfunction, however, has not been explored in detail in T1DM. Hence, it is unknown whether the same pattern of β-cell dysfunction is also found in T1DM, as proven in T2DM. In T1DM, β-cell loss has classically been attributed to apoptosis and it is believed that 70-90 % of β-cells are lost at disease diagnosis (Morgan and Richardson, 2016). However, within this thesis, presence of insulin-producing β-cells even in long-duration cases of T1DM was observed. A few other studies have also been reporting presence of insulin-containing islets and residual insulin secretion in T1DM pancreas (Davis et al., 2015, Oram et al., 2015, Steenkamp et al., 2017).

An important goal of the study was to quantify changes in islet hormone expression profiles in T1DM and non-diabetic pancreata. This study also highlights the differences in hormone expression profiles in two different cohorts of T1DM: a slow progressing, less aggressive type in Cohort 1 (>13-years old at diagnosis) and highly aggressive Cohort 2 (<7-years old at diagnosis). The most striking and novel observation was the maintenance of endocrine cell number per islet, despite the known reduction of pancreatic β-cell mass and whole pancreas volume in T1DM (Campbell-Thompson et al., 2015). This study supports the already established finding of a clear loss of insulin-expressing β-cells, but shows perhaps for the first time that the maintenance in number of endocrine cells per islet may be attributed to the significant increase in number of non-β-hormone (glucagon, somatostatin & PP) producing cells. This underlines the impact of changes in islet endocrine constitution that may disrupt islet cytoarchitecture which has been shown central to normal islet function (Cabrera et al., 2006). Moreover, an increase in non-β-population in the islets can also affect glucose homeostasis. For example, increased glucagon expression in isolated T1DM islets (Brissova et al., 2018) and abnormal secretion in response to meals in recent-onset T1DM patients (Brown et al., 2008) has been reported to disturb the fine balance between insulin and glucagon secretion which may underlie T1DM development.

Moreover, ‘abnormal’ cells like insulin and non-β-hormone co-expressing cells (indicative of β-cell plasticity) and CPHN cells (indicative of β-cell regeneration) were observed in T1DM pancreas indicating existence of alternative, intermediate phenotypes in T1DM aetiology. Co-localization of insulin and non-β-cell hormones was more frequent in $ChrA^+$ cells in T1DM compared to controls. This could mean that during disease development, β-cells gain non-β identity as a mechanism to escape the

immune attack. A similar finding has been reported in a human study involving T2DM (Cinti et al., 2015). Presence of cells co-expressing insulin and non-β hormones was relatively rare in Cohort 2 in comparison to Cohort 1, which is explained by an exaggerated loss of β-cells (over 80 %) observed in this Cohort. The presence of poyhormonal cells, as hypothesised, correlates with the extent of insulin-expressing cells. After stripping and re-staining of these tissue sections, it was observed that these polyhormonal cells were insulin-expressing cells that co-expressed either somatostatin or glucagon, mainly insulin and glucagon co-expressing. However, an unresolved issue is the functionality of such bi-hormonal cells. If such cells do exist in normal pancreas (as was seen in our study), it is important to understand their role in normal physiology of glucose regulation, i.e. what hormone do these cells secrete in response to meal ingestion? Is it insulin, or a non-β-hormone (glucagon), or both? Also, is this just a temporary state in response to a certain physiological trigger, or have these cells always been bi-hormonal. Presence of these poly-(/bi-) hormonal cells was found to be two-fold higher in younger pancreas of control donors compared to older pancreas. In foetal pancreas, there is evidence of polyhormonal cells that express various co-expressing combination of insulin, glucagon, somatostatin, and PP, but these polyhormonal cells disappear by 20-weeks of gestation (Riopel et al., 2014). Can these be evidence of a subset of multipotent precursor cells thought to exist in adult pancreas?

A review article (Habener and Stanojevic, 2012) based on various studies have hypothesized a role for α-cells in the neogenesis of β-cells during embryonic development and restoration of β-cells in times of extreme β-cell loss (following an injury). It is understood that all endocrine cells, including α- and β-cells, of the pancreas originate from the same progenitor cells (Abed et al., 2012). According to Habener and Stanojevic (2012), α-cells in adult pancreatic islets have the ability to convert to β-cells via a progenitor population called pro-α-cells. In times of β-cell injury, α-cells trigger production of GLP-1 which is a β-cell growth and survival factor. Also, production and secretion of stromal cell derived factor-1 (SDF-1) is induced following β-cell injury. SDF-1 leads to de-differentiation of nearby α-cells to pro-α-cells which then, trans-differentiate into β-cells by production of GLP-1. This demonstrates a model of islet cell plasticity, which may be happening in T1DM. The present data indicate an increase in the non-β-cells in the T1DM islets, with an increase in number of cells co-expressing insulin and glucagon. However, in T1DM, such trans-differentiation of α-cells to β-cells

may lead to increased autoimmune attack, thereby, killing the newly-formed β-cells and unsuccessful attempt at restoration of lost β-cell mass.

Presence of CPHN cells was also detected in T1DM pancreata. CPHN cells could either indicate loss of β-cell identity (and regression to a progenitor–like state) or could be representative of β-cell regeneration as a response to immune attack. However, presence of CPHN cells was rarer in the islets compared to the single, scattered cells in the exocrine pancreas. A similar observation of CPHN cells in scattered cells, was found in a study by the Butler group who also reported that the pattern of distribution of these cells was similar to that in neonatal pancreas suggesting possibility of attempted β-cell regeneration (Md Moin et al., 2016). However, the presence of CPHN cells in both normal and diseased donors under 7-years of age (Cohort 2) suggests attempted regeneration which is evident even after birth until puberty under normal physiology.

Future work is needed to understand the functionality and origin of these polyhormonal and CPHN cells. Staining with endocrine progenitor marker, NGN3, and other β-cell specific genes is essential to confirm the origin of these cells. Moreover, histological evaluation of the T1DM tissue should be carried out to characterize insulitis by assessing immune cells within and around the islets. Also, evaluation of the whole tissue would be helpful to examine lobular patterns of islet damage and assessment of loss of islets that is demonstrated in T1DM. Deeper molecular phenotyping in human pancreas can be achieved by examination of GLP-1, and SDF-1 signalling. Finally, β-cell heterogeneity is demonstrated in human pancreas, and four subtypes of β-cells have been identified in adult islets (Dorrell et al., 2016). These subtypes present different antigens and have distinct gene expression profiles with different extents of glucose-stimulated insulin secretion responses. Characterization of these different types of β-cells should be performed, to understand if any subtype is more susceptible to autoantigen detection and attack in T1DM.

## 6.2 Development and validation of an automated method of cell quantification and analysis

A need for an automated method for cell counting and analysis was confirmed following extensive, time-consuming manual analysis of tissue hormone expression undertaken in Chapter 3. Thus, a method for automated scanning and image acquisition using Vectra 3.0 and phenotypic analysis by inForm® software was sought to determine if it can provide comparable results to manual analysis. While manual

assessment is considered as ‘gold standard’ due to accuracy and quality of results, the major advantage of using such an automated method was that analysis of the whole tissue, including all the islets on the tissue section, is possible in a shorter time. The results obtained matched closely to manual analysis. Where larger (>50) number of islets were analysed by automated assessment, results were not significantly different to manual analysis. This highlights the importance of a minimum number of islets needed to produce results that would be representative of the whole tissue. Analysis of 50 islets by manual assessment gave comparable results to the automated assessment of all islets on the section indicating that 50 islets may be robust number to get a representative estimate of whole tissue.

Bland-Altman plots and paired Student’s t-tests were used to compare results obtained by manual and automated assessment. In most instances, both statistical tests indicated no significant difference between the two methods. In cases where there was a difference, it was due to lower (<50) number of islets analysed by automated measurement. Thus, an important conclusion of the study was that the number of islets analysed bears an impact on the overall accuracy of the data. This should be considered as an important parameter when decision on a minimum sample size for any quantitative study has to be made.

Analysis of single hormone expression within islets was relatively easily achieved by the automated method. However, determination of certain cell phenotypes that were occasionally or rarely found in the tissue was difficult i.e. polyhormonal and CPHN cells. The challenge was that due to fewer cells available for training the software and also quality of staining, the software was unable to differentiate and identify these cells. If these issues can be successfully addressed, this method of automated image analysis could be independently used for cell quantification without the need for manual assessment. This would help to avoid human bias / inconsistency and also may be more time-efficient. In the future, automated analysis could also be used as a way of second-counting to confirm data obtained from manual assessment.

## 6.3 Characterising changes in endocrine and exocrine compartment of CF and CFRD pancreata

Cystic fibrosis-related diabetes (CFRD) is the most common comorbidity in people with CF, occurring in 40-50 % of adults with this condition. Islet destruction, secondary to fibrosis, causes significant reductions in β-cell mass (Kayani et al., 2018). Although pancreatic insufficiency at birth is a strong risk factor for CFRD development

later in life (Norris et al., 2019), there appears to be no direct correlation between presence of pancreatic fibrosis and CFRD prevalence in CF patients, indicating a β-cell specific dysfunctional component to the pathogenesis. Whilst it is established that β-cell dysfunction in cystic fibrosis (CF) leads to diabetes, the mechanism by which the CF transmembrane conductance regulator channel influences insulin secretion remains debated. Currently, three major hypotheses have been proposed: (1) Intrinsic CFTR-dependent pathways of insulin secretion, (2) Pancreas-extrinsic CFTR defects, and (3) Remodeling of islets following loss of exocrine tissue due to inflammation (Sun et al., 2017). Since the contribution of each to the pathogenesis of CFRD remains largely unknown, we sought to determine CFTR localisation within human pancreas using novel and highly sensitive approaches. IF staining of pancreatic tissues indicated co-localisation of CFTR in KRT7+ ductal cells, but not in ChrA+ endocrine cells. These observations were confirmed by combined CFTR ISH and IHC (ChrA and insulin), which demonstrated the absence of CFTR RNA in the human islets. Employment of these highly sensitive techniques definitively demonstrated the absence of CFTR within β- or any other islet endocrine cell types. This is in line with recent observations in isolated human islets, and strongly indicates that CFTR may impact on β-cell function through non-cell autonomous derived factors.

Further, CF pancreata with and without CFRD, were studied to understand morphological changes compared to normal pancreas. H & E staining revealed extensive acinar damage following fibrotic lesions with and without deposition of lipids and fatty tissue. There was an age-dependent increase in the size of islets in CF pancreas. Whether these are smaller islets coming together due to pancreatic insufficiency or an increase in islet size due to infiltration of interstitial fatty tissue is yet to be determined. In any case, the islets were relatively spared even after extreme acinar damage. Despite the presence of ‘relatively-normal-looking’ islets, loss of insulin due to β-cell dysfunction leads to development of CFRD. Quantification of hormone expression shed light on a significant reduction in insulin only and no changes in non-β-cell hormones. Taken together the findings of the present study would support a role of faulty CFTR–mediated exocrine-derived, pathways for β-cell damage.

While various factors have been implicated in the loss of β-cells in CF and CFRD (as already discussed), effects of lipotoxicity have not been explored. Fatty replacement of exocrine tissue is characteristic of CF pancreas. As noted in Chapter 1, fat deposition in pancreas is directly associated with β-cell dysfunction. Moreover,

adipokines, like leptin, that are secreted from adipocytes are shown to have a pro-inflammatory cytokine-like action on β-cells (Cernea and Dobreanu, 2013). Leptin receptors are expressed by β-cells, and hence, leptin exposure can lead to reduction of glucose-stimulated insulin secretion and decreased levels of pre-proinsulin (Cernea and Dobreanu, 2013).

A more characteristic finding of the study was the presence of $ChrA^+$ cells lining the ducts, a phenomenon not commonly observed in control pancreas. Preclinical studies have demonstrated the differentiation of ductal cells to endocrine islet cells in response to inflammation and injury (Bonner-Weir et al., 2008). The $ChrA^+$ cells around ducts and islet-like structures (ductuloinsular complexes) adjacent to the ducts could represent a population of newly formed endocrine cells in response to continuous fibrotic environment. The present data also points towards endocrine neogenesis from the pancreatic ducts in CFRD as evident by the presence of CPHN cells around ducts. Most of the endocrine cells found in ducts were $glucagon^+$, with a small population co-expressing ductal marker KRT19. Recent studies have reported *in vivo* conversion of genetically modified α-cells into β-cells. The $glucagon^+$ cells around ducts may suggest a possible route for restoration of β-cells via an α–cell intermediate.

On the basis of data observed, it was hypothesized that endocrine cell regeneration from a population of progenitor cells residing in the ductal epithelium may occur in CF pancreas due to tissue injury imposed by extensive fibrosis and islet remodelling. These ductal progenitors give rise to $glucagon^+$ cells, which, as explained earlier, can trans-differentiate into insulin-producing β-cells (and possibly other non-β-cells too) as they move away from the ductal epithelium and cluster to form new islets. However, under the constant fibrotic and inflammatory signal, maturation of the new islet is hampered thereby leading to an overall loss of β-cells.

However, further work is required to robustly test the hypotheses generated by this study. Firstly, assessment of these endocrine cells from the ducts should be conducted using several other markers including, Ki67 (proliferative cell marker) and de-differentiation markers such as NGN3. Also, the potential of endocrine single cells to regenerate needs to be explored using a combination of deep molecular phenotyping in human tissue and well-validated preclinical models.

## 6.4 Conclusions

Collectively, the studies comprising this thesis provide some new insights into T1DM and CF pancreata. The results draw attention to the working of endocrine and exocrine pancreas as a single unit. In T1DM, changes in endocrine constitution have an effect on the exocrine pancreas as is demonstrated by loss of pancreatic mass and exocrine tissue loss. On the other hand, CF is a classic example of how changes in exocrine tissue may affect islet function and subsequent remodelling of islet leads to diabetes (a disease primarily considered to affect endocrine pancreas). Thus, in the future, more efforts to assess the inter-effects of these two distinct (or not so distinct) compartments should be made. Results from such a study would be helpful in translating more meaningfully *in vitro* models of disease development.

In 1938, pathologist Shields Warren wrote, “The pancreas in diabetes is not simply the scarred field of an old battleground, but is the actual field of conflict. It does not submit without a struggle to injury, but endeavours to regenerate.” Data presented in this thesis demonstrates the struggle faced by pancreas in response to stressors like autoimmunity (T1DM), inflammation and fibrosis (CF). Evidence of ductal-derived endocrine cell neogenesis in CF also sheds light on the regenerative capacity of pancreas. This gives hope that by controlling different stressors central to the disease development, restoration and renewal of β-cells is not impossible but rather a goal requiring active pursuit towards truly curative therapies for this devastating disease.

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