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IMMUNOHISTOCHEMICAL ANALYSIS OF STRUCTURAL REMODELLING AND STEM CELL PHENOTYPES DISTRIBUTION IN HUMAN DIABETIC PANCREAS

Coordinatore:
Chiar.mo Prof.: **CARLO FERRARI**

Tutor:
Chiar.mo Prof.: **FEDERICO QUAINI**
Co-Tutor:
Dott.ssa **GALLIA GRAIANI**

Dottorando: **ALESSANDRA ZECCA**

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Alla mia famiglia,

Amore puro e incondizionato.

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RIASSUNTO

I dati epidemiologici evidenziano che l'incidenza del diabete mellito, entro il 2025, raggiungerà il 5,4% della popolazione mondiale adulta e che la maggior parte delle morti sarà attribuibile a patologie cardiovascolari ad esso associato.

Il diabete potrebbe rappresentare una condizione di partenza per stabilire se la patologia d'organo sia primitivamente dovuta a modificazioni nelle proprietà funzionali delle cellule staminali, delle cellule parenchimali o di entrambe le popolazioni. La condizione di iperglicemia cronica caratteristica del diabete si associa ad un danneggiamento a lungo termine, disfunzione, fino all'insufficienza multiorgano, attraverso complicanze micro- e macro-vascolari che colpiscono cuore, cervello, vasi sanguigni periferici, retina, reni e sistema nervoso periferico.

La variabilità della risposta tissutale all'iperglicemia causata dal diabete rende difficile, però, attribuire una causa univoca dello scompenso multiorgano associato alla malattia. Esiste una letteratura significativa riguardante i difetti funzionali dei progenitori endoteliali sia midollari che circolanti (EPCs), e numerose osservazioni del nostro gruppo hanno documentato la presenza di microangiopatia nel midollo e una alterazione delle nicchie osteoblastiche dovuta ad un difetto del sistema nervoso simpatico nel diabete.

L'ipotesi che si vuole dimostrare nel corso di uno studio molto più ampio è che, il diabete, oltre all'insulto metabolico diretto alle cellule parenchimali, possa anche determinare uno scompenso funzionale delle cellule staminali progenitrici residenti e circolanti, tali da portare ad alterazioni dell'omeostasi e disfunzione di organi bersaglio.

Poiché in letteratura sono scarsi i dati che descrivono in un organo complesso quale il pancreas, la distribuzione sia di cellule organo-specifiche (alfa e beta cellule) sia di cellule caratterizzanti le nicchie staminali in corso di diabete, ci siamo proposti di studiare morfologicamente e morfometricamente le componenti strutturali dell'organo al fine di descrivere possibili alterazioni indotte dalla malattia.

La casistica comprende 35 campioni di pancreas umano di cui 15 provenienti da pazienti normoglicemici e 20 da pazienti con diabete mellito di tipo 2.

Dopo aver quantificato la componente fibrotica del tessuto mediante colorazione tricromica di Masson, avvalendoci di tecniche di immunoistochimica, è stato effettuato il riconoscimento di cellule alfa secernenti glucagone e cellule beta secernenti insulina. E' stata, poi, effettuata una valutazione mediante immunofluorescenza della distribuzione della rete vascolare, riconoscendo i profili positivi ad actina muscolare liscia (arterie), CD34 (venule e capillari) e D2-40/podoplanina (vasi linfatici).

Mediante tecniche di immunofluorescenza abbiamo identificato la presenza e la localizzazione di cellule che presentassero fenotipi di staminalità adulta (c-kit, CD133, CD34 e CD44) in assenza di altri marcatori di differenziazione. Dal momento che i mastociti esprimono alti livelli di c-kit abbiamo effettuato una doppia colorazione per Tryptase.

Infine, su un numero limitato di campioni di pancreas ottenuti da pancreasectomia, abbiamo isolato e caratterizzato le cellule endoteliali e le abbiamo testate sottoponendole a diverse concentrazioni di glucosio, paragonandole alle cellule HUVEC, cellule usate come controllo nello studio di popolazioni endoteliali.

La prima valutazione, volta alla descrizione della distribuzione delle varie componenti strutturali ha evidenziato che circa il 60% del pancreas è occupato dal tessuto parenchima funzionale (esocrino ed endocrino), 18% da fibrosi mentre per il 22% da stroma vascolare; percentuali che si modificano in presenza di diabete con una riduzione della componente funzionale ed un aumento della deposizione di collagene, soprattutto di tipo interstiziale e perivascolare.

Per quanto riguarda la porzione endocrina, nel diabete le isole risultano essere di minore dimensione e con una interessante riduzione sia di cellule positive per insulina sia

di quelle positive per glucagone. Contrariamente, è stata ritrovata una popolazione di cellule singole positive per i due marcatori distribuite nel parenchima pancreatico che, invece, è risultata arricchita nei pazienti con diabete.

La valutazione della rete vascolare documenta una riduzione della densità venulare e capillare nelle sole isole di Langerhans diabetiche mentre la densità di arteriole α-SMA^{pos} è ridotta nel parenchima, ma con un aumento dello spessore della parete arteriolare nei soggetti diabetici. Tuttavia, la riduzione del parenchima funzionale, come conseguenza del diabete mellito, tende a depletare il rapporto vasi /cellule in confronto con il parenchima di controllo. Anche per i vasi linfatici si documenta un trend di riduzione nei campioni con presenza di patologia diabetica.

Si è quindi proceduto al riconoscimento delle cellule progenitrici, costituite da una popolazione di cellule positive per c-kit, recettore di stem cell factor. Queste cellule c-kit^{pos} rappresentano lo 0,058±0,015% della popolazione totale nei pancreas normoglicemici, risultando prevalentemente (>50%) localizzate all'interno degli isolotti; nei campioni diabetici, invece, la percentuale si riduce drasticamente e in maniera più evidente nel parenchima pancreatico. Il fenotipo staminale CD133 mostra invece un andamento opposto, con un incremento anche se non significativo nei campioni patologici. È interessante notare che le cellule progenitrici positive per CD34 sono risultate significativamente ridotte ($p<0,05$) nei pancreas dei pazienti diabetici confrontati con i controlli normoglicemici.

Per quanto riguarda lo studio *in vitro,* una volta isolate le cellule endoteliali dai pezzi a fresco di pancreas umano, è stata condotta un'analisi funzionale sul comportamento di queste cellule in condizioni di iperglicemia, volte a mimare il microambiente diabetico. Inaspettatamente, il saggio di vitalità effettuato a 12 e 72ore ha mostrato un aumento della proliferazione delle cellule endoteliali pancreatiche (PanEC) nel medium condizionato con

i più alti livelli di glucosio (22mM) mentre le HUVEC risentono dell'effetto tossico dell'iperglicemia. I saggi di Wound Healing e angiogenesi su matrigel hanno mostrato un rallentamento della migrazione e della tubulogenesi nella nostra popolazione di interesse (PanEC) in tenore di alto glucosio.

Questo studio offre le basi per indagini successive volte a comprendere l'impatto del diabete mellito sul riarrangiamento strutturale e sul possibile coinvolgimento di cellule progenitrici nei meccanismi di disregolazione dell'omeostasi tissutale e funzionale.

Pur non avendo stabilito se queste modificazioni siano una causa o una conseguenza del danneggiamento multiorgano tipico del diabete, il nostro approccio potrebbe offrire nuovi spunti nella comprensione della angiopatia diabetica tessuto-specifica.

ABSTRACT

Epidemiologic data show that the incidence of diabetes mellitus will reach 5.4% of the adult world population by 2025 and that the majority of deaths will be attributable to the associated cardiovascular diseases.

Diabetes mellitus may represent a unique condition to establish whether organ pathology is primarily due to changes in the functional properties of stem or parenchymal cells or both. Chronic hyperglycemia is associated with long-term damage, dysfunction, and failure of different organs, especially the eyes, kidneys, nerves, heart, and blood vessels.

The variability in tissue response to chronic hyperglycaemia makes it difficult to sustain a unified hypothesis to dissect the mechanism of diabetes associated multiorgan damage. A significant literature exists on the functional impairment of endothelial progenitors' cells (EPCs) and several observations by our group have documented diabetic microangiopathy in the bone marrow and demonstrated an alteration of osteoblastic stem niches by changes in the sympathetic autonomic nervous system.

The present study is on line with the view that diabetes, is directed not only to parenchymal cells, but can also affect stem cell populations, in terms of distribution, number and function. Alterations in stem cell homeostatic control lead to dysfunction of target organs.

Robust data on the structural changes in organ-specific cells (alpha and beta cells) and stem cells phenotypes in the course of diabetes have been poorly described. Thus, we set out to study morphologically and morphometrically the structural components of the organ in order to describe alterations induced by the disease.

Our study includes 35 samples of human pancreas, 15 from normoglycemic patients and 20 from patients with type 2 diabetes mellitus.

After the quantification of the fibrotic component by Masson's trichrome staining, alpha-secreting glucagon and beta-secreting insulin cells were immunohistochemically detected. The immunofluorescence evaluation of the vascular network distribution was performed, by the identification of smooth muscle actin positive arteries, CD34 positive venules and capillaries and D2-40/podoplanine labelled lymphatic vessels.

Using immunofluorescence techniques, we identified the presence and localization of adult stem cells phenotypes (c-kit, CD133, CD34 and CD44) in the absence of other differentiation markers. Since mast cells express high levels of c-kit we performed a double staining for Tryptase.

Finally, on a limited number of pancreatic samples obtained from pancreatectomy, endothelial cells were isolated, characterized and exposed to different glucose concentrations. HUVEC were employed as control.

The first evaluation, aimed at describing the distribution of the various structural components, showed that nearly 60% of the pancreas is occupied by the functional parenchymal tissue (exocrine and endocrine), 18% by fibrosis, while 22% is occupied by vascular stroma. Diabetic tissue showed a reduction of the functional component and an increase in collagen deposition especially in the interstitial and perivascular location.

In diabetes the endocrine islands resulted of smaller size and with an interesting reduction of both insulin and glucagon positive cells. In contrast, a population of individual cells positive for the two markers distributed in the pancreatic parenchyma was found and was enriched in patients with diabetes.

Evaluation of the vascular network documented a reduction of venular and capillary density in diabetic Langerhans islets while the density of arterioles α-SMA^{pos} was reduced in the parenchyma, although the thickness of the arteriolar wall was increased in diabetic subjects. However, the reduction of the functional parenchyma, as a consequence of

diabetes mellitus, tended to deplete the vessel/cell ratio in comparison with non-diabetic control parenchyma. Lymphatic vessels also showed a trend to decrease in diabetic samples.

We candidate c-kit, the receptor of the stem cell factor (SCF), as one of the markers to identify the population of stem and progenitor cells resident in the pancreas. c-kit^{pos} cells represented 0.058±0.015% of the total population in the normoglycemic pancreas, resulting predominantly (>50%) located within the islets; on the other hand, in diabetic samples the percentage was drastically reduced and to amore extent in pancreatic parenchyma. CD133^{pos} stem cell phenotype showed an opposite trend, although without statistical significance. It is interesting to note that progenitor cells positive for CD34 were significantly reduced ($p<0.05$) in the pancreas of diabetic patients compared to normoglycemic controls.

Regarding the in vitro study, once the endothelial cells were isolated from fresh human pancreas, a functional analysis was carried out. We tested the behavior of these cells in hyperglycemia, mimicking the diabetic microenvironment.

Unexpectedly, the vitality test performed after 12 and 72h of treatment showed an increase in the proliferation of pancreatic endothelial cells in conditioned medium with the highest levels of glucose (22mM) while HUVECs were affected by the toxic effect of hyperglycemia. Wound healing and angiogenesis assay showed an inhibition of migratory and tubulogenesis capacity in PanECs in high-glucose medium.

This study provides the basis for subsequent investigations on human pacreas to understand the impact of diabetes mellitus on the structural rearrangement and on the possible involvement of progenitor cells in the mechanisms of dysregulation of tissue and functional homeostasis.

Although we did not establish whether these structural alterations were the consequence, or a cause of diabetes associated multiorgan damage, our approach may offer new insights on the understanding of the diabetic paradox of a tissue specific angiopathy.

INTRODUCTION

1. DIABETES MELLITUS

1.1 GENERAL ASPECTS

Diabetes mellitus (DM) is one of the most common chronic diseases in the world. According to the International Diabetes Federation, diabetes affected approximately 425 million people in 2017, and this number is expected to become much higher by 2045. Many factors, like diet, urbanization and obesity, increase the prevalence of diabetes[1,2].

Diabetes mellitus is a heterogeneous association of disorders characterized by hyperglycemia due to deficit in insulin performance[3]. It is a metabolic disorder with multiple etiologies complicated with disturbances in carbohydrate, fat, and protein metabolism[4]. The great numbers of people with diabetes are injured by type 2 diabetes. It is more common in adults but take place in children also[3]. There are two primary type of the disease: type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM).

1.2 TYPE 1 DIABETES MELLITUS (T1DM)

T1DM results from a spontaneous or environmentally discharged autoimmune attack on β-cells in pancreas, which leads to β-cells deficiency and dysfunction. Its onset is to age 20–30, which is why T1DM is also referred to as juvenile diabetes[5,6]. The exact etiology of T1DM is still unknown, but it is thought that there is a genetic predisposition, with a strong link with specific HLA (DR and DQ) alleles[7].

T1DM is also known as diabetes mellitus insulin-dependence. As long as the pancreatic β-cell mass is normal, even the blood sugar remains normal. At a certain point, it begins the destruction of the cells and, for a period of time, the blood sugar continues to be normal, because the amount of insulin produced by the cells remained intact is sufficient to maintain normal blood glucose levels. When the number of β-cells destroyed is such that

the amount of insulin is so decreased, the levels of blood glucose increase considerably, and the individual must take insulin.

1.3 TYPE 2 DIABETES MELLITUS (T2DM)

Conversely, T2DM is not due to the destruction of β-cells of the pancreatic islets, so much so that, for a certain time, the insulin is present or even increased. T2DM has been viewed as resulting from insulin resistance in key target organs, like liver, skeletal muscle and adipocytes[8]or defected in insulin secretion by the β cells[9].

At least initially, and often throughout their lifetime, these individuals do not need insulin treatment to survive.

There are probably many different factors of this form of diabetes. Although the specific etiologies are not known, autoimmune destruction of cells does not occur[10]. This form of diabetes frequently goes undiagnosed for many years because the hyperglycemia develops gradually and the classic symptoms of diabetes, as polydipsia, polyuria, weight loss, are not recognized clearly. Nevertheless, in many patients there is an increased risk of developing macrovascular and microvascular complications[11], together with the diabetic foot, which is an amount of micro and macro vascular effects[12].

In patient with T2DM, insulin levels may appear normal or even elevated, so the higher blood glucose levels would be expected rise up even higher insulin levels. But this phenomenon not occurs, because the insulin functionality is compromised[13]. Thus, insulin secretion is defective in these patients and insufficient to restore the balance for insulin resistance. Insulin resistance may improve with weight reduction and/or pharmacological treatment of hyperglycemia but is difficult to come back to normal[3].

In T2DM other hormones abnormalities such as reduced secretion of the incretin glucagon-like peptide 1 (GLP-1), hyperglucagonaemia, and higher concentrations of other

counter-regulatory hormones also contribute to insulin resistance, reduced insulin secretion, and hyperglycemia in type 2 diabetes[14,15,16,17]. Also, obesity contribute to insulin resistance through several causes, including an imbalance in the concentrations of hormones, like an increased level of leptin and glucagon and reduced levels of adiponectin, increased concentrations of cytokines (tumor necrosis factor α, interleukin 6), suppressors of cytokine signaling and other inflammatory signals. Vascular complications such as coronary artery disease, peripheral arterial disease, stroke, nephropathy, neuropathy, and retinopathy are increased by insufficient blood glucose control[14,18,19].

Variety of mechanisms have been proposed to be associated with pathogenesis of T2DM, including glucotoxicity, lipotoxicity, oxidative stress, endoplasmic reticulum stress, and amyloid deposition. The disease has recently been divided into three subtypes: subtype 1 is associated by higher risk of developing neuropathy and retinopathy, subtype 2 with increased risk of onset of cancer malignancy and various cardiovascular complications, and subtype 3 with increased risk of neurological and cardiovascular disease[20,21].

1.4 INSULIN ROLE IN DIABETES

Insulin is the unique hormone involved in the storage and in the control of release of the energy from metabolism. It is synthesized in the beta cells of pancreatic islets and after secretion; insulin enters the portal circulation and is carried to the liver, its prime target organ. About 50% of secreted insulin is degraded in the liver, the residue is metabolized by the kidneys. C-peptide is the single chain precursor of insulin. It is produced in equal amounts to insulin and can therefore be used to assess endogenous insulin secretion[22].

Insulin is a major regulator of intermediary metabolism, but its actions are influenced by other hormones. Insulin role in the fasting and postprandial states is different. In the fasting state, its main action is to regulate glucose release by the liver, and in the postprandial state, it promotes glucose uptake by fat and muscle tissue[23].

Insulin has no effects on glucose only, but also on increased glycogen synthesis in the liver and muscle, and on lipogenesis in adipose tissue and liver. In addition, at the muscular level, it also stimulates protein synthesis. The inhibition of gluconeogenesis is the most important effect of insulin in the liver.

The peripheral insulin-resistance seems to be due to alterations in insulin receptor. In particular, in T2DM, the presence of toxic substances produced in excess by adipose tissue can lead to abnormal phosphorylation of insulin receptor (obesity is in fact a very important risk factor).

There are also evidences that mitochondrial oxidative stress contributes in rat pancreas, to islet cell apoptosis and insulin secretory defects[24].

A defect in insulin secretion by β-cells represents a typical feature of diabetes. Normally a subject reacts to glucose by producing insulin or glucagon, while individuals with diabetes are not able to produce appropriate quantity of insulin. Furthermore, diabetic individuals often have an excess of glucagon and the complex balance of the opposing effects of these two hormones is dysregulated.

Cell membranes are not inherently permeable to glucose. A family of specialized glucose-transporter (GLUT) proteins carries glucose through the membrane into cells: *GLUT-1* is a transporter enables basal glucose uptake into many cells not mediated by insulin; *GLUT-2* transports glucose into the beta cell, that acts for glucose sensing, and is also present in the renal tubules and hepatocytes; *GLUT-3* is enables non-insulin-mediated glucose uptake into brain neurons and placenta, and *GLUT-4* is responsible of much of the peripheral

action of insulin. It is the channel through which glucose is taken up into muscle and adipose tissue cells following stimulation of the insulin receptor[22].

Insulin receptor consists of a dimer with two α-subunits, which include the binding sites for insulin, and two β-subunits, which straddles the cell membrane. Insulin binds to the α-subunits and it induces a conformational change in the β-subunits, resulting in initiation of a cascade response involving a host of other intracellular substrates. One consequence of this mechanism is migration of the GLUT-4 glucose transporter to the cell surface and increased transport of glucose into the cell. The insulin-receptor complex is then internalized by the cell, insulin is degraded, and the receptor is recycled[25].

1.5 COMPLICATION IN DM

Diabetes is associated with a large number of complications. Acute metabolic complications associated with mortality include diabetic ketoacidosis from exceptionally high blood glucose concentrations (hyperglycemia) and coma as the result of low blood glucose (hypoglycemia)[26].

In T1DM, the most severe metabolic complication is diabetic ketoacidosis, which is accumulation of ketones in the blood. This is because fatty acids cannot be converted into glucose at steady state. Ketones make the blood acidic and slow down all body functions. This also leads to a coma and eventually death[27,28].

In the Type 2 diabetic, ketoacidosis rarely occurs while hyperosmolar coma may supervene.

Long-term complications of diabetes are mainly due to poor control and elevation of blood glycemia, which leads to damage of blood vessels (angiopathy). In diabetes, the resulting complications are grouped in “microvascular disease” (due to damage to small blood vessels) and “macrovascular disease” (due to damage to the arteries).

Microvascular complications include eye disease or "retinopathy," kidney disease termed "nephropathy," and neural damage or "neuropathy". The major macrovascular complications include accelerated cardiovascular disease resulting in myocardial infarction and cerebrovascular disease manifesting as strokes. Although the underlying etiology remains controversial, there is also myocardial dysfunction associated with diabetes which appears at least in part to be independent of atherosclerosis. Other chronic complications of diabetes include depression, dementia, and sexual dysfunction[28]. For all these reasons, diabetes mellitus results as a multiorgan disease.

Retinopathy. Diabetic retinopathy is characterized by a variety of lesions within the retina and is the major cause of blindness among adults from 20 to 74 years[29,30]. Changes in vascular permeability, capillary microaneurysms, capillary degeneration, and excessive formation of new blood vessels (neovascularization), also occurs. Clinically, diabetic retinopathy is separated into non-proliferative and proliferative stages. In the early stages, hyperglycemia can lead to pericyte death and thickening of the basement membrane, which contribute to changes in the integrity of blood vessels in the retina, altering the blood-retinal barrier and vascular permeability[28,31].

Nephropathy. Diabetic nephropathy represents the major cause of end-stage renal failure in Western societies[31]. Clinically, it is characterized by the development of proteinuria with a subsequent decline in glomerular filtration rate. Importantly, macrovascular complications like heart attacks and strokes are driven by kidney disease as risk factor[32]. Hypertension[33] and poor glycemic control frequently bring to diabetic nephropathy, although a subset of patients develop nephropathy despite good glycemic control and normal blood pressure.

Neuropathy. Diabetic neuropathy is a syndrome which strikes both the somatic and autonomic peripheral nervous system[34]. The neuropathy is a major cause in the impaired

wound healing, erectile dysfunction, and cardiovascular dysfunction in diabetic patients. Disease progression in neuropathy was traditionally clinically characterized by the increase of vascular abnormalities, such as capillary basement membrane thickening and endothelial hyperplasia. Advanced neuropathy due to nerve fiber deterioration in diabetes is characterized by altered sensitivities to vibrations, which progress to loss of sensory perception[28]. Peripheral neuropathy in diabetes may occurs in several forms, including sensory and autonomic neuropathies. More than 80% of amputations after foot ulceration or injury can result from diabetic neuropathy[13,34].

Several pathological mechanisms are proposed to explain why diabetic patients develop microangiopathies. Aldose reductase is the initial enzyme in the polyol pathway and may participate in the development of diabetes complications. This pathway involves the conversion of glucose into glucose alcohol (sorbitol). The flux of sugar molecules through the polyol pathway is increased by high glucose levels, which causes sorbitol accumulation in cells. Osmotic stress from sorbitol accumulation has been indicated as an underlying mechanism in the development of diabetic microvascular complications. Glycoproteins is also known to injure cells. High glucose concentrations can promote the non-enzymatic formation of advanced glycosylated end products (AGEs). Oxidative stress may also play an important role in cellular dysfunction from hyperglycemia. High glucose levels can stimulate free radical production and reactive oxygen species formation. Animal studies have suggested that treatment with antioxidants, may attenuate some vascular dysfunction associated with diabetes, but treatment has not yet been shown to alter the progression of retinopathy or other microvascular complications of diabetes[35].Growth factors, including vascular endothelial growth factor (VEGF), growth hormone, and transforming growth factor, have also been postulated to play important roles in the

diabetic retinopathy. In animal models, suppressing VEGF production is associated with less progression of retinopathy[40,36].

Macrovascular disease. In type 1 diabetes, it is usual to see progression to cardiovascular disease (CVD) with an impairment in kidney function[37,38]. In type 2 diabetes, kidney disease remains a major risk factor for premature CVD, in addition to dyslipidemia, poor glycemic control, and persistent elevations in blood pressure[39]. Cardiovascular disorders in diabetes include atherosclerosis, manifest as myocardial infarction and stroke. Atherosclerosis is a complex phenomenon resulting from chronic inflammation and injury to the arterial wall of peripheral or coronary vessels and involving numerous cell types and important interactions between cells. After endothelial injury and inflammation, lipids from LDL particles build up in the endothelial wall. Angiotensin II may promote the process of oxidation in these particles. The arterial wall is then infiltrated by monocytes that differentiate into macrophages, which accumulate oxidized lipids to form foam cells. Once formed, foam cells attracted T-lymphocytes that, induce smooth muscle proliferation in the arterial walls and collagen accumulation. The result of the process is the formation of a lipid-rich atherosclerotic plaques that destabilize and rupture, bringing to myocardial infarction or strokes[40,13].

2. PANCREAS GROSS ANATOMY

Pancreas is an important structure of gastrointestinal system. It's a gland and consists in two portions with different function and structure. In general, pancreas is designate to digestive enzymes secretion into the intestine, and to hormones secretion into the blood. This role, as endocrine organ, is mainly involved in glucose metabolism and energy storage[41] control.

Pancreas is an elongated and flattened gland 12 to 20 cm in length. The head takes place behind the peritoneum of the posterior abdominal wall and has a lobular structure. The pancreas is protected by a fine connective tissue, without a true capsule. The head of the pancreas is on the right side and lies within the curvature of the duodenum. The neck, body, and tail of the pancreas is obliquely in the posterior abdomen, with the tail extending as far as the gastric surface of the spleen[42].

2.1 EXOCRINE AND ENDOCRINE PANCREAS

2.1.1 EXOCRINE PANCREAS

The pancreas, histologically, has two separated elements, exocrine and endocrine. Exocrine portion is consisting of numerous little glands, calls acini, clustered in lobules with a tubular network. Centroacinar cells are typically located at the junction of an acinus or acinar tubule with a small ductule, but they may be interspersed within an acinar tubule[42].

Epithelial cells are disposed around a central lumen. Each lobule has its own ductile. Many ductules join to form intralobular ducts and then interlobular ducts that drain into branches of the main pancreatic duct. Small microvillus protrudes from apical surfaces of secreting cells into gland lumen.

Exocrine cells are intensely basophilic for its abundance of granular endoplasmic reticulum and ribosome; Golgi's complex is well developed, and, like endoplasmatic reticulum, it is in basal region. Apical portion consist, instead, of zymogens granules into vesicles.
Exocrine cells produce lot of different digestive enzymes that, through Santorini duct, spill into the duodenum. This secretory function is regulated by volume of intestinal content and mediated by humoral and neurological factors.

2.1.2 ENDOCRINE PANCREAS

Endocrine portion is consisting of Islets of Langerhans that are crucial 'micro-organs' inserted in the glandular exocrine pancreas that regulate essentially glucose metabolism. Smaller islets are dispersed throughout the acinar lobules and larger islets takes place along the main and interlobular ducts of the pancreas. Most islets are spherical or ellipsoidal, but they can be irregular in shape, sometimes conditionate by an adjacent structure, often a duct. Several reports provide support that there is a higher number of islets in the tail of the pancreas than in the head and body.

Islets emerge via the aggregation of four main discrete endocrine cell types that are intimately associated with endothelial cells and neuronal processes becoming a single functional unit[43].

The α-cells produce glucagon, a hormone of 29 amino acids, that induces hyperglycemia because it has a glycogenolysis action on liver. Hepatic glucose production maintains basal blood glucose concentrations in a normal range during the fasting state[44].

In contrast, β-cells produce insulin (51 amino acids) that induces hypoglycemia: when blood glucose levels rise, insulin is secreted, lowering blood glucose by increasing its uptake in cells and stimulating the liver to convert glucose to glycogen, in which form it can be stored. The β cells of the pancreas have a transporter GLUT2[45] (a facilitated

diffusion) for the transport of glucose. When blood sugar increases, GLUT2 enters more glucose in the β cells, by diffusion; this glucose is metabolized and is formed ATP, which inhibits a K^+ channel, causing a depolarization of the membrane; this cause the opening of Ca^{2+} voltage-dependent channels. The Ca^{2+} induces the fusion of insulin-containing vesicles with the plasma membrane and the consequent release of insulin outside the cell. When blood sugar decreases, β-cells return to the idle state. Alterations in β-cells induce a defective secretion of insulin, that cause diabetes mellitus[46,47].

Another endocrine cells population is the δ-cells, producing somatostatin which is able to repress secreting of both insulin and glucagon.

Last main cells population is represented by PP-cells, secreting pancreatic polypeptide which exerts various effects on the gastrointestinal system, such as the stimulation of gastric secretion and inhibition of intestinal motility[48].

2.1.3 HISTOLOGY OF ISLETS IN DM

Islets in the type 2 diabetic pancreas do not appear histologically different from those of the nondiabetic pancreas, except for the presence of amyloid IAPP. Pathologists have been described an "hydropic degeneration" as vacuolization in islets from diabetic persons.

Also, fibrosis, particularly along the islet micro-vasculature, has been reported in patients but has been found equally in nondiabetic pancreas. As already highlighted, beta-cell dysfunction is a fundamental characteristic in type 2 diabetes together with insulin resistance and several studies have shown that the beta-cell mass and are reduced of 30% in T2DM[48]; also, the only insulin-resistance in non-diabetic subjects causes an insular remodeling with an average growth of the insular diameters and an increasement of the number of alpha and beta cells due to neogenesis and transdifferentiation mechanisms.

Alpha-cells may represent a population of cells that could proliferate and transdifferentiate in order to replace the loss of beta-cells in DM[49].

3. EMBRYONIC PANCREAS DEVELOPMENT

The pancreas is derived from the inner germ layer, the endoderm.

Embryogenesis, it depends on a lot of extrinsic, inductive signals, as well as the intrinsic regulation of gene expression by stage-specific DNA binding transcription factors, have been shown to direct lineage specification and organ development[50,51,52].

Many authors[52] affirm that embryonic stem cells (ESCs) are first induced to form 'definitive endoderm', the germ layer from which the gut-associated organs thyroid, lung, liver, pancreas, and the epithelia of the entire gastrointestinal tract are derived.

The pancreas arises from two diametrically juxtaposed anlagen located on the dorsal and ventral portions of the developing foregut endoderm.

In mouse and chick, signals from notochord promote the exclusion of Sonic Hedgehog (Shh), a member of the Hedgehog family of secreted signaling molecules, in the pancreatic endoderm prior to dorsal bud formation. The absence of Shh in this area allows the expression of pancreatic and duodenal homeobox factor 1 (Pdx1), a transcription factor essential for pancreas development[50].

Other transcription factors, including Ptf1a, Gata4, and Sox9 also mark pancreas specification, and their importance in human pancreas development is evidenced by several reports of pancreatic agenesis and permanent neonatal diabetes mellitus (PNDM) caused by mutations in these genes.

The next major event in pancreas development is the separation of multipotent progenitor cells (MPCs) into distinct 'tip' and 'trunk' domains[7]. The tip progenitors possess the ability to become all pancreatic epithelial cell lineages, including acinar, duct, and endocrine cell

types. Subsequently, cells that remain at the distal tip of the epithelial structure going through acinar differentiation, while cells proximal to the tips develop into bipotent (endocrine/duct) trunk progenitors[50,53]. Transcription factors such as Ngn3 and NeuroD1 are essential for early endocrine lineage determination[53].

Furthermore, sequential expression of Nkx2.2, Pax4, Nkx6.1, MafA, Pax6 and Pdx1 determine insulin-producing β-cell fate, whereas Brn4, Arx1, Nkx6.2 and MafB expression promote the development of α-cell producing glucagon (Fig.3). Pdx1 expression becomes restricted during the first stages of development of endocrine population but at later stages during β cells formation its expression is upregulated because the protein enhances β cell function and is involved in insulin secretion[52].

During early stage of fetal life, a majority of the hormone expressing cells are isolated or in small aggregates adjacent to the ductal tree and often co-express a specific marker of ductal cells, cytokeratin 19 (CK19), suggesting that ductal cells may represent a population of pancreatic progenitors. Later in fetal development, insulin-positive cells begin to cluster with glucagon-positive cells and then, these cells, along with other hormone-expressing cells, aggregate into larger immature islet that are invaded by blood vessels. The close association of endocrine cells with ducts has been observed not only during human pancreatic progress but also in the adult human pancreas[6]. The understanding of the transcription factor hierarchy and the various growth factors involved in pancreatic maturation has proved useful in reprogramming other cell types into pancreatic cells.

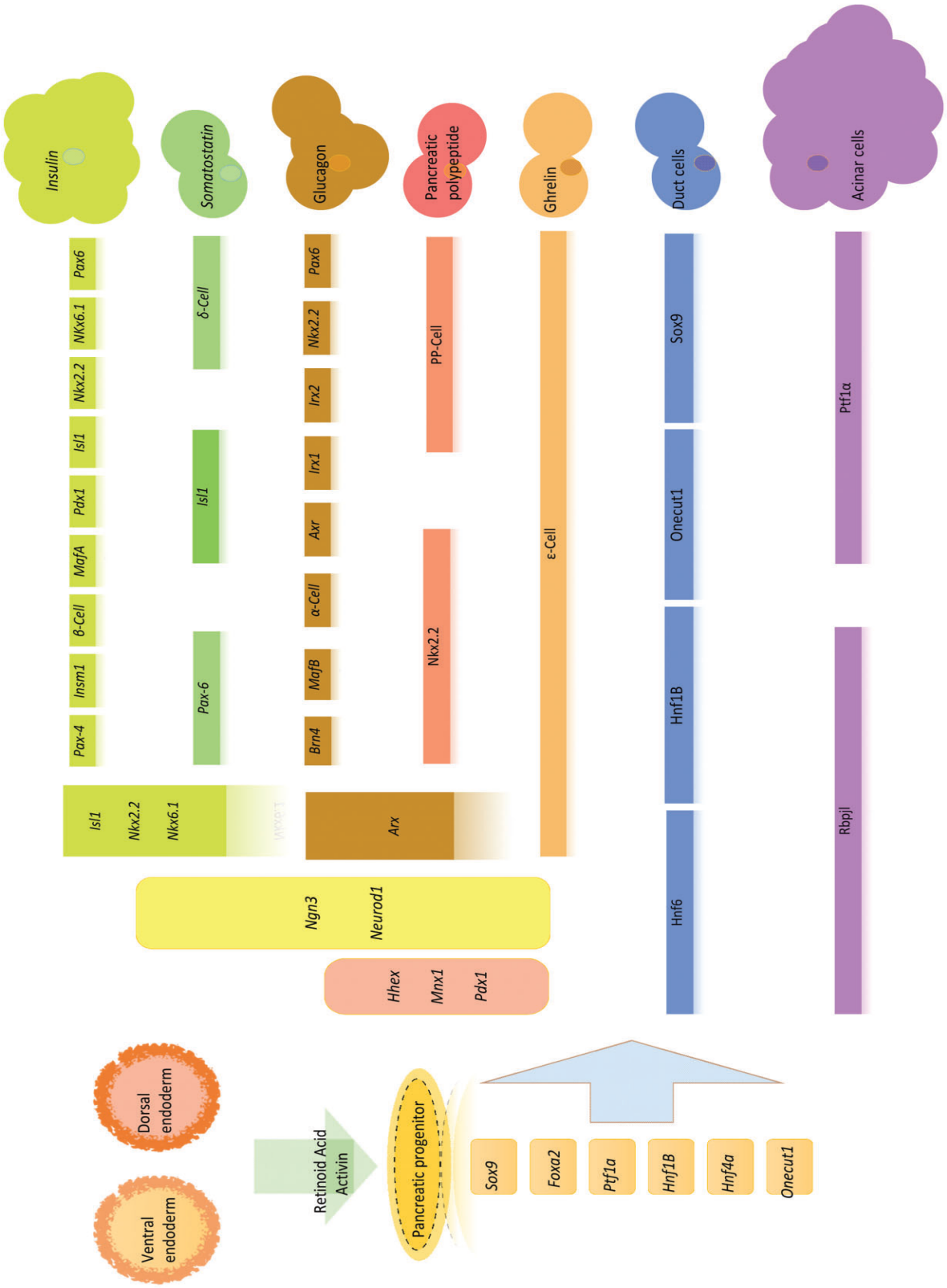


Figure 1. Diagram of pancreatic progenitors toward differentiated lineages.

After the activation of PDx1, the pancreatic fate is induced from endoderm progenitors. Pancreatic progenitors give rise to acini, ductal, and endocrine progenitors. Endocrine progenitors then differentiate into specific hormone secreting cells: α, β, δ, PP. Key transcription factors involved in each differentiation step and the time they are expressed are indicated.

4. STEM CELLS IN DIABETES AND PANCREAS

Stem cell is an undifferentiated cell, which can self–renew to replicate itself as well as give rise to the differentiated cells under appropriate conditions[54].

There are distinct populations of stem cells conforming to their differentiation potential[55].

Pluripotent stem cells are capable of becoming all cells of all tissue types[56]. Examples of pluripotent stem cells are embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC). Multipotent stem cells are able to differentiate into many cell types, but within a specific lineage. For example, the hematopoietic stem cells (HSC) can produce erythrocytes, platelets and lymphocytes. Progenitor cell, instead, lacks the self–renew and often is capable to differentiate itself into one cell type (unipotent).

Adult stem cells, which exist in the postnatal organism and in adult organs, could be either multipotent or unipotent[57].

Several data have been reported on the regulatory role of stem cells in multiple solid organs[58]. The possibility to identify and characterize multipotent cells, progenitors, precursors, committed and differentiated populations is also well documented.

One of the treatment strategies for diabetic patients is pancreatic islet cell transplantation[59] but in this method there are some limitations, like the lack of donor organs as well as low possibility of becoming completely insulin free[60]. Stem cell therapy is another strategy.

Different types of stem cells have been considered for diabetes treatment.

The first report of insulin producing cells from mouse embryonic stem cells was published in 2000, but these cells had short life[61]; more studies have been performed in this field and after manipulation of culture, separated Nestin positive cells, using Pdx1 and Pax4 that are translation factors associated with beta cells, encouraging results were obtained[62,63].

Different types of Mesenchymal cells, which show the ability of differentiation into insulin-producing cells in vitro, are from many departments and include: mesenchymal cells derived from bone marrow[64], adipose tissue and mesenchymal cells of pancreas[65].

Stem cell therapy has been used for treatment of some kinds of diabetic complications. Promising results have been obtained using fetal CD133 positive cells[66], autologous bone marrow stem cells, mesenchymal stem cells and autologous peripheral blood mononuclear cells[67].

Pancreatic stem cells have been proposed as a potentially source of β-cells for transplantation.

However, in the adult pancreas the existence and the exact nature of stem cells has been considerable controversy. It has been demonstrated that the pancreas has a very limited regenerative capacity. These evidences have led to the proposal of potential candidates for adult pancreatic stem cells. They include pancreatic ductal cells, exocrine cells, Ngn3-positive cells, Nestin positive cells and oval cells[61].

Immunohistochemically localization of single β-cells near and within ductal structure, following pancreatic injury, was originally presented as evidence that new β-cells in the adult could originate from stem cells within the ductal tissue[68]. Furthermore, it is possible to generate islets containing α-, β- and δ-cells from cells obtained by adult pancreatic ductal epithelial cells in long-term culture with multipotent capacity[69]. However, rapid proliferation of ductal cells could make lose their ductal phenotype and revert to multipotent cells that ridifferentiate into other cell types[70].

Nestin-positive cells within the pancreatic islets and ducts were also proposed to be presumed multipotent stem cells that could provide precursors for neogenesis of endocrine cells[71,72]. Nestin is an intermediate filament protein that is a marker of neural stem cells. The nestin-positive cells in pancreas result negative for endocrine hormones and ductal

markers, but, their capacity to expand themselves *in vitro*, bring them to insulin- and glucagon-producing cells[71,72].

Although the possibility to use adult pancreatic stem cells would be revolutionary in diabetes therapy. It is difficult clinically to define real pluripotent pancreatic stem cell and at the same time to identify the activity, isolate and characterize this kind of cells is still on going.

The intracellular mechanisms and interactions with the extracellular environment able to determine the fate of stem cells is complex. However, the identification of phenotypic characteristics and their distribution within the tissue is of high relevance.

Evidence has been provided on the possible involvement of a cancer stem cell in pancreatic tumor[73,74] as evidence exists on stem cells involvement in tissue repair in pancreatitis[75]. Cancer stem cells (CSC) are assumed to proliferate slowly, to self-renew but also to give rise to distinct cell populations[76].

Clearly, a more detailed functional analysis of alfa and beta cells is required and should include: analysis of key functional proteins and metabolic enzymes responsible for glucose sensing and secretory coupling; metabolomics analysis of mitochondrial activity and secretory coupling factors; mechanistic studies of the electrical and exocytotic machinery; assessment of secondary stimulatory pathways; and precise characterization of actual glucose sensitivity (both graded and dose-response)[77].

Particular attention should be given to the expression of c-kit and CD133 as repeatedly implicated in the identification of normal stem phenotypes. Prominin-1 or CD133 is an antigen frequently associated with phenotypes of normal progenitor cells[78].

c-kit (CD117) is a receptor tyrosine kinase of the stem cell factor and is expressed by stem cells from different tissues and species[79].

The binding of c-kit to its ligand, stem cell factor (SCF), results in its activation, which mediates survival, migration and proliferation in multiple cell types, including pancreatic beta cells. In mouse's pancreas, the great presence of c-kit expression in the early stages of pancreatic development indicates that it may be involved in stabilize the endocrine cell precursor pool in fetal mouse pancreas[80].

SCF–c-kit interactions mediate β-cell differentiation and proliferation and have been demonstrated across multiple species in vitro[81].

5. THERAPY: STATE OF ART

Since the complications of diabetes are quite important and that the disease has a high incidence, research has been carried out in this field. Innovations in regenerative medicine allowed proposing two possible therapies for β-cell mass restoration:

1) β-cell replacement, including transplantation of insulin-producing cells;

2) β-cell regeneration, by differentiation of ESCs and iPSCs or, transdifferentiation from, non-pancreatic adult cells into insulin-producing cells [4,82].

Hypothetically, β-cell mass replacement is an appropriate strategy for DM because it restores the natural response to modifications in glucose blood levels, and monitors insulin production and functionality. New treatments include whole pancreas or pancreatic islet transplantation, especially in T1DM patients.

Although islet transplantation could represent a definitive therapy only for type 1 diabetes, multiple issues have precluded its widespread use, including chronic immunosuppressive therapy, inadequate supply of cadaveric organ donors, and limited duration of graft function.

As previously described, alternative approaches concern the possibility to use pancreatic progenitors to generate insulin-producing β-cells in the hope of treating

diabetes, but actually studies documenting a robust glucose responsiveness of β-like cells are lacking. Moreover, the highly competitive research on differentiation of embryonic stem cells (ESCs) into insulin-producing cells has not reach a reproducible clinical application.

The pharmacological therapy of type 2 diabetes is extremely heterogeneous and consists of several medications nowadays available. These are biguanide (metformin), sulfonylureas, meglitinides, thiazolidinediones, DPP4 inhibitors, GLP1 agonists, SGLT2 inhibitors, together with insulin therapy if needed. Metformin, anyway, remains thefirst-line medication for the treatment ofT2DM[83].

The management of the cardio-vascular risk is also needed, using ACE inhibitors, aspirin, statin.

6. DIABETES AND CANCER

Diabetes and cancer are two diseases with several impact on health worldwide. Epidemiologic evidence suggests that people with diabetes have significantly higher risk for many forms of cancer. Recently, the results of studies have been combined for meta-analytic study, indicating that some cancers develop more commonly in patients with type 2 diabetes[84]. It remains unclear if the association between diabetes and cancer is direct and due to hyperglycemia, or diabetes is a group of biologic factors that increase cancer risk, or the cancer-diabetes association is indirect and due to common risk factors, such as obesity, diet, tobacco smoking and alcohol consumption. Cancer risk seen to be influenced by duration of diabetes and also complicated by pharmacological therapy often necessary for diabetic patients' treatment[85].

Insulin and insulin-like growth factor (IGF) receptors are expressed in most of the cancer cells and is capable of stimulating their proliferation and metastasis. After

interaction between the receptors and their ligands various pathways are activated. These signaling pathways stimulate multiple cancer characterize including proliferation, protection from apoptosis, invasion, and metastasis. It is also clear that insulin/ IGF may stimulate normal cells that are recruited in cancer progression. For example, hyperglycemia let IGF- I encourage vascular smooth muscle cell proliferation and migration[91]. IGF-I is more potent mitogenic and anti-apoptotic than insulin[86]and could act as a growth stimulus in pre-neoplastic and neoplastic cells that express its receptors[87]. Human tumors commonly over-express the insulin and IGF-1 receptors, and many cancer cell lines have been shown to be responsive to the physiological concentrations of IGF-I[86].

Hyperglycemia is often related to cancer, but not necessarily glucose mediates this association; rather, hyperglycemia may intervene to lead to hyperinsulinemia.

Given the molecular heterogeneity of cancers, it's not possible to exclude that may exists a subset of cancer cells for which hyperglycemia confers a growth advantage and instead an appropriate diabetic therapy limits tumor progression[88]. For example, Metformin is the most commonly used therapy in patients with T2DM. In laboratory studies, metformin has been shown to have anti-tumoral effects, like inhibition of cell proliferation, reducing colony formation, and induction of partial cell cycle arrest in cancer cell lines. Results of a growing number of human studies suggest that treatment with metformin is associated with reduced risk of cancer[89]or cancer mortality[90].

However, data suggest that the activation of insulin receptor may be more important than hyperglycemia in determining tumor growth[91].

Adipose tissue is recognized as an active endocrine organ producing free fatty acids, interleukin-6, monocyte chemoattractant protein, plasminogen activator inhibitor-1, adiponectin, leptin, and tumor necrosis factor[92], which could influence regulating malignant transformation or cancer progression. In some cases, the role for these molecules

is well characterized. For example, IL-6 is known to enhance cancer cell proliferation, survival, and invasion while also suppressing host anti-tumor immunity[93].

6.1 DIABETES AND PANCREATIC CANCER

Pancreatic cancer remains one of the deadliest tumor types. The early stages of this cancer do not usually produce symptoms, so the disease is generally advanced when it is diagnosed. Despite intensive research efforts to better understand its tumor microenvironment, the prognosis of pancreatic cancer remains depressing[94].

Most of the pancreatic cancers are ductal adenocarcinomas (PDAC) types that arise in the head of the gland. This type appears as firm, sclerotic and poorly defined masses that replace the normal lobular architecture of the gland[95]. Microscopically, infiltrating ductal adenocarcinomas are invasive malignant epithelial tumor with ductal differentiation and without a predominant component of any of the other carcinoma types[96].

Several data show that individuals with DM have a greater relative risk of developing pancreatic tumor than non-diabetics[97]. The interpretation of the association is complicated by the fact that abnormal glucose metabolism may be a consequence of pancreatic cancer[89]. In general, risk for diabetes-associated pancreatic tumor were superior in individuals with short durations of diabetes than in those with long durations. This is like a paradox, since usually, if the time of exposure to a risk factor for a disease is increased, it is expected that the incidence of the disease would be increased too[96]. This inverse association may be explained by various supposition, like the lifestyle changes after diabetes diagnosis or use of certain antidiabetic medications may be associated to decreasing risk of pancreatic cancer with increasing duration of diabetes. Diabetes has developed more often in patients with carcinoma of the head of the pancreas than in those

with carcinoma in other organ portion, in patients with pancreatic cancer at early stage. However, why diabetes develops in patients with pancreatic cancer remains unclear.

The majority of patients with pancreatic cancer have normal β-cell function[98], and diabetes in pancreatic cancer cases is characterized by peripheral insulin resistance. Because the islet mass destroyed by the tumor is only a small proportion of the whole islet mass, the islet dysfunction is unlikely to be the result of decreased total islet volume[99].

6.2 THE ROLE OF THE ISLETS OF LANGERHANS IN PANCREATIC CANCER

Over the last few years, interesting speculations have been done about the connections between the endocrine pancreas and cancer. It is for sure believed that ductal cells are the cells of origin ductal adenocarcinomas of pancreas. However, this assumption could be questioned. First of all, intrainsular ductules have been described in tissues from patients with pancreatic cancer or chronic pancreatitis, the first morphological changes are the appearance of ductular structures within or around the islets[100]. Immunohistochemical studies have shown the abnormal colocalization of hormones and the presence of endocrine cells in invasive regions of pancreatic cancer[101].

With the observation that islet cells can transform into duct cells it is easy to say that at least some human pancreatic adenocarcinomas may originate within islets via transdifferentiation[102].

Several cell culture tests have been performed to explain which population may underlie pancreatic cancer. In same case, when islets cells are put in culture, acinar cells and ductular structures develop primarily within the center, and these structures express ductal specific cell markers and at the same time antigen also expressed in most adenocarcinomas[103]. The islet cells don't have a great proliferation potential and tend to

lose their endocrine granules in culture, containing a mixture of insulin and glucagon that seem to imitate the embryonic pancreas. Islet cells are also capable of transdifferentiation into acinar cells, hepatocytes or mucinous and oncocyte-like cells[104]. Indeed, acinar, ductal and islet cells can both act as facultative stem cells or transdifferentiate in each other under reactivation of PDX1 and finally redifferentiate into cells with a stem cell character[105].
Is still not clear and needs to be determined if there is an activation of pancreatic stem cells or transdifferentiation of endocrine cells.

Some important information has also been gained from different animal models. Syrian golden hamsters treated with a specific carcinogen for inducing pancreatic cancer, call N-nitrosobis(2-oxopropyl) amine (BOP), develop pancreatic adenocarcinoma that is similar to the human[106]. In this model, treatment with streptozotocin bring to destruction of ß- cells and seem to inhibit pancreatic tumor development, while stimulation of islet cell proliferation augments pancreatic carcinogenesis after BOP treatment[107].

Transplantation of hamster islets into the submandibular gland of Syrian golden hamsters followed by BOP treatment led to the development of ductal pancreatic adenocarcinoma in this site[108]. Cancer was not observed after transplanting ductal or acinar cells into this gland, strengthening the idea of the origin of endocrine-related cancer.

Animal models indicate that there are several possible ways to develop pancreatic cancer, either from a particular stem cell or through transdifferentiation. It appears that, at a certain stage, all three cell types have the plasticity to dedifferentiate and could be the cell of origin of pancreatic adenocarcinoma. This suggests that differentiation is not an irreversible process and that islet, duct and acinar cells are facultative stem cells, able to gain stem cell properties back during the process of trans- and dedifferentiation. In this process of re-entering the cell cycle, PDX1, which is the transcription factor in pluripotent progenitor cells in the embryonic pancreas, becomes reactivated[109]. It seems that a

particular cell state and increased proliferation rate, combined with the effect of a carcinogen is much more important than the cell type for the development of pancreatic adenocarcinoma[103].

6.3 STEM CELLS AND PANCREATIC CANCER

Several studies suggest that stem cells or progenitors are the cells of origin of cancer[109]. The proliferation and differentiation of stem cells are strictly regulated but otherwise, dysfunction of these systems would result in disorganized proliferation and expansion, typical of malignant disease. Although studies indicate that progenitor cells contribute to the regeneration and tumorigenesis of the pancreas, it is likely that pancreatic regeneration depends in part on the trans differentiation of mature cells[103]. Consequently, it is not yet established if stem/progenitor cells or differentiated mature cells are responsible for the initiation of malignant disease[110].

The pancreatic Cancer Stem Cells (CSC) are identified positive for CD133, CD24, CD44, EPCAM, ESA, c-Met, Aldh1[111]. CD44 is a multistructural and multifunctional transmembrane glycoprotein, it is a receptor for hyaluronic, a major component of the ECM, and a co-receptor for many growth factors and cytokines[112]. CD44 has been characterized as an adhesion receptor engaged by migrating T cells and it is able to regulate binding and rolling interactions with vascular endothelial cells that express hyaluronic acid[113]. In addition to regulating adhesion during T-cell migration, it is also apparent that CD44 has underappreciated roles in regulating effector T-cell responses [114]. Accumulating evidence indicates that CD44 are cancer stem cell markers and critical players in regulating the properties of CSCs. The stem cell niche surrounding CSCs provides a regulatory micro-environment for CSCs, allowing them to regenerate the most tumor cells while maintaining self-renewal potential[115]. The molecular structure of CD44

functions as a receptor for HA and other ECM components, enabling CSCs to sense environmental changes and mediate signaling transduction to regulate CSC stemness properties. Consequently, CD44 binding regulates CSC survival, self- renewal, maintenance, and chemoresistance[95], which at least in part explains why CD44 is critical for disseminated cancer cells to adapt to new environments and why CD44 is required for metastatic colonization[116].

6.4 IMMUNOTHERAPY IN PANCREATIC CANCER

Personalized medicine is one of the goals of the new oncology medicine, especially immunotherapy. Immunotherapy has emerged as a promising treatment modality in a variety of malignancies, including pancreatic cancer[117], which is unique from an immunological perspective.

There are some limits in immunotherapy applicated in pancreatic cancer. First, intratumoral effector T-cells are rare, in contrast to many other solid tumors for which infiltration of effector T-cells is often prominent[118]. Second, the RAS oncogene drives an inflammatory program that establishes immune privilege in the pancreatic tumor microenvironment[119]. Third, pancreatic cancer is associated with a massive infiltration of immunosuppressive leukocytes into the tumor microenvironment. Fourth, the development of pancreatic cancer is associated with a strong desmoplastic reaction that consists of multiple cell types, molecular factors, and extracellular matrix[120]. This dense desmoplastic stromal reaction is one of the hallmarks of pancreatic cancer and plays a vital role in promoting angiogenesis while evading from immune cells[121]. Studies have uncovered a rich communication between stellate cells (fibroblasts) and cancer cells. The abundance of PDGF (platelet derived growth factor), fibronectin, proteoglycans and hyaluronic acid distorts the normal pancreatic architecture and transforms it into a complex, abnormal

configuration of seemingly impenetrable walls. Accordingly, this extensive stroma is not only a passive barrier for the immune system but rather interacts with cancer cells and participates in its progression and invasion[120]. There are different types of cells that determine the immune response against cancer, divided into two main categories: effector immune cells, such as the Natural killer cells (NK), the CD8 Cytotoxic and CD4 helper T-cells or Tumor infiltrating lymphocytes (TIL), and the Dendritic cells (DC); Suppressor immune cells, as the tumor associated macrophages (TAMs), T-regulatory cells (CD4 +, CD25+ FoxP3+) (Tregs), Myeloid Derived Suppressor Cells (MDSCs), fibroblasts, and mast cells[121]. An important role may be played also by the Co-inhibitory Receptors and ligands, as program-death (PD1) and cytotoxic T-lymphocyte associated protein 4 (CTLA4)[122,123]. Treatments that bypass tolerance and induce a specific antitumor immune response in the periphery still are ineffective owing to the immunosuppressive tumor microenvironment (TME), which includes Tregs, tolerogenic antigen-presenting cells, MDSCs, immunoregulatory molecules, and immunosuppressive cytokines. In particular, Tregs and MDSCs were shown to be increased in mouse and patient tumors, inhibiting protective $CD8^+$ T-cell responses, and promoting PDA growth. Treatment strategies that target these suppressive populations enhance cancer-specific T-cell activation in preclinical and clinical studies. Treg depletion facilitates tumor eradication by increasing the effector T- cell.

7. RATIONALE

Diabetes mellitus may represent a unique condition to establish whether organ pathology is primarily due to changes in the functional properties of stem or parenchymal cells or both. The impairment of tissue homeostasis is still incompletely understood.

The variability in tissue response to chronic hyperglycaemia makes it difficult to sustain an unified hypothesis to dissect the mechanism of diabetes associated multiorgan damage. A significant literature exists on the functional impairment of endothelial progenitors' cells (EPCs) and several observations by our group have documented diabetic microangiopathy in the bone marrow[81] and demonstrated an alteration of osteoblastic stem niches by changes in the sympathetic autonomic nervous system[82].

The present study is on line with the view that diabetes, is directed not only to parenchymal cells, but can also affect stem cell populations, in terms of distribution, number and function. Alterations in stem cell homeostatic control lead to dysfunction of target organs. Similarly, diabetes by altering tissue microenvironment, affects the cross talk between stem cells and their niches.

8. AIM OF THE STUDY

Few data are available on the complex structure of the human pancreas because the access to the human pancreatic samples is limited and routine biopsy of the pancreas is unrealistic[124], and the diabetic environment is still completely unknown.

Thus, we performed a morphometric study for the immunohistochemical characterization of the structural components and stem phenotypes of healthy and diabetic human pancreas. The possibility to provide basic observations documenting alteration in stem cell number and distribution of different type of cell populations in diabetic disease, open new opportunity in therapeutic strategies.

Thus, since diabetes causes damage to blood vessels (micro and macro angiopathy), we performed also a characterization of endothelial pancreatic cells in hyperglycemia from human diabetic and not diabetic pancreatic tissue.

PhD student, under the Principal Investigator supervision, carried out the development and the set-up of the protocols for the immunohistochemical analysis on pancreatic tissue, the collection of the data and the interpretation of the results.

MATERIALS AND METHODS

1. PATIENT POPULATION

To establish the role of diabetes mellitus on morphological modification in human pancreas, we analysed 30 autoptic pancreases, collected from death in the minimal interval of law. In this case, informed consent for research purposes is not necessary, as detailed by protection commissioner (***Autorizzazione n. 9/2013 - Autorizzazione generale al trattamento dei dati personali effettuato per scopi di ricerca scientifica -*** *Gazzetta Ufficiale n. 302 del 27 dicembre 2013)*.

In particular, population is composed by 30 pancreases (head and body) obtained from 12 non-diabetic subjects (5 males and 10 females) and 18 diabetic subjects (6 females and 12 males), age ranging from 63 to 75 years.

The material has been collected in the Department of Pathological Anatomy of Azienda Ospedaliero-Universitaria of Parma.

Pancreas was sampled for diagnostic purposes and a portion of the organ, apparently free from alterations, was processed for the subsequent investigation. The adequacy of the tissue sampling was confirmed in the subsequent histological examination. This procedure was carried out by the medical staff of Pathological Anatomy, ensuring the priority of the specimens for diagnostic purposes.

Other 5 pancreatic samples were obtained after pancreasectomy surgery performed in the Unit of Gastrointestinal Surgery of Azienda Ospedaliero-Universitaria of Parma. Patients were enrolled after informed consent to the employment of biologic samples for research purpose. In particular, 3 pancreatic samples were obtained from non-diabetic patients (1 female and 2 male) and the other 2 from diabetic patients (1 female and 1 male). Pancreatic tissue was collected and transported under sterile condition to the Department of Pathologic Anatomy, where it was sampled under hood at laminar flow.

Portions of distal and neoplastic (if present) or pancreatic tissue from diabetic and normoglycemic patients were sampled by the medical staff ensuring the priority of their use for diagnostic purposes.

Tissue obtained was put in sterile PBS in order to perform cell isolation or fixed for ultrastructural analysis. Immunohistochemically analysis were performed to confirm the correct tissue sampling and to compare the data with *in vitro* studies.

2. SAMPLING AND TISSUE PROCESSING

The biological material thus collected was immediately fixed in buffered formalin 10% and subsequently treated by following the ordinary protocol processing for histological study, which include dehydration in increasing alcoholic series, clarification in xylene and inclusion in paraffin. From each sample were obtained serial sections of 5μm thick, which, then, were mounted on glass slides and stained with haematoxylin and eosin, Masson trichrome or used for immunohistochemical staining.

It was then conducted a morphological investigation in order to assess the suitability of the preparation and morphometric investigations.

3. MORPHOMETRICAL ANALYSIS

A section obtained from each case was stained with Masson's Trichrome in order to recognize fibrotic tissue and describe principal structure organization. Microphotographs of the entire section were captured by an optical microscope connected to a digital camera. Number and extension of fibrotic foci was computed by image analysis software (Image

Pro-plus 4.0, Media Cybernetics, USA) keeping separate the diffused damage by perivascular and periductal fibrotic accumulation present in the pancreatic tissue.

In particular, histological sections stained with Masson's Trichrome were analysed at a final magnification of 20X, taking advantage of a stereological grid that defines an area of 0.014 mm^2 and containing 42 points, each of which corresponds to an area of 0.0033 mm^2; this evaluation was carried out on a large number of microscopic adjacent fields (>50 for each section). The volume fraction occupied by fibrous tissue was calculated by counting the number of points that fell on the diffuse fibrosis, perivascular and ductal portion and expressed as a volume fraction compared to all the points examined.

4. IMMUNOHISTOCHEMICAL ANALYSIS

4.1 ENDOCRINE CELLS DISTRIBUTION

The endocrine portion of tissue was studied to evaluate alfa- and beta-cells amount and distribution. To this purpose one section of each sample after antigen unmasking by water bath (pH=9, 70°C, 40') was incubated respectively with polyclonal rabbit anti-glucagon (ROCHE, Basel, Switzerland) and rabbit polyclonal anti-insulin (30'RT, ROCHE) antibody. The reactions were then revealed by immunoperoxidase technique and nuclei visualized by Hematoxylin and Eosin staining.

In particular, from each sample were captured several microphotographs, documenting the whole tissue section with final magnification of 20X (microscope LEICA DMD108 with integrated photo camera) and analysed by image analysis software (Image Pro-plus 4.0,Media Cybernetics, Bethesda, MD, USA).

Number and density of isle was computed and number and density of glucagon or insulin positive cells positive measured for each isle. Data were expressed as a percentage or density compared to the number of total cells counted in analysed area. Islets diameter was also determinate to establish diabetes modifications in islets size because of diabetes.

4.2 DUCTAL SYSTEM

Another section for each sample was then incubated with citokeratin19, specific pancreatic marker for the duct system. The identification of this specific epithelial cells was obtained by incubation with anti- CK19 antibody (A53-B/A2.26 mouse, ROCHE), after appropriate antigen unmasking (water bath).

The evaluation was performed by counting the positive profiles for CK19, keeping separated small (intracinar) and large (interlobular) structures. Values were then expressed as density per mm^2.

4.3 VESSELS DISTRIBUTION

The capillary network is very important to allow the normal tissue performance. With this purpose the proportion of vessels within the fragments was determined.

In detail, sections were exposed to the mouse monoclonal anti-α-SMA (1:1000, Sigma, St. Louis, USA) antibody in order to recognize artery and arterioles edge.

Capillaries and venules vessels were detected by incubation with anti CD34 antibody (mouse monoclonal, 1:50, QBEND BIOGENEX, Fremont, CA, USA). Nuclei recognized by Hematoxylin staining.

The evaluation was performed keeping separated parenchymal, insular and periductal vascular structures.

At the same time, lymphatic vessels were detected by incubation with D2-40 antibody (mouse monoclonal, 1:50, BioCare Medical; Pacheco, CA, USA).

4.4 STEM CELLS PHENOTYPES IDENTIFICATION

For the first time we hypothesize that stem cells have a crucial role in the etiopathogenesis of diabetes. For this reason, we performed immunohistochemical reactions to identify this cell lineage, quantify its number and its distribution to document healthy tissue and to compare this data with diabetic pancreas.

We candidate c-kit, receptor of the stem cell factor (SCF), as a marker to identify the population of stem and progenitor resident in the pancreas. The sections were incubated with a rabbit polyclonal anti-c-kit (CD117, 1:30, DAKO, Glostrup, Denmark) antibody, and then with anti-rabbit FITC secondary antibody anti rabbit (1:20, Jackson Laboratories, West Grove, PA, USA). Since CD117 marker is expressed also by mast cells, the same sections were incubated with a specific mast cell antigen: tryptase (Try), this enzyme is particularly abundant in these cells, but completely absent in stem cells. We investigated the abundance of c-kit^{pos}Try^{neg} cells in diabetic and non-diabetic samples.

c-kit^{pos}Try^{neg} cells were considered stem cells by assessing the distribution within the tissue and their number computed. Values were then expressed as density per mm^2.

Progenitor cells compartments were also analysed in terms of abundance of expression of CD133. Pancreas sections were incubated with rabbit polyclonal antibodies anti-CD133 (1: 100, Abcam, Cambridge, UK). Nuclei were visualized by counterstaining with DAPI, following the protocol previously described.

Slides were observed by fluorescence microscope Olympus BX60 using a 40X objective to count the fluorescent signals.

Using morphometric methods well established in our laboratory, the quantitative analysis of progenitor populations and their distribution was expressed as the number per mm^2 of tissue analysed and as a percentage compared to the number of total cells counted and as the number of progenitor cells in both research groups.

CD44 antigen can be considered both as a cell adhesion molecule, playing a role in the cross-talking between cells and microenvironment and as a stem cell marker. The pancreas sections were incubated with rabbit polyclonal antibodies anti-CD44 (1:100, o.n. 4° C, Santa Cruz, Dallas, TX, USA) followed by incubation with secondary anti-rabbit antibody (CY3-conjugated, 1:20, 60' at 37° C, Jackson Laboratory). For each section photos of 20 fields were captured. Fluorescence emission corresponding to $CD44^{pos}$ cells was quantified using software 'Image pro plus 4.0' and expressed as Integrated Optical Density (IOD).

5. ULTRASTRUCTURAL ANALYSIS

Human pancreases from fresh samples were analyzed by transmission electron microscopy (TEM) to detect structural and subcellular alterations.

Briefly, the specimens of interest were fixed in Karnovsky solution (4% formaldehyde, 5% glutaraldehyde) for 3 hours at room temperature. The tissues were then postfixed in 1% osmium tetroxide (OsO_4) for 90 minutes and dehydrated by increasing concentrations of alcohol. Following this procedure, samples were washed with propylene oxide and embedded in epoxy resin embedding media.

Sections of 0.5μm thickness were stained with methylene blue and safranin to select morphologically the field of interest. Subsequently, ultrathin sections were collected on a

300-mesh copper grid and, after staining with uranyl acetate and lead citrate, were qualitatively examined under a transmission electron microscope (Philips EM 208S).

Since the close connection between diabetes and pancreatic cancer, in particular pancreatic adenocarcinoma, the samples were analyzed by evaluating the content of inflammatory infiltrate and the structures alterations in the presence of both diabetes and cancer.

6. *IN VITRO* STUDY

6.1 ENDOTHELIAL CELL ISOLATION

Pancreatic tissue fragments, obtained from pancreasectomy of patients with and without diabetes, were processed following the protocol below.

In particular, portions of distal and neoplastic (if present) or pancreatic tissue from diabetic and normoglycemic patients sampled by the medical staff were put sterile PBS in order to perform cell isolation.

In sterile conditions, the biologic material was minced using surgical scissors and the micro-fragments put in a solution of collagenase/dispase (1mg/ml; ROCHE) for 60 minutes in a shaking bath at 37°C.

The digested sample was then purified from debris using a nylon filter with pores of 100µm.The cell suspension was centrifuged at 210g for 5 minutes.

The pellet was then resuspended in culture medium EGM-MV (Lonza, Basel, Switzerland) added with 5% FBS, and seeded in collagen-coated 6 well plates.

Twenty-four hours after plating the debris and the non-adherent cells (erythrocytes, leukocytes, death cells) were removed by washing twice with PBS. Fresh culture medium was then added to cell culture.

Cell monolayer was daily observed using an inverted microscope (Olympus CK40, Japan) and fresh culture medium was changed twice a week. Within 5-7 days was observed a heterogeneous cell population mainly constituted of fibroblasts, stromal, epithelial and endothelial cells.

In order to obtain a cell population constituted of endothelial cells, isolated from healthy and diabetic pancreas, a sorting, using immunomagnetic beads, was performed.

Briefly, the heterogeneous cell population was detached by Trypsin/EDTA (Sigma Aldrich) and resuspended in 60µl of EGM-MV, added with 20µl of FcR Blocking Reagent and 20µl of paramagnetic microbeads covered with antibodies against the CD31 antigen (Myltenyi Biotec.).

After 20 minutes of incubation at 4°C, the cell suspension was transferred in a MS Column exposed to a magnetic field produced by OctoMACS SeparatorTM and then eluited with 2ml of EGM-MV. The cell population thus sorted consists of all the cells that don't express the endothelial marker CD31.

Afterwards, the MS Column was removed from the OctoMACS SeparatorTM and eluited with 2ml of EGM-MV in order to recover the $CD31^{pos}$ cell fraction.

The $CD31^{pos}$ cell population was seeded in a collagen-coated $25cm^2$ flask with culture medium EGM-MV added with 10% FBS.

6.2 IMMUNOCYTOCHEMICAL ANALYSIS

In order to confirm the purity of the $CD31^{pos}$ cell population an immunocytochemical analysis was performed. The cells cultured on suitable chamber slides were fixed with 4% paraformaldehyde, blocked, and permeabilized.

The samples were then stained with primary antibodies anti-CD31 (mouse monoclonal, prediluited 4°C o.n., DAKO) and anti-vWF antibody (rabbit polyclonal, 1:200 4°C o.n.,

DAKO), markers typical of endothelial lineage. Nuclei were counterstained by DAPI and cover slips mounted with Vectashield (Vector, USA).

6.3 CELL VIABILITY ASSAY

In order to understand the behavior of pancreatic endothelial cells under hyperglycemia, simulating diabetic condition, different functional analysis was performed. Ninety-six-well tissue culture flat-bottom plates (Corning, 3595) were coated with collagen solution type 1. Pancreatic endothelial cells (PanEC) were seeded at $5x10^3$ cells per well in 100 µl in EGM-MV with 5% FBS.

After adhesion, cell lines were exposed to various glucose concentrations (5.5 mM-normoglycemic, 11 mM, 16.5mM, and 22mM) at 12 and 72hours. Cell viability under normal growth conditions and hyperglycemia treatment was assessed by adding 100 µl of 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) at 1 mg/ml (Sigma-Aldrich, M2128) to each well. Plates were further incubated at 37°C for 1 hour. At the end of the incubation, the formazan was then dissolved using 100 µl of dimethyl sulfoxide (DMSO; Sigma-Aldrich, D2650) per well. Each condition was repeated five times. The optical density was measured at 595 nm with a microplate reader (BioRad, Hercules, CA, USA). The absorbance values are proportional to the quantity of formazan produced and therefore to metabolic activity and cell viability. Data were analyzed comparing the absorbance values obtained at the different glucose concentrations with the group control (Normoglycemic).

6.4 MATRIGEL ASSAY

To evaluate endothelial cell tube formation, 100 µl of the Matrigel® matrix solution was transferred into each well of a 96-well plate. The matrix was allowed to solidify at

37°C and 5% CO_2 for 45 minutes. The endothelial cells were then trypsinized and seeded (2×10^4cells/well) in 100 µl fresh medium with experimental glucose concentration, as described before.

After 2h and 24h of incubation, the tubular structures were visualized and photographed under an inverted light microscope at 4x magnification (Olympus, Japan).

Quantitative assessment of the tube formation was carried out by using the Image Pro-Plus 4.0 software (Media Cybernetics, Bethesda, MD, USA) by measuring the area of the tubular structures. This area was divided by the total frame area to obtain the quantitative measurement of tube forming ability.

6.5 CELL MIGRATION ASSAY

In order to evaluate if the hyperglycemia impairs the migration cells capacity, PanECs were analyzed for "wound healing" assay.

Endothelial cells isolated were seeded (20000 cell/cm^2) in 6-well plates. After confluence, cells were starved by adding Endothelial Basal Medium (EBM, Lonza, Basel, Switzerland) added with 0.5% FBS.

After 12 hours of starvation a wound was made with a 1000µl pipette tip and culture medium at different concentration of glucose was added.

The closure of the wound area by migrating cells, was observed at 0 and 24 hours after seeding using an inverted-phase contrast microscope (Olympus CK40, Japan) coupled with a camera and images were recorded at each time point. The images were analyzed by Image Pro-Plus 4.0 software (Media Cybernetics, Bethesda, MD, USA).

Open wound area was measured for normo and hyperglycemic condition and compared with the area value obtained for each condition at 0h. Data were express in percentage of closing area in hyperglycemia condition relating to migrating cells in control medium.

6.6 REAL TIME PCR

Quantitative PCR (qPCR) assays were used to assess pro-inflammatory marker gene expression in hyperglycemia (Normoglycemic, 22mM glucose and osmotic control with mannitol) for 6 h and 12 h. Cells were lysed total RNA was extracted using miRNeasy Mini Kit (Qiagen Ltd., West Sussex, UK) and quantified by NanoDrop (NanoDrop Technologies, Wilmington, DE, USA). iScript Reverse Transcription Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to obtain cDNA, starting from 250 ng of total RNA.

Interleukin IL-6, MCP-1, ICAM and VCAM gene expression was assessed with TaqMan primers and probes on a CFX Connect Real-Time (Bio-Rad), as already reported. Specific thermal cycling conditions were used: 98 °C for 30 s, followed by 40 amplification cycles (95 °C for 3 s; 60 °C for 20 s).

Gene expression values were calculated based on the ΔΔCt method using GAPDH as reference gene. Three independent experiments were performed, and samples were analyzed in triplicate.

7. STATISTICAL ANALYSIS

The results were expressed as mean ± standard error. The statistical significance for the comparison between two measurements was determined with the "t" test of Student. Statistical tests were performed using the SPSS package (SPSS, Chicago, IL, USA). The normal distribution of the data has been verified by applying the Kolmogorov-Smirnov test. Values with $p < 0.05$ were considered significant.

RESULTS

1. PATIENT POPULATION

As described in the methodology, we analysed 35 human pancreases.

The study was conducted on 20 diabetic and 15 ages matched non diabetics patients (Table 1).

Main Inclusion Criteria: tissue samples obtained from individuals who at the time of pancreatic surgery were affected by DM or specific pathologies different from pancreatic cancer were included. Subjects were included regardless of the time of onset of DM.

Main Exclusion Criteria: patients affected by diseases that, independently from diabetes and pancreatic cancer, may compromise the function and structure of the organ were excluded. Samples that were not adequately preserved or correctly processed were also excluded.

As shown in Table 1, patients were affected by diabetes from at least 4 years, suggesting a long-term alteration of pancreatic function. Since most samples were obtained from autopsy, detailed clinical information were available only from 9 out of 20 patients.

Table 1: Study Population

	Non-Diabetic	Type 2 Diabetes
Male (M)	4	13
Female (F)	11	7
Hba1c, %	n.a.	4.78±1.55
Duration of Diabetes, years	n.a.	10±6
Age, years (Mean±St.Dev)	72.87± 2.62	72.12±1.02
Treatment	n.a.	Insulin (3) Oral Anti-Diabetic Drugs (6)

2. MORPHOMETRIC ANALYSIS OF THE STRUCTURAL COMPOSITION OF THE HUMAN PANCREAS

Following the criteria described in the methodology, the structural components of diabetic and non-diabetic tissue were morphometrically quantified on histologic sections.

The analysis of the different tissue compartments was performed taking into consideration the high variability of the histologic pattern and the actual difficulties to obtain sample with similar orientation in paraffin embedded preparations of human pancreas.

In order to evaluate and quantify tissue composition of diabetic and non-diabetic pancreas, on each sample Hematoxylin-Eosin (Figure 1A) and Masson Trichrome (Figure 1B) stained sections were utilized to perform microscopic analysis.

The tissue area sampled for this evaluation ranged from 8 to 48 mm^2.

As documented diabetes did not significantly affect the tissue fraction occupied by parenchymal compartment of the pancreas, as shown in bar graph (Figure 1C). Intriguingly, the percentage of adipose tissue in diabetic patients (13.47±2.28%) was moderately reduced respect to non-diabetics (17.53±5.37%, n.s.).

Morphometric analysis on Trichrome Masson's stained sections (Figure 2A and Figure 2B) documented a predominant component of functional parenchyma, collagen accumulation and vascular stroma in both groups of analyzed human pancreases. Collagen deposition appeared to be influenced by diabetes. In particular, samples of diabetic patients showed higher amount of fibrosis (27.75±2.55%) if compared to non-diabetic (19.15±1.44%, $p<0.05$) (Figure 2C) and a consequent decrease in parenchymal tissue (54.82±2.96%, vs ND samples 59.40±1.36%, $p<0.05$). Quantitatively, three different patterns of fibrosis were analyzed: diffuse or interstitial, periductal and perivascular.

Diffuse fibrosis representing the major form of collagen accumulation was mainly expressed in diabetic samples (20.05±2.02%) compared to non-diabetic (12.57±1.65%, $p<0.05$). Similarly, perivascular fibrosis resulted significantly higher in diabetic samples. (D: 3.62±0.41% vs ND: 2.61±0.30%, $p<0.05$, Figure 2D).

3. IMMUNOHISTOCHEMISTRY

3.1 PANCREATIC DUCTS

The ductal structures were studied in order to evaluate how these fundamental components of the pancreatic parenchyma participate to the structural remodeling in diabetes. Cytokeratin 19 (CK19, Figure 3, panel A and B) is a specific marker for epithelial ductal cells and was detected by immunoperoxidase technique to study the number and distribution of ductal structures. As documented in Figure 3C, the distribution of $CK19^{pos}$ profiles was evaluated on structures of different dimension considering diameters with threshold limit of 20μm and according to their intracinar and interlobular location. Data documented that small $CK19^{pos}$ structures were predominant and their distribution was similar in the two groups (Bar graph, Figure 3 C).

Overall, 30.04±5.06% of pancreatic tissue was occupied by $CK19^{pos}$ structures. The quantitative analysis of these pancreatic structures did not show significant differences between non-diabetic and diabetic samples.

3.2 ISLET OF LANGHERANS

Pancreatic islets were characterized by qualitative and quantitative methods, using immunohistochemistry to detect insulin and glucagon, the two main hormones produced by

the endocrine component of the pancreas. In particular, insulin is produced by β-cells, whereas glucagon is generated by α-cells, representing the main endocrine compartments in pancreatic tissue. As illustrated in Figure 4A and 4B, endocrine islets show the typical compact and elliptical shape.

In samples obtained from diabetic patients the incidence of glucagon^{pos} islets resulted modestly decreased (5.89±0.29 n/mm^2) compared to non-diabetic patients (6.58±0.44 n/mm^2, n.s., figure 4C). No significant difference was documented between the density of insulin^{pos} islets in diabetic and non-diabetic subjects (Figure 4C).

Since diabetes may alter the number and function of endocrine cells, the density of cells expressing the two different antigens, reflecting the principal hormonal production, was computed both in diabetic and non-diabetic groups. Interestingly, a significant decrease in glucagon^{pos} cells density and insulin^{pos} cells density was observed in diabetic patients (Insulin D: 375.04±31.03 n/ mm^2; Glucagon D: 371.88±32.69 n/ mm^2) compared to non-diabetic patients (Insulin ND: 456.51±39.39 n/ mm^2, $p<0.05$; Glucagon ND: 474.80±55.25 n/mm^2, $p<0.05$) (Figure 4D).

The subsequent analysis to establish the functional components within each individual islet, documented a significant lower fraction of glucagon^{pos} cells/islet in diabetic patients than in non-diabetic ones (D: 30.81±3.43% vs ND: 39.14±1.23%, $p<0.05$) and, similarly, a lower percentage of insulin positive cells in diabetic than in non-diabetic subjects (D: 50.22±2.17% vs ND: 58.89±1.27%, $p<0.05$, Figure 4E).

Intriguingly, individual endocrine cells, not associated with insular structures and scattered throughout the pancreatic tissue were also observed (Figure 5). With this intent data were represented in the histogram of Figure 5C. Interestingly, both individual glucagon^{pos} cells (3.04±0.70 n/mm^2) and insulin^{pos} cells (4.46±0.87 n/mm^2) distribution in

diabetic pancreas was significantly higher than in non-diabetic samples. (Insulin ND: 2.47±0.30 n/mm^2, $p<0.05$; Glucagon ND: 2.90±0.78 n/mm^2, $p<0.05$) (Figure 5, Panel C).

An additional important parameter to understand whether the endocrine function was altered in the presence of diabetes was represented by the pancreatic islet's diameters (Figure 6 B, C). Islet dimension was computed by measuring the geometric mean of longitudinal and transverse diameters.

The measured values of islet diameters were classified in classes of frequency. As appreciable by scattered plot graph, the most abundant class of islets dimensions was represented by diameter included in 15-50μm range in both group of analysis. In particular diabetes was associated with a rise in the number of islets of this class, Figure 6A. In the other classes of frequency, no remarkable differences were present.

3.3 VESSELS DISTRIBUTION

In order to evaluate the potential implication of vascular structures in pancreatic diseases, the distribution of this tissue component was assessed. To this purpose, antibodies that specifically recognize endothelial cells (*CD34*) and smooth muscle cells (α-*SMA*) were used by immunohistochemistry. It is known that diabetes causes a profound alteration of vascular networks and these changes, called diabetic microangiopathy, lead to the complications of the disease. Capillaries, venules and arterioles were analyzed in the two studied groups. The quantitative and qualitative distribution was investigated evaluating the amount of these structures relative to their location within the tissue (parenchymal, Figure 7A, insular, Figure 7B). In addition, lymphatic vessels were immunohistochemically assessed.

As expected, capillary density was 5-times higher with respect to venules density in both groups. Capillaries and venules density in intracinar location did not significantly

differ between diabetic and non-diabetic patients, whereas both vascular structures located within endocrine islets were decreased in diabetic patients compared to non-diabetics. Indeed, islets (ENDO) capillary density was lower in diabetic patients (10.92±1.27 n/mm^2) than in non-diabetic samples (18.91±2.06 n/mm^2, $p<0.05$). Similarly, venules were decreased in diabetics samples (4.78±0.62 n/mm^2) compared to ND (8.12±1.03 n/mm^2, $p<0.05$) (Figure 7C and 7D). This structural alteration may represent the cause or the consequence of β-cell functional impairment, which is one of the patho-physiologic mechanisms of T2DM onset.

Furthermore, we evaluated the density of the arteriolar network in the two groups, focusing our attention to the localization of these vessels within the pancreatic tissue. Arteriolar density was decreased in parenchyma (EXO) in diabetic compared to non diabetic patients (D: 1.66±0.14 n/mm^2 vs ND: 1.82±0.36 n/mm^2; Figure 8B). However, in insular position (ENDO), arteriolar density was slightly increased in samples from diabetic pancreas, although were less represented than in EXO portion, (Figure 8B, D: 0.14±0.05 n/mm^2 vs ND: 0.09±0.02 n/mm^2).

Arteriolar diameters were also computed, in order to establish whether diabetic microangiopathy involved a shift in the frequency distribution of this vascular parameter. Thus, the internal diameter of arterioles was measured in α-SMA^{pos} structures located within the pancreatic parenchyma. The collected values were divided into nine classes of frequency and it was first observed that the most common classes in all groups are the ones with smaller diameters, similarly to what noticed for the islets size. Diabetes determined an upward shift in the frequency of small and large arterioles of pancreatic tissue (Figure 8C). When arteriolar wall thickness was measured, a significant increase was detected in diabetic samples compared to non-diabetic (Figure 8, Bar graph D, EXO D: 5.52±1.21μm vs ND: 4.65±0.45μm, $p<0.05$; ENDO D: 2.82±0.31μm vs ND: 2.61±0.17μm).

Conversely, no significant changes were detected in external diameters of arteriolar profiles located within the insular structures (Bar graph E).

It should be pointed out that both wall thickness and external diameter values resulted significantly lower in ENDO portion compared to EXO.

As a final approach to study pancreatic vasculature, lymphatic vessels abundance was investigated. Immunofluorescence technique was applied to recognize lymphatic profile, as illustrated in Figure 9A.

This analysis was performed exclusively on parenchyma portion, whereas no positive immunostaining was detected in either periductal or insular areas. Lymphatic vessels density was only mildly impaired by diabetes (D: 0.65±0.08 n/mm^2 vs ND: 0.76±0.10 n/mm^2, Figure 9B).

4. IDENTIFICATION OF STEM CELL ASSOCIATED PHENOTYPES IN HUMAN PANCREAS

The structural rearrangement of the pancreatic tissue taking place in several diseases, prompted us to investigate the number and distribution of progenitor cells implicated in the control of tissue homeostasis. Importantly, available literature on the morphometric quantification of this cell population in pancreatic tissue is lacking.

To this purpose, c-kit, the receptor of Stem Cell Factor, was selected as this antigen is consistently expressed in stem cells of different tissues and different species. Since c-kit is expressed by mast cells, the same sections were incubated with tryptase (Try), an enzyme highly expressed by mast cells and absent in stem cells (Figure 10).

The quantitative evaluation documented that 11.79% of c-kit^{pos} cells co-express tryptase (c-kit^{pos}Try^{pos}). Thus, only c-kit^{pos}Try^{neg} cells were considered in our quantitative analysis.

Small round c-kit^{pos}Try^{neg} cells were present in human non diabetic pancreatic tissue (0.058±0.01%) and this fraction was significantly reduced in diabetic samples (0.0281±0.01%, $p<0.05$, Figure 10 Graph bar D). In particular, c-kit^{pos}Try^{neg} cells were predominantly distributed in islets while a reduced incidence was measured in interstitial space in diabetic patients (0.016±0.1%) compared to non-diabetic ones (0.36±0.1%, $p<0.05$) (Figure 10, Panel E).

In order to have a more complete panel describing progenitor cells present in human pancreas, cells expressing CD133 (Figure 11A) were documented.

CD133, identified as Prominin, is expressed by progenitor cell populations detected in several normal (bone marrow, kidney, colon, pancreas and skin) and neoplastic (brain, colon, lung) tissues (Figure 11, Panel C).

Quantitative analysis of CD133^{pos} cell in human pancreas documented a trend to increase in diabetic samples (ND: 0.24±0.10% vs D: 0.43±0.25%, ns).

CD133^{pos} cells distribution was variable in different areas of pancreatic tissues. Similarly, to c-kit^{pos} cells distribution, CD133^{pos} cells were more represented in insular area. (Figure 11B).

In order to establish the impairment of vascular compartment, we also assessed the amount of endothelial progenitor cells. In addition to labelling microvascular lumens, CD34 is also associated to hematopoietic and endothelial progenitors phenotypes (Figure 12, Panel A).

Quantitative analysis showed a decrease in CD34^{pos} cells density in diabetic pancreas (D:1.53 ±0.11 n/mm^2 vs ND:1.95±0.21n/mm^2, $p<0.05$), an observation which is in line with the reduced vessels distribution with diabetes (Figure 12B).

We finally evaluated, from either a qualitative and quantitative point of view, the expression of CD44 in the two groups of patients by immunofluorescence analysis of

pancreatic tissue. Our interest was attracted by the discovery that expression of a variant isoform of CD44 (CD44v) induced a metastatic phenotype in locally growing tumor cells. CD44 in association to Insulin immunostaining (Figure 13A and 13B) was detected. However, the specific distribution of CD44 within the different structural and cellular components of pancreatic parenchyma was not assessed.

The quantification of CD44 expression was performed by immunofluorescence and expressed as Integrated Optical Density (IOD). From Figure 13C, it is possible to appreciate a reduction in the expression of CD44 in diabetic pancreatic samples compared to non-diabetic ones. Being CD44 an adhesion molecule, the downregulation of this receptor may be related to the early incidence of cancer in diabetic patients and the high risk of metastatization and invasiveness typical of pancreatic tumor.

5. ULTRASTRUCTURAL ANALYSIS

As a final approach to describe morphologic characteristics of pancreatic tissues, the ultrastructural analysis was performed through Transmission Electron Microscope (TEM). Ultrastructural aspects of the human pancreas were evaluated. A pancreatic insula located in the distal area of the sample is composed by different endocrine cells including α-cells filled with glucagon granules and a centrally located duct-like structure (Figure 14A and 14B).

6. IN VITRO ANALYSIS

6.1 IMMUNOCYTOCHEMISTRY

Blood endothelial cell populations obtained from human pancreatic tissues were analyzed *in vitro* by immunocytochemistry. The purity of primary culture was determined according to morphologic criteria (Figure 15, A and B) and ensured by immunocytochemistry using the pan-endothelial marker CD31 (Panels C and D) and the dot-like cytoplasmatic labelling of von Willebrand factor (Panels E and F). This investigation was performed also on HUVEC cells for comparison.

6.2 CELL VIABILITY ASSAY

In order to understand the behavior of pancreas-derived endothelial cells (PanEC) under hyperglycemia, simulating diabetic condition, the MTT assay was performed following the method previously described.

After adhesion, PanECs were exposed to various glucose concentrations (5.5 mM-normoglycemic, 11 mM, 16.5mM, and 22mM glucose) for 12h and 72 hours. Cell viability under normal growth conditions or hyperglycemia was assessed.

Interestingly, hyperglycemia increased the proliferation rate of PanEC lines. In particular, proliferation with 22mM of glucose was significantly higher than control group in pancreatic endothelial cells after 12hours of treatment (Cell viability, 22mM-glucose: 117% vs CTRL: 100%, $p<0.05$), while, as expected, high concentration of glucose was cytotoxic for HUVEC cells (data not shown) (Figure 16).

6.3 ANGIOGENESIS ASSAY

A Matrigel®-based tube formation assay was performed to assess the *in vitro* angiogenic properties of human PanECs in hyperglycemia. Tubular structures were measured at 24 hours after plating.

The quantitative analysis showed that the tube forming ability reaches its peak 24 hours after cell seeding. Inhibition of tubular formation by EC lines was more evident following 22mM glucose concentration, with the generation of thicker tubule structures and in reduced number compared to control condition (Figure 17, Panel A and B).

6.4 CELL MIGRATION ASSAY

In order to determine whether isolated endothelial cells differ in terms of cell migration, scratch assays were performed in EC exposed to hyperglycemia.

The capacity of migration was evaluated on confluent monolayer of PanEC after 22mM glucose treatment for 12, 24, and 72 hours. A cell-based scratch assay followed by the quantification of the wounded area was performed. High concentrations of glucose tended to inhibit migration capacity of PanEC cells, although without statistically significant impact (Healing area after 72hours of treatment, 22mM-glucose: 61% vs CTRL: 64%, Figure 17, panel C).

6.5 REAL TIME-PCR

To better understand these cellular events, a molecular analysis on endothelial pancreatic cells was performed. Gene expression of pro-inflammatory markers, IL-6, MCP-1 and adhesion molecules as ICAM and VCAM, was assessed in hyperglycemia condition (Normoglycemic and 22mM glucose) after 6, 12 and 72 hours.

RT-PCR analyses are ongoing but Figure 18 shown the preliminary analysis performed on one sample.

At early time, after 6 hours of treatment, PanECs showed an increased expression of IL-6 and MCP-1 when cultured in 22mM glucose concentration compared to normoglycemic condition.

In particular, IL-6 reached a 3-time higher expression than control condition, although its expression returned on control values at 72hours. Similarly, MCP-1 doubled the value at early time.

Conversely, the expression of ICAM and VCAM had the opposite trend. In fact, activation of ICAM took place after 72hours of treatment, tripling its expression compared to normoglycemic condition while VCAM reached its peak at 12 hours with a 20% increase to decline at 72 hours.

DISCUSSION

Diabetes is a complex group of heterogeneous diseases that do not recognize a unique pathogenetic clue. Many organs are affected by diabetes, but the mechanism of diabetes associated multiorgan damage is still unclear.

Pancreas is the main target organ of diabetes; however its structural alterations are poorly investigated. Few data are available on the complex structure of the human pancreas because the access to the human pancreatic samples is limited and routine biopsy of the pancreas is unrealistic[124], rendering the diabetic environment still poorly defined.

Thus, we performed a morphometric study on the immunohistochemical characterization of the structural components and stem phenotypes of healthy and diabetic human pancreas, to postulate specific features resulting from the alteration in number and distribution of different cell populations. Our investigation may provide the basis to better understand the pathogenetic mechanisms of the disease, and prospectively propose new therapeutic targets.

Diabetes did not show a significant impact on the gross structural composition of pancreatic parenchyma although induced alterations of vascular and endocrine compartments together with evidence of tissue injury. As expected and stated by the literature, the degree of fibrosis increased as a consequence of diabetes, in particular in terms of diffused/parenchymal and perivascular collagen accumulation. The density of insulin^{pos} and glucagon^{pos} cells was impaired by diabetes as well as the percentage of glucagon^{pos} cells per islet was decreased. Diabetes induced an increase in the number of islets of small dimension that may be interpreted as a compensation for the extensive loss of endocrine cells with diabetes.

Evidence of transdifferentiation of alpha glucagon-producing cells in beta insulin-producing cells as a consequence of pancreatic injury has been widely proposed. The higher number of individual glucagon positive cells found in diabetic than in non-diabetics

samples and, similarly, a higher density of individual insulin positive cells in control pancreatic tissue parenchyma, may be attributed to a compensatory mechanism in response to the drop of endocrine cells in diabetic pancreas[125, 126].

Long-term complications of diabetes are mainly due to poor control and elevation of blood glycemia, which leads to systemic damage of blood vessels (angiopathy). In diabetes**,** the resulting complications are grouped in "microvascular disease" (due to damaged small blood vessels) and "macrovascular disease" (due to damaged arteries) [33,40]. The vascular pancreatic network was impaired by diabetes and the lowest density of capillaries and venules was found in diabetic patients. On the other hand, the rise of arteriolar density in diabetic patients was less significant. Interestingly, also pancreatic lymphatic vessels were altered by diabetes.

These data seem to be in line with the presence of microangiopathy also at level of the pancreatic islets, a phenomenon not fully described in the literature.

Several data have been reported on the regulatory role of stem cells in the homeostatic control of multiple solid organs[58]. The possibility to identify and characterize multipotent cells, progenitors, precursors, committed and differentiated populations has been widely documented.

The involvement of stem cells in regenerative medicine concerning diabetes has been repeatedly reported. One of the treatment strategies of diabetic patients is pancreatic islet cell transplantation[59] although this approach suffers of some limitations, as the lack of organ donors as well as the low possibility for the patients to become completely insulin free[60].

Although the use of adult pancreatic stem cells would be revolutionary in diabetes therapy, it is difficult to define a clinically relevant population of multipotent pancreatic stem cells as serious limitations exist in their identification, isolation and characterization

Evidences have been provided on the involvement of stem cells in diabetic conditions; specifically the impairment of endothelial progenitor cells and alterations of osteoblastic stem niches in diabetic bone marrow[81,82].

The present study is in line with the view that diabetes is directed not only to parenchymal cells, but can also affect stem cell populations, in terms of number, distribution and function. Accordingly, in diabetic pancreas we found a reduction of antigenic markers, as $CD117^{pos}$ cells and $CD34^{pos}$ that define cells with stem characteristics.

c-kit (CD117) is a receptor of the stem cell factor and is expressed by stem cells from different tissues and species[79]. In mice, the great presence of c-kit expression in the early stages of pancreatic development, indicates that it may be involved in stabilize the endocrine cell precursor pool in fetal mouse pancreas. c-kit interaction with its ligand mediate β-cell differentiation and proliferation and have been demonstrated across multiple species in vitro[80,81]. c-kit-expressing cells may also be involved in promoting beta cell regeneration; in fact, transplanted adult bone marrow derived c-kit-expressing cells reduce hyperglycaemia in diabetic mice[127]. For the reasons above, the reduction of $c\text{-}kit^{pos}$ cells we found in human diabetic pancreas, could explain the inability of the pancreatic system to counteract the persistent damage driven by diabetes.

Interestingly, $CD133^{pos}$ cells were increased in diabetic sample, while CD44 expression resulted down-regulated in diabetes. CD133 and CD44 are often associated with cancer stem cells markers. Being a marker of Cancer Stem Cells (CSCs), CD44 can promote the spread of cancer cells and its expression may be linked to patient prognosis.

The lowest levels of CD44 were observed in diabetic patients, while in the literature, high serum concentrations of CD44 are associated with insulin-resistance.

The close association between pancreatic cancer and diabetes is still under intense debate, although it is not clear whether diabetes leads to cancer predisposition.

Endothelial dysfunction is considered an early event in diabetes-related microvascular disease and is associated with accelerated endothelial senescence and ageing[128]. As previously described, since diabetes causes damage to blood vessels (micro and macro angiopathy), we isolated and characterized pancreatic-derived endothelial cells.

A substantial literature[129, 130] exists describing a toxic effect of hyperglycemia in different endothelial populations cells, as HUVEC cells. However, pancreatic endothelial cells displayed a different behavior when exposed to hyperglycemia. Indeed, pancreatic endothelial cells showed resistance to high levels of glucose. Thus, our preliminary results support the contention of an organ-dependent microcirculation.

In conclusion, we provide basic morphometric data on the immunohistochemical characterization of parenchymal and undifferentiated cell compartments of the human pancreas under diabetes. Moreover, pancreatic endothelial cells exposed to culture conditions mimicking diabetes were described. Evidence has also been documented of a differential distribution of stem cell associated phenotypes in human pancreatic tissue, highlighting their main distribution within the insulae.

This study provides the basis for subsequent investigations on human pancreas aimed at understanding the impact of diabetes mellitus on the structural rearrangement and on the possible involvement of progenitor cells in the mechanisms of dysregulation of tissue and functional homeostasis.

Although we did not establish whether these structural alterations were the consequence, or a cause of diabetes associated multiorgan damage, our approach may offer new insights on the interpretation of the diabetic paradox, which may involve a tissue specific angiopathy.

FIGURES

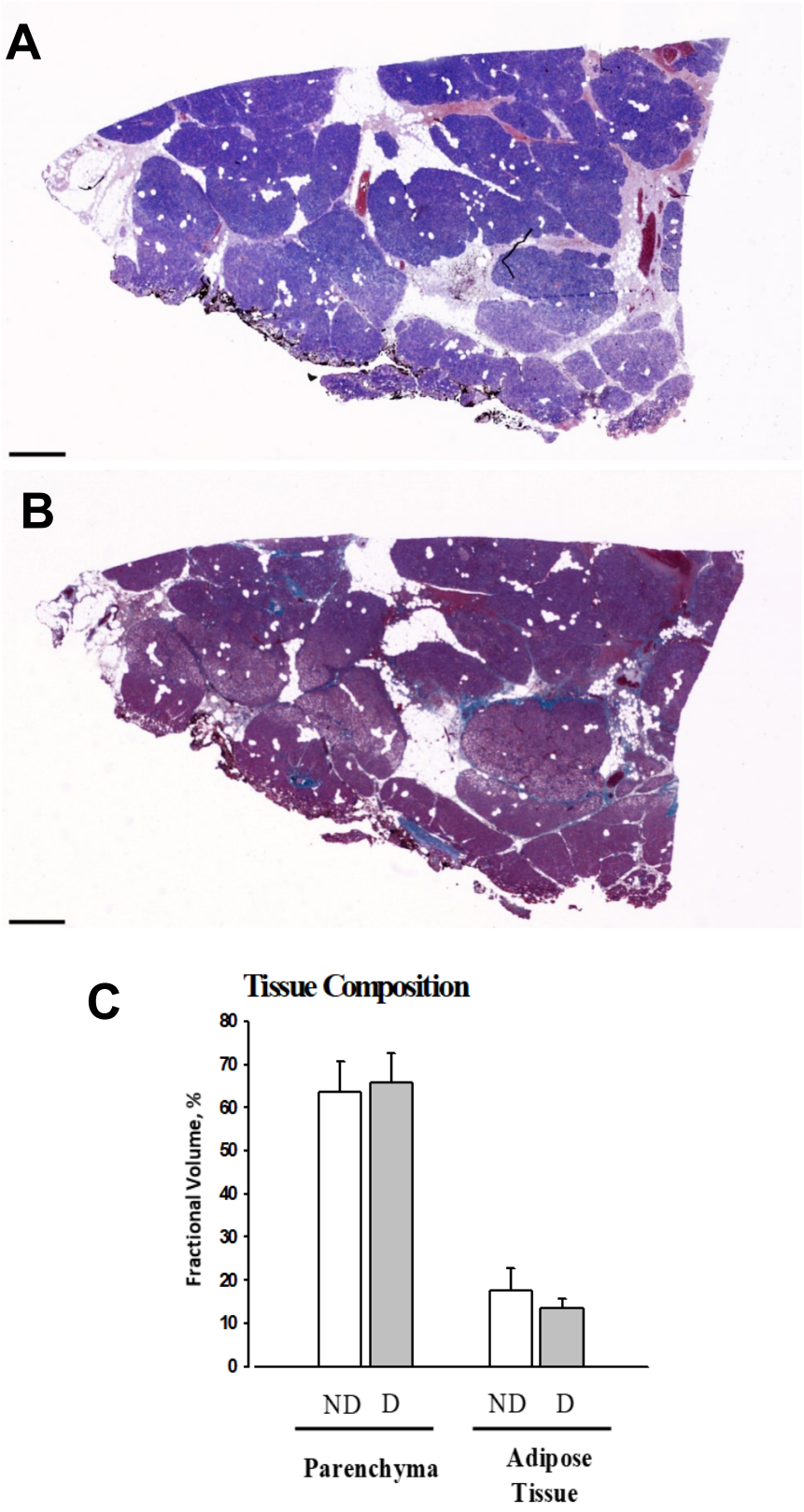


Figure 1. Tissue Composition. Serial sections of human pancreatic tissue stained with Hematoxylin and Eosin (**A**) and Masson's Trichrome (**B**). In **B** the functional parenchyma is recognized by reddish color, while greenish corresponds to fibrotic tissue.
(Optical microscopy, O.M., Panel A and B scale bars=1mm).
Bar graph (**C**) documenting the tissue composition expressed as fractional volume of parenchyma and adipose tissue in non-diabetics (ND) and diabetics (D) samples.

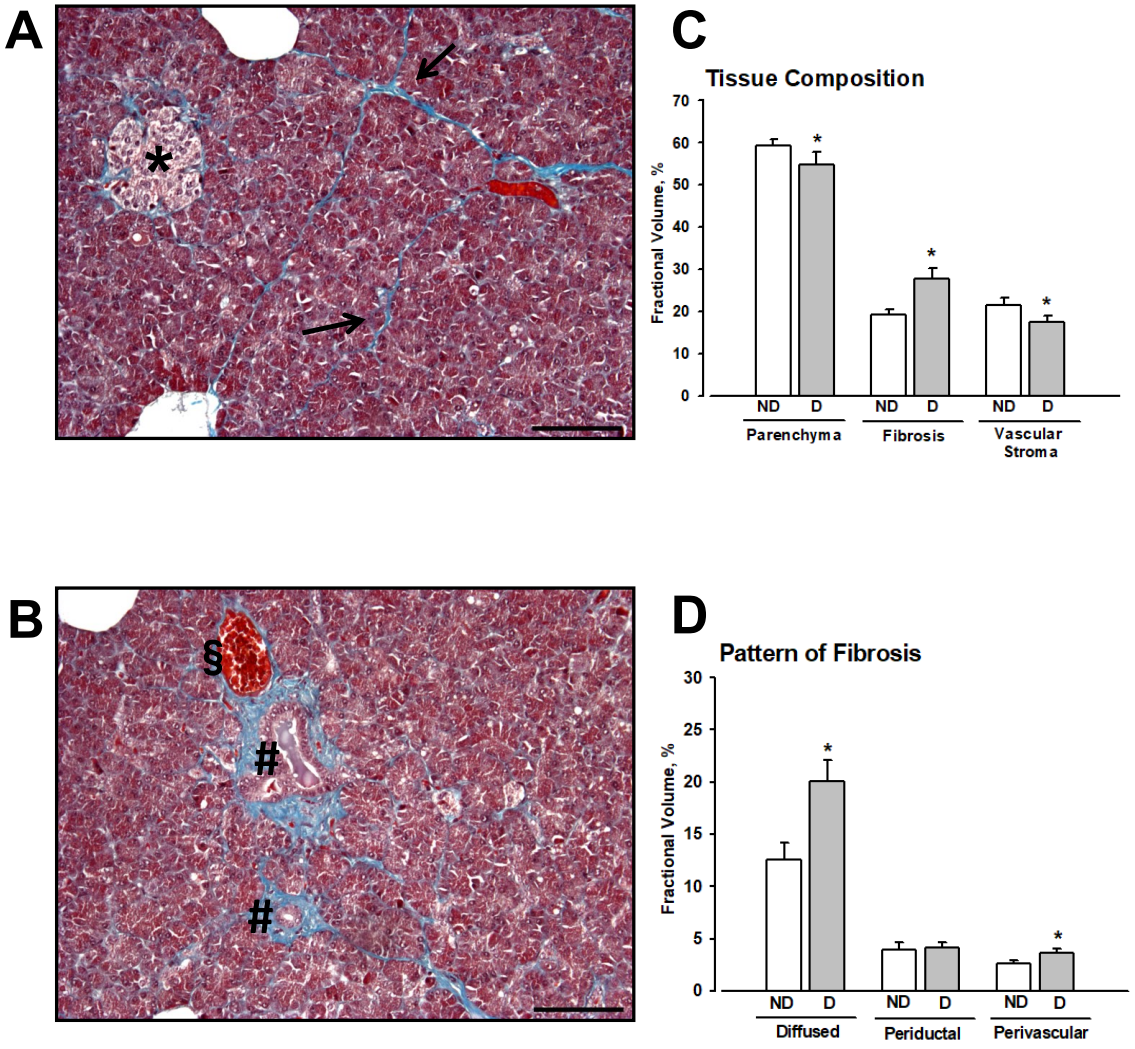


Figure 2. Different Patterns of Collagen Deposition in Human Pancreas. Representative microscopic images of Masson's Trichrome stained non diabetic sections illustrating the different distribution of fibrotic tissue. **A**: example of collagen deposition in peri-insular area (asterisk). Diffuse interstitial fibrosis is documented by collagen deposition widening the interstitial space (arrows). **B**: The typical accumulation of fibrotic tissue surrounding small pancreatic ducts (#) and collagen perivascular deposition (§) are illustrated.
(Panel A, B scale bars=50μm)
C: Quantitative evaluation of the different tissue compartments in human non diabetic (ND) and diabetic (D) pancreas, expressed in percentage. Fibrotic tissue increases significantly in D, while parenchyma and vascular stroma are reduced compared to ND.
D: Bar graph illustrating the percentage of different patterns of fibrotic tissue in human pancreas. Diabetes is associated to significant increase in diffused and perivascular fibrosis.
(*=$p<0.05$ vs Non Diabetic, ND).

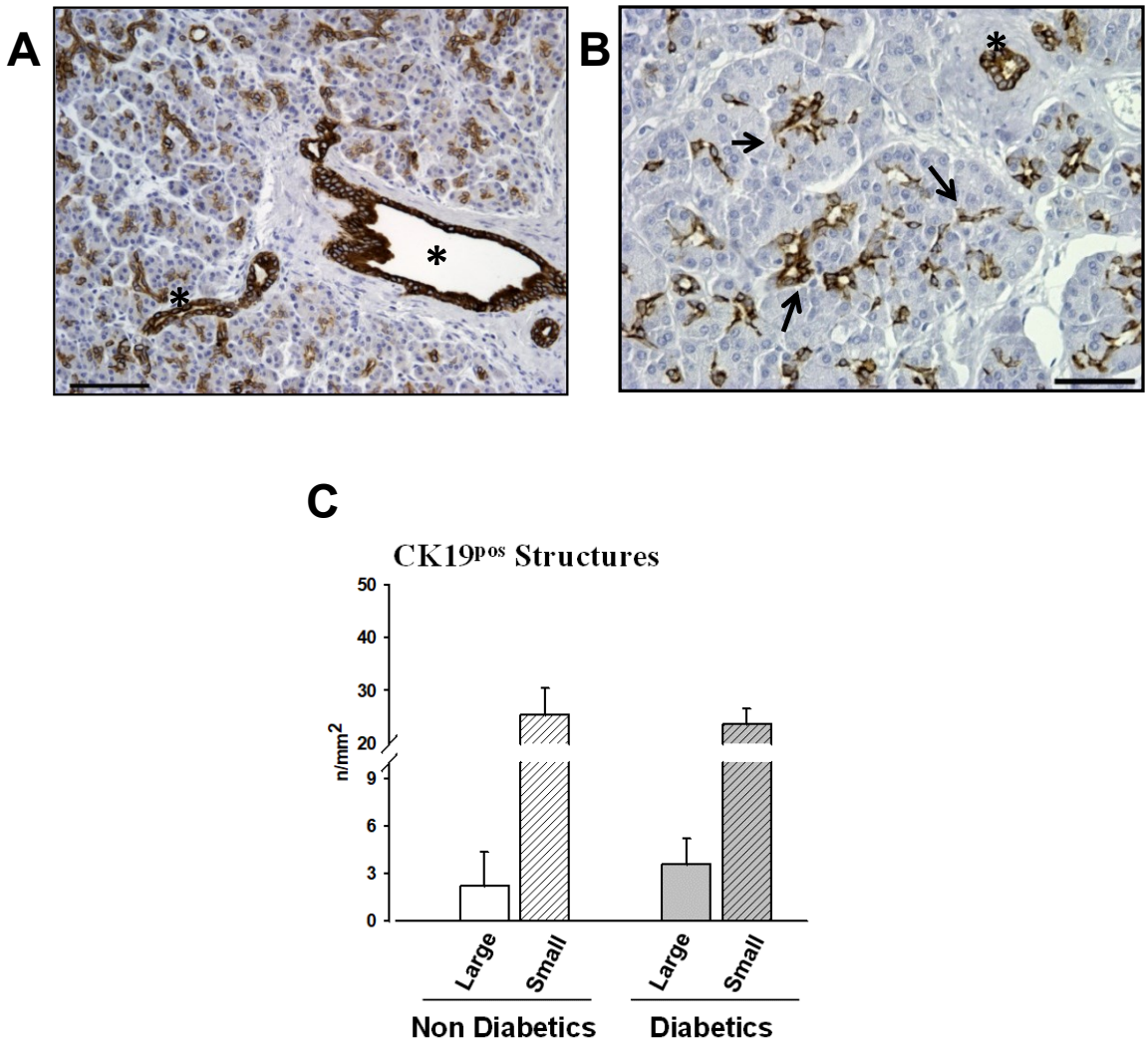


Figure 3. Excretory Structures. A and **B:** Representative images of CK19 expression in human pancreatic tissue. Panel **A** shows two large longitudinally oriented interlobular pancreatic ducts (asterisks) labelled by CK19 (brownish) surrounded by small acinar structures. **B**: Higher magnification of small intracinar tubules (arrows) immunostained by CK19. A transversally oriented duct surrounded by thick fibrotic tissue (asterisks) is also documented.
(Scale bar: A= 100 μm, B= 50 μm)
C: Bar graph illustrating the numerical density of $CK19^{pos}$ ducts with different size from both diabetic and non diabetic patients. **Small**= intracinar ducts; **Large**= interlobular ducts.

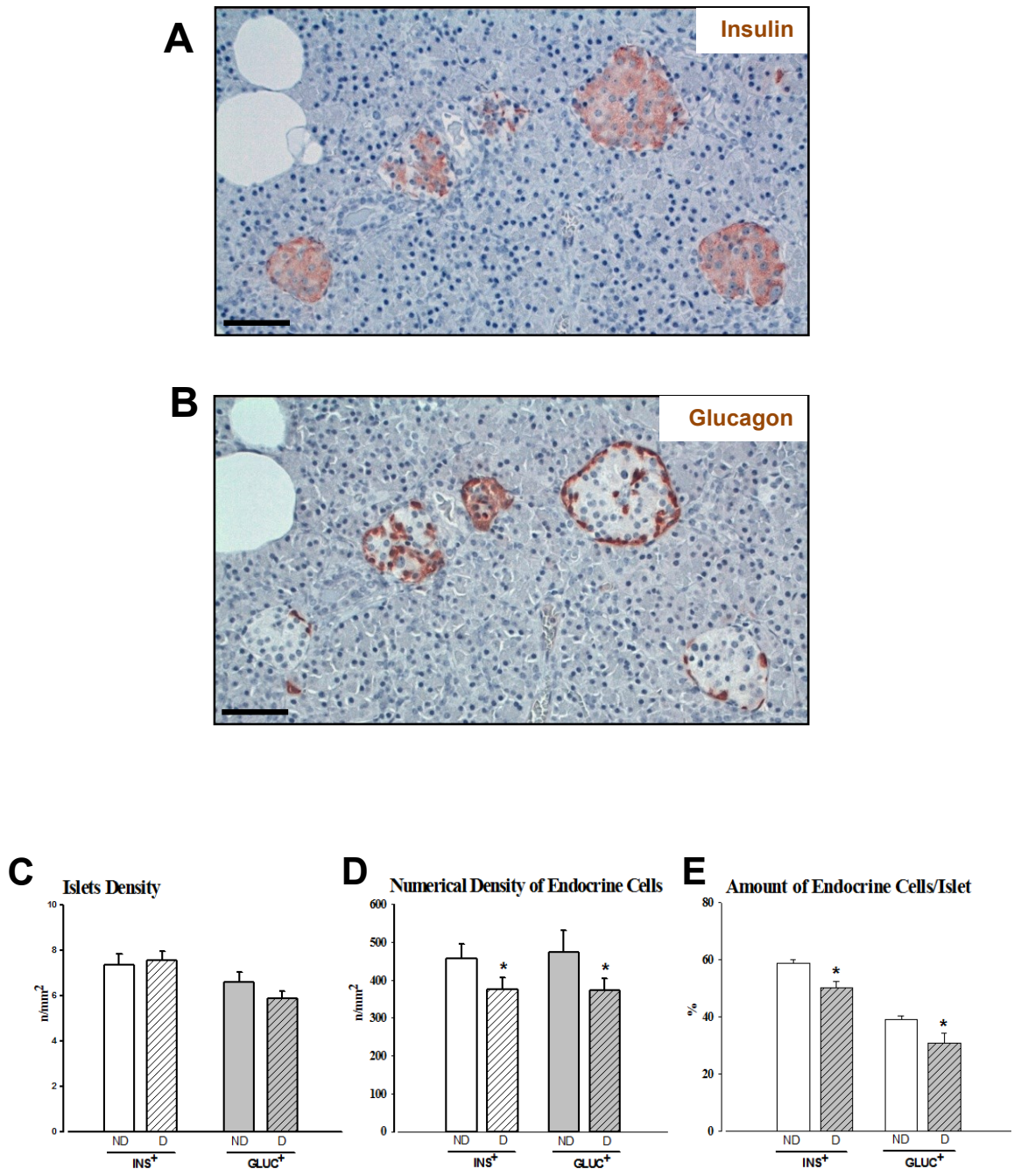


Figure 4. Quantitative evaluation of endocrine islets. Serial sections of human pancreatic tissue from a control case illustrating on the same microscopic field by immunoperoxidase (brownish), insulin^{pos} (**A**) and glucagon^{pos} (**B**) cells within pancreatic islets. (Scale bars= 50µm)
Bar graphs: C: the density of glucagon^{pos} islets was only slightly decreased (n.s.) in diabetic samples. **D**: a significant reduction in both insulin^{pos} and glucagon^{pos} cells density is documented in diabetic pancreas . **E**: quantification of the fraction of endocrine cells within the insulaes. Diabetes reduces the fraction of both insulin^{pos} and glucagon^{pos} cells.
(* = $p<0.05$ vs Non Diabetics, ND).

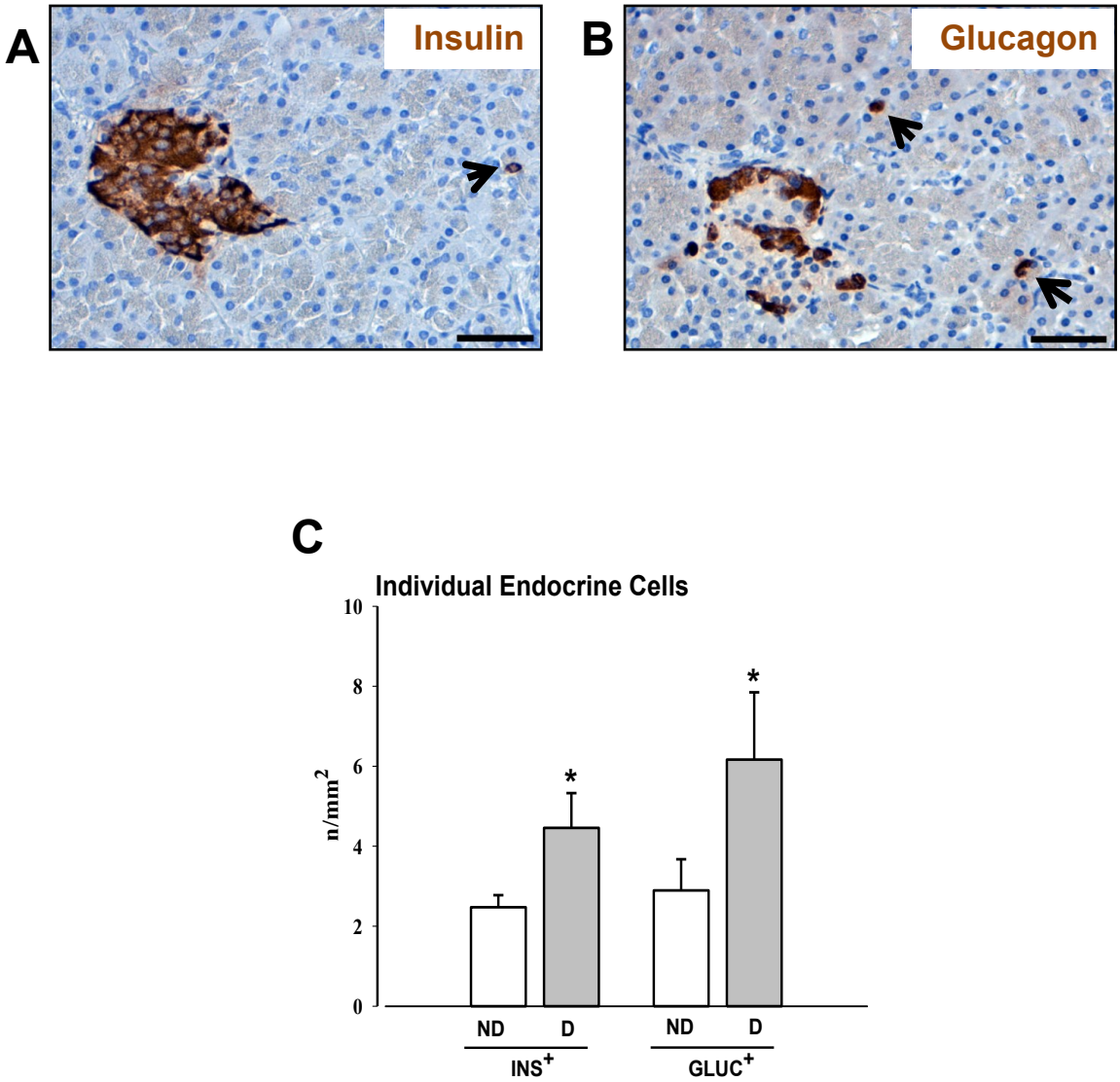


Figure 5. Individual Endocrine Cells. Sections of human pancreatic tissue illustrating insulin^{pos} (**A**) and glucagon^{pos} (**B**) insulaes and respective individual endocrine cells (arrows) recognized by the brownish staining of immunoperoxidase. (Scale bars= 50µm)
C: histogram shows a significant increase in individual insulin^{pos} and glucagon^{pos} cells in diabetes compared to control samples. (* = $p<0.05$ vs Non Diabetics, ND).

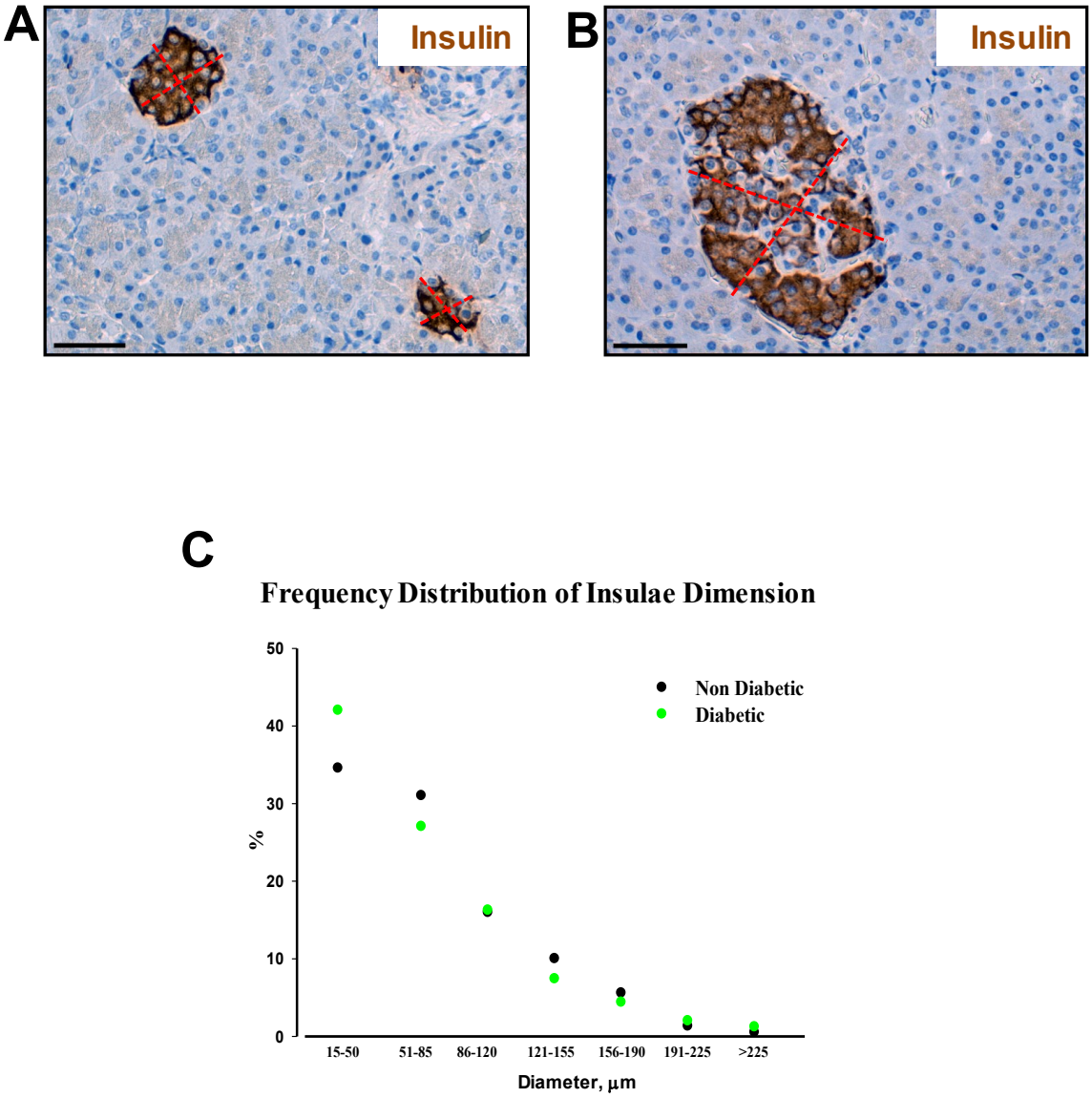


Figure 6. Size Distribution of Insulae. **A** and **B**: Representative images of Langerhans islets differing for dimension recognized by insulin antibody. Islets dimension is computed by measuring the geometric mean of longitudinal and transverse diameters, as shown in panels by red dotted lines. (Scale bar=50µm)
C: Quantitative evaluation of the size distribution of islets in the two studied groups, expressed as class frequency. An increased incidence of small islets in diabetic samples was observed, while no difference in medium and large islets is detected.

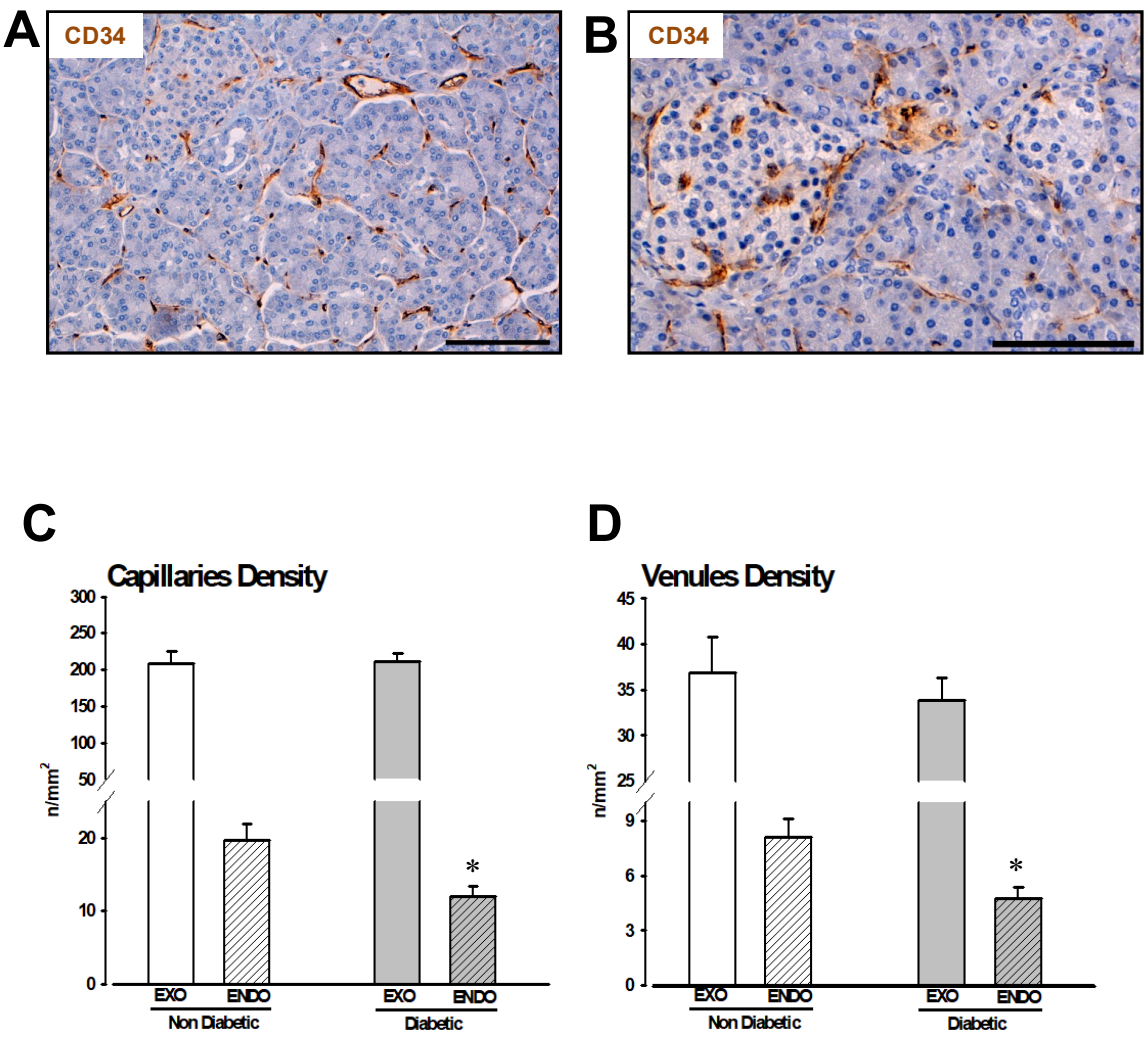


Figure7. Distribution of Vascular Structures in Human Pancreas. Microscopic images obtained from histologic sections of pancreatic tissue showing by immunoperoxidase the expression of $CD34^{pos}$ capillaries and venules in parenchymal (**A**) and insular (**B**) location.
(Optical microscopy, O.M., Panel A, B scale bar= 100 μm)
Bar graphs showing vessels distribution in exocrine (**EXO**) and insular (**ENDO**) parenchyma. The quantitative estimation in the overall population documented that, compared to non diabetic, the incidence of capillary (**C**) and venules (**D**) significantly decreased only in insular portion of diabetic pancreas.
(*=$p<0.05$ vs Non Diabetic, ND)

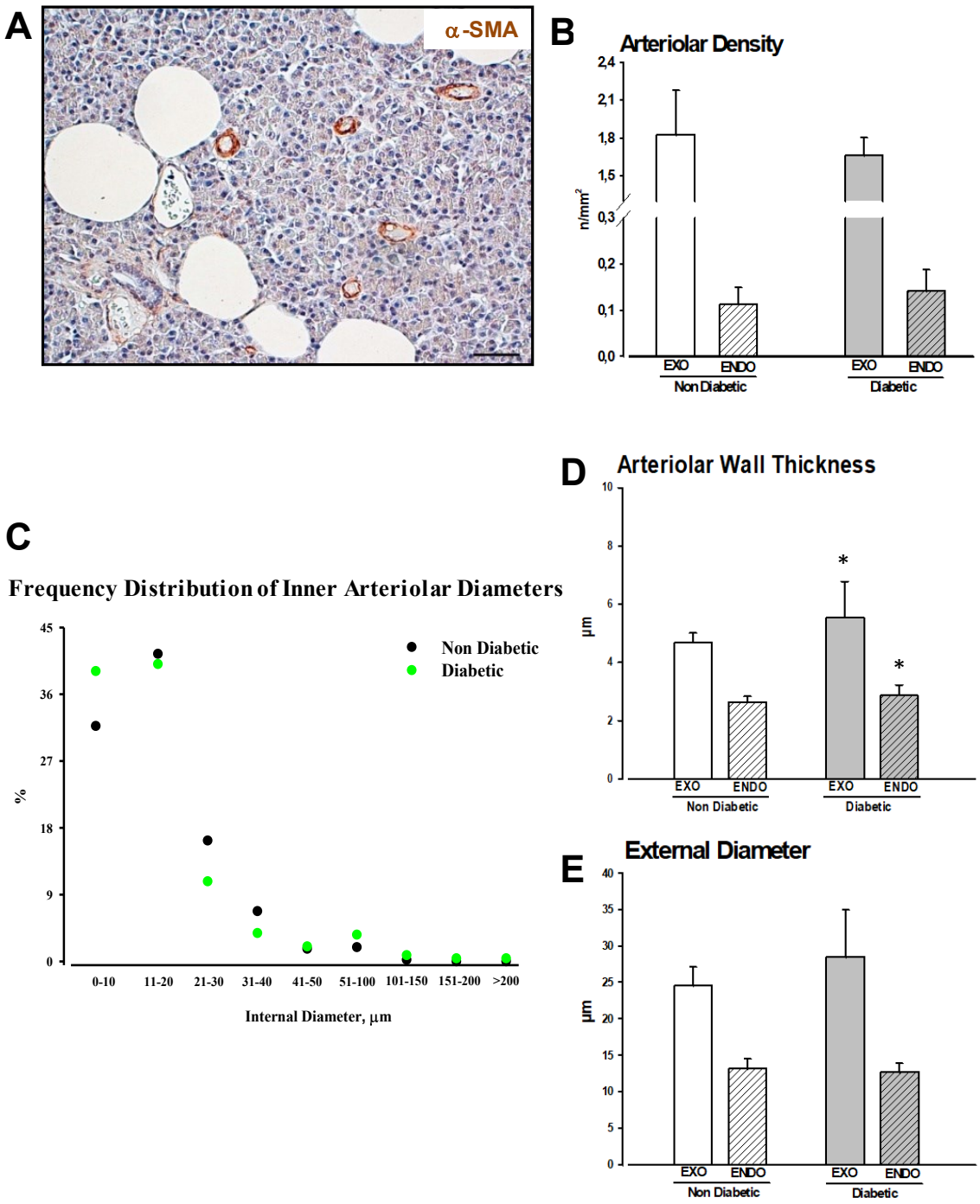
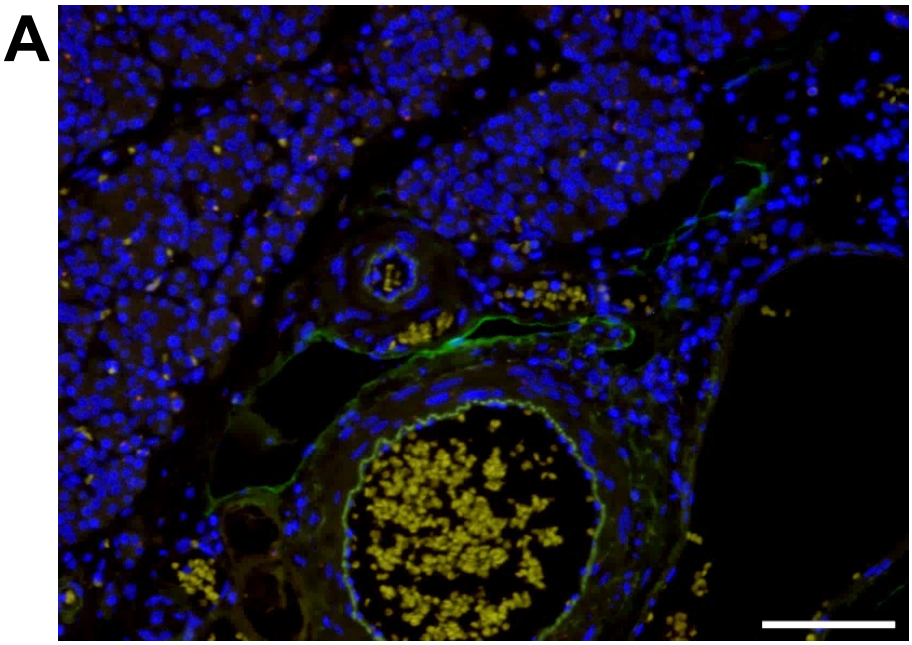


Figure 8. Arteriolar Network. **A**: Microscopic images obtained from a histological section of pancreatic tissue showing arteriolar profiles by the expression of α-SMA^{pos} recognized (brownish). (Scale bar= 50 µm)
B: Bar graph showing arteriolar density in exocrine (**EXO**) and insular (**ENDO**) parenchyma of pancreatic tissues obtained from diabetic and non diabetic patients.
C: Scatter plot graph showing the size distribution of arteriolar internal diameters according to nine classes of frequency in pancreatic tissues from Non Diabetic (Black Plot) and Diabetic (Green Plot) patients. **D**: Bar graph showing arteriolar wall thickness measured in EXO and ENDO portion of non diabetic and diabetic samples. **E**: bar graph documenting arteriolar external diameters in EXO and ENDO portions of non diabetic and diabetic pancreas. (*=$p<0.05$ vs Non Diabetic)



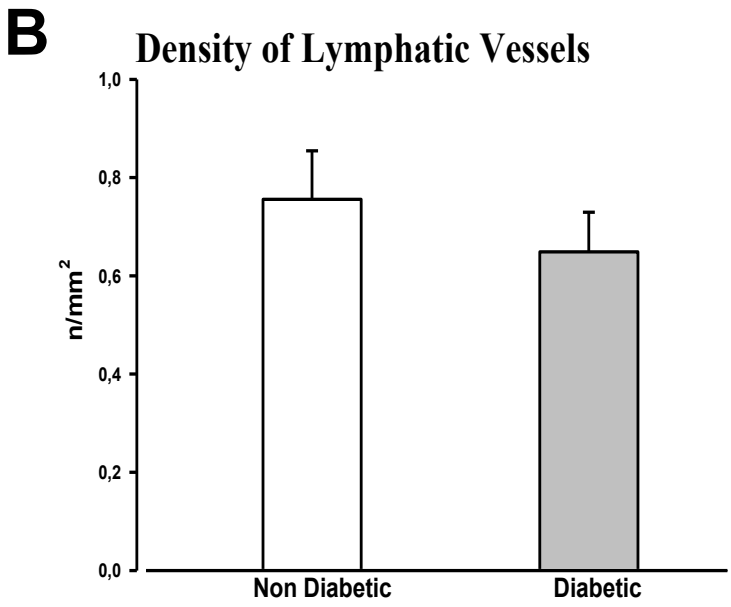


Figura 9. Lymphatic Vessels in Human Pancreas. **A:** Sections of human pancreas immunostained with D2-40 antibodies (green) to detect lymphatic vessels. Nuclei are shown by the blue fluorescence of DAPI. Yellowish fluorescence corresponds to autofluorescence of red blood cells. (Fluorescence microscopy, Scale bar: 100 μm)
B: Bar graph illustrating the quantification of lymphatic vessels in Non Diabetic and Diabetic pancreas. Diabetes was associated with a slight reduction (n.s.) of lymphatic vessels density.

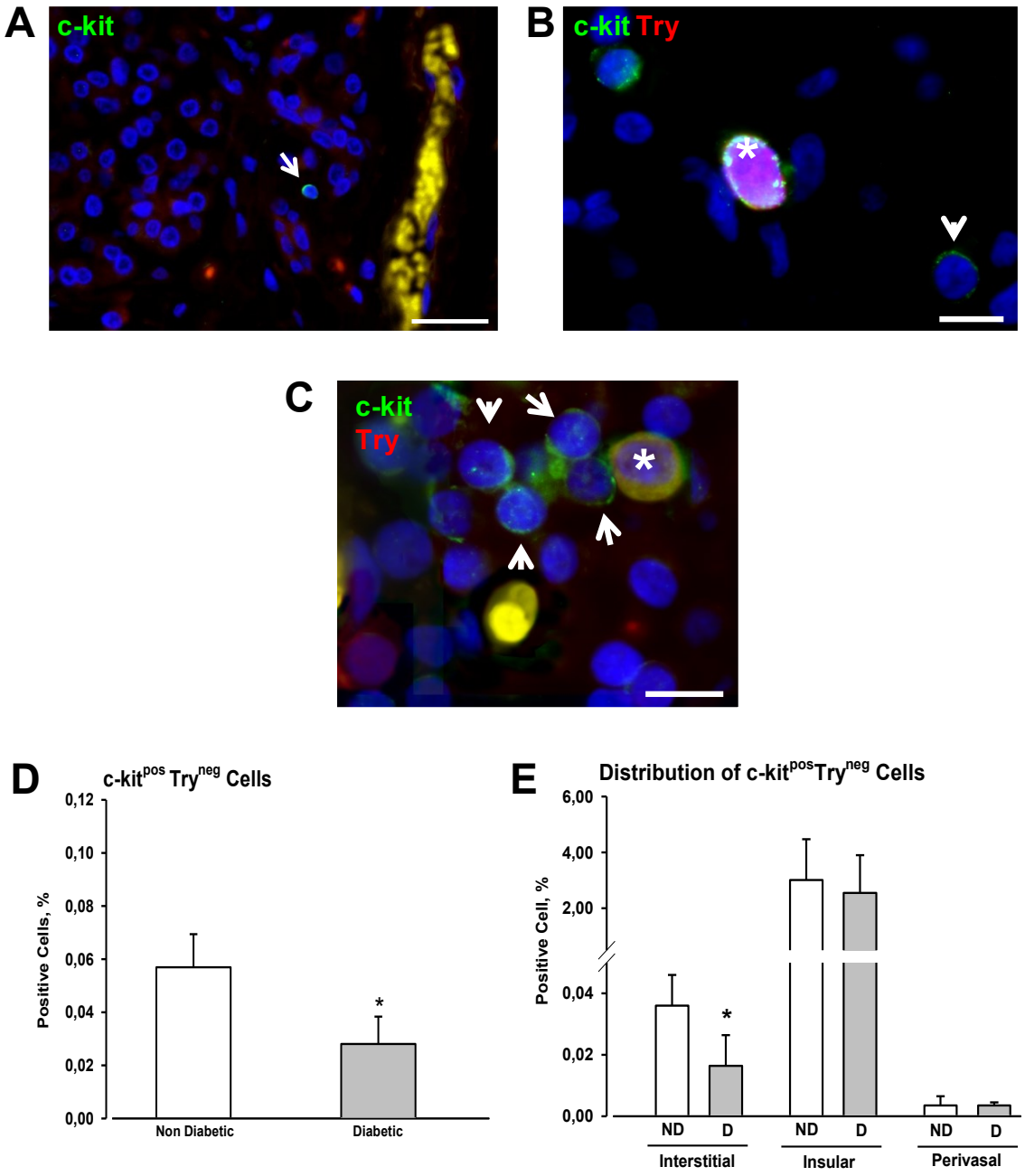


Figura 10. Quantitative Analysis of c-kit^{pos}Try^{neg} Cells. **A**: Representative image of c-kit surface expression (green) in a small cell located in proximity of a blood vessel.
(Scale bar=50µm).
c-kit^{pos} cells (green, white arrows) are present as individual element (**B**) or nested in cluster (**C**) and are negative for Tryptase (Try) (red fluorescence). Mast cells (asterisks) are recognized by the yellowish fluorescence corresponding to the co-expression of c-kit (green) and Tryptase (red). Nuclei are counterstained by DAPI (blue). Yellowish autofluorescence of red blood cells is apparent. (Scale bars: B=10µm; C=30µm).
D: Bar graph of the quantification of c-kit^{pos}Try^{neg} cells in human pancreas, documenting a reduced incidence in diabetic samples. **E**: Bar graph illustrating the distribution of c-kit^{pos}Try^{neg} cells according to the different localization within the pancreatic tissue. c-kit^{pos}Try^{neg} cells are mainly distributed in insulae. Interestingly, a significant reduction in the fraction of c-kit^{pos}Try^{neg} cells in interstitial space was documented in diabetic samples.
(*=$p<0.05$ vs Non Diabetic).

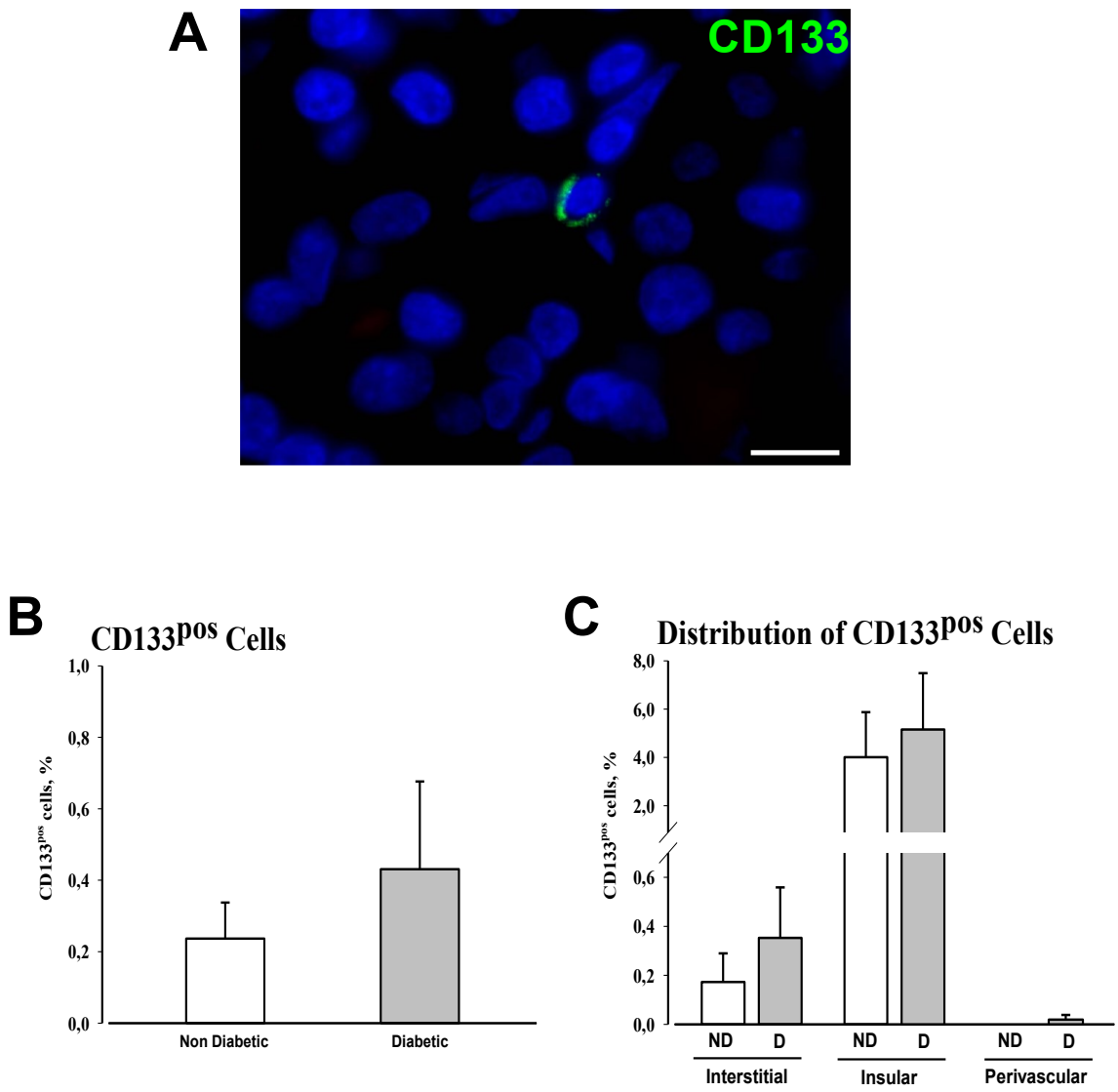


Figura 11. Quantitative Analysis of CD133 (prominin) in Human Pancreas.
A: Microphotograph showing an example of a CD133^{pos} cell (green) by immunofluorescence in human pancreas. Nuclei are counterstained by DAPI (blue).
(Fluorescence Microscopy, Scale bar=10µm).
B: Quantitative analysis of CD133^{pos} cell documenting a trend to increase in diabetic samples (n.s.).
C: Bar graph illustrates CD133^{pos} cells distribution in interstitial, insular and perivascular portions of Non Diabetic and Diabetic pancreatic tissue. Similarly to c-kit^{pos} cells, CD133^{pos} cells were more represented in insular area.

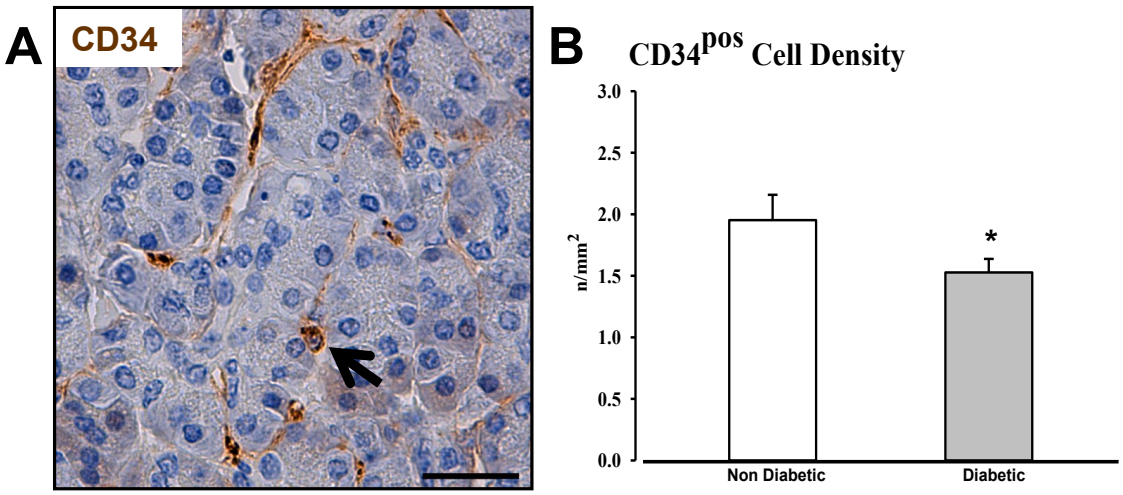


Figura 12. Endothelial Progenitor Cells. **A**: Representative image of the immunohistochemical detection of CD34 (brownish) in human pancreas. An individual $CD34^{pos}$ cell in proximity of a CD34 labelled microvascular lumen is recognized (arrow).
(Optical Microscopy, Scale bar=40μm).
B: Bar graph documenting a reduction of $CD34^{pos}$ cells density in diabetic pancreas.
(*=$p<0.05$ vs Non Diabetic).

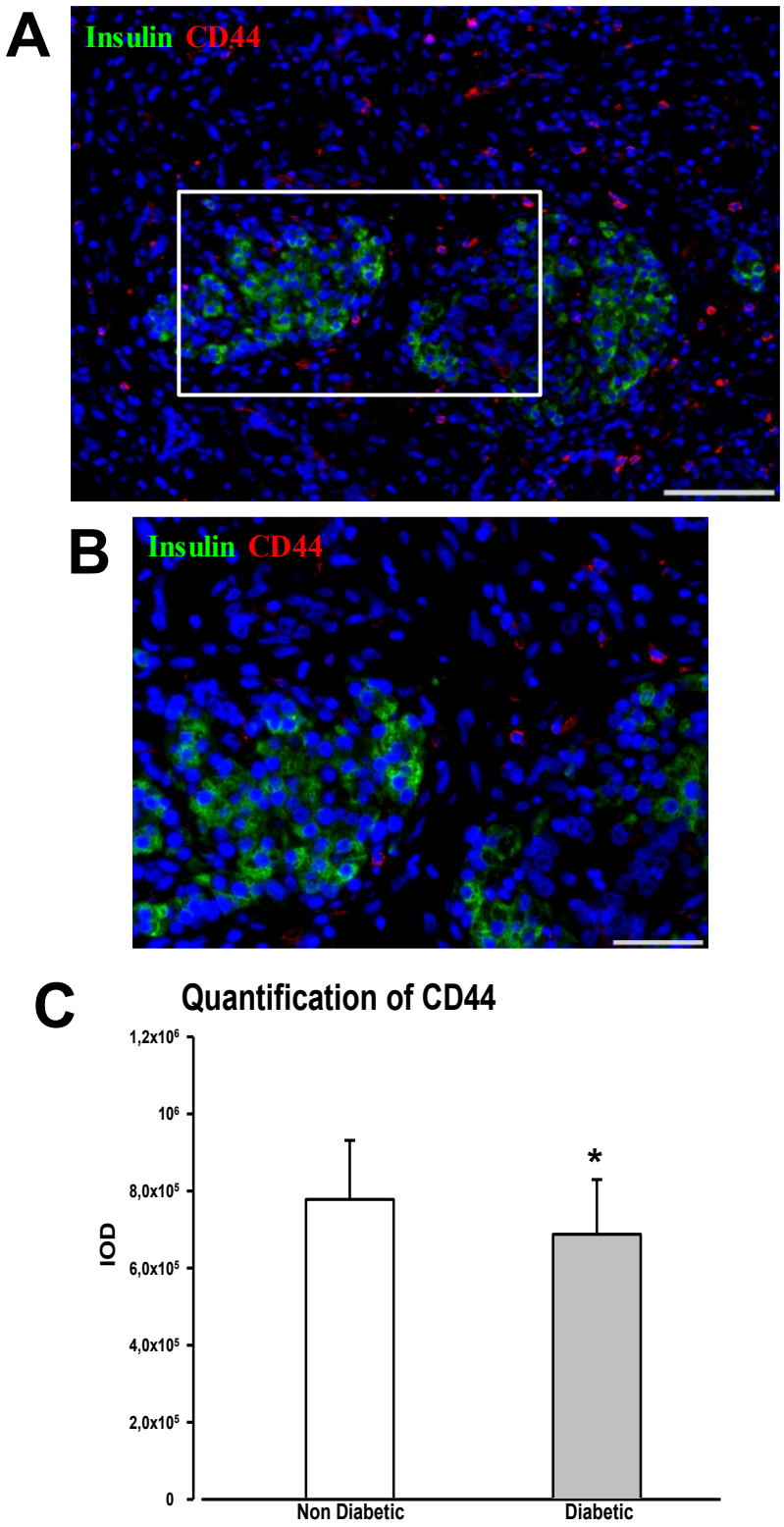


Figure 13. CD44 Distribution. **A** and **B**: Microphotographs of pancreatic samples showing the expression of CD44 (red) and Insulin (green) revealed by immunofluorescence. Nuclei are shown by the blue fluorescence of DAPI. The expression of CD44 in cells surrounding insulin labeled islets is documented. The white rectangle inscribes an area shown at higher magnification in **B**. (Fluorescence Microscopy, Scale bar, A=100µm; B= 50µm)
C: Bar graph showing the significant reduction of $CD44^{pos}$ cells in samples from diabetic patients. (*= $p<0.05$ vs Non Diabetic).

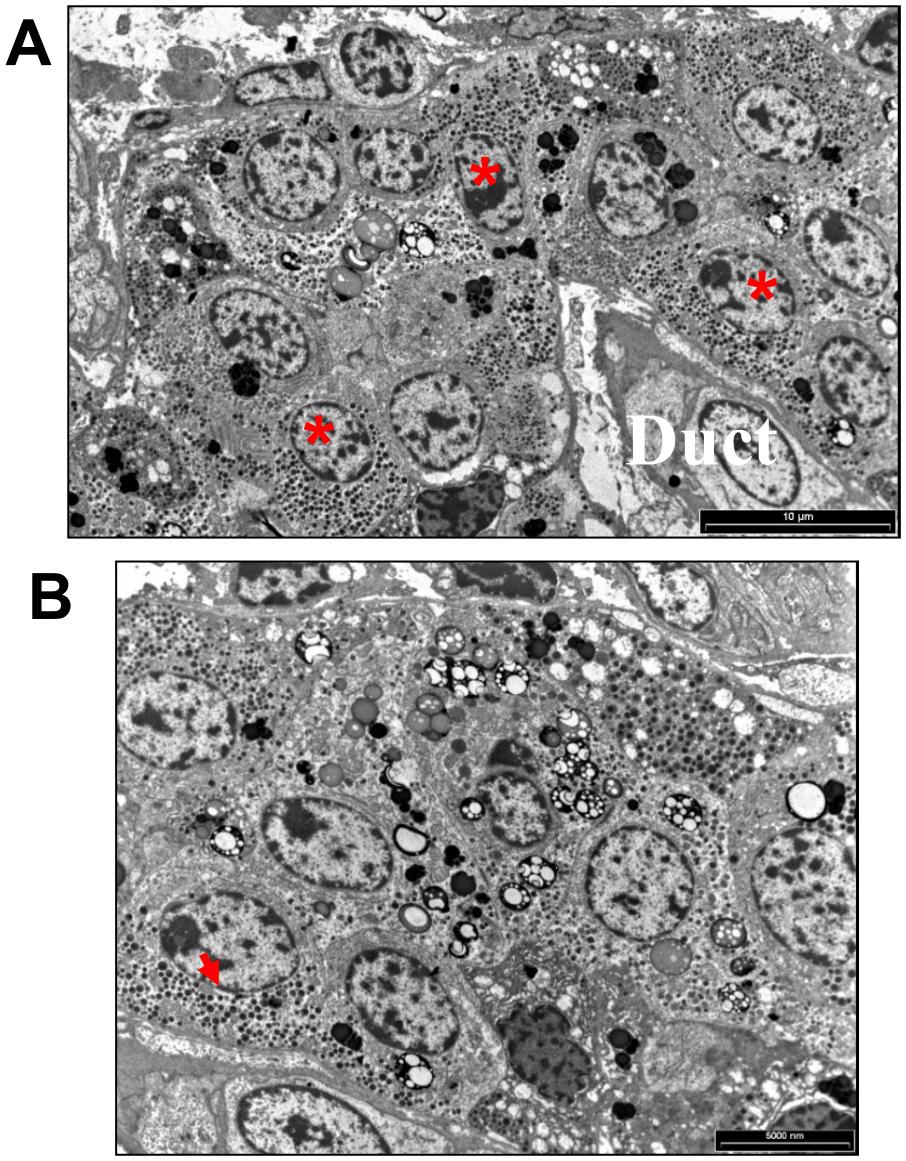


Figure 14. Ultrastructural Analysis of Human Pancreas. TEM images illustrating the ultrastructural characteristics of an islet of Langerhans in human pancreas.
A: Low magnification TEM image illustrating a pancreatic insula composed by different endocrine cells, including α-granules (*) carrying cells and a centrally located ductule-like structure (Duct).
B: α-cells filled with glucagon (arrow) are shown at higher magnification.
(Scale Bars: A: 10 µm; B: 5 µm).

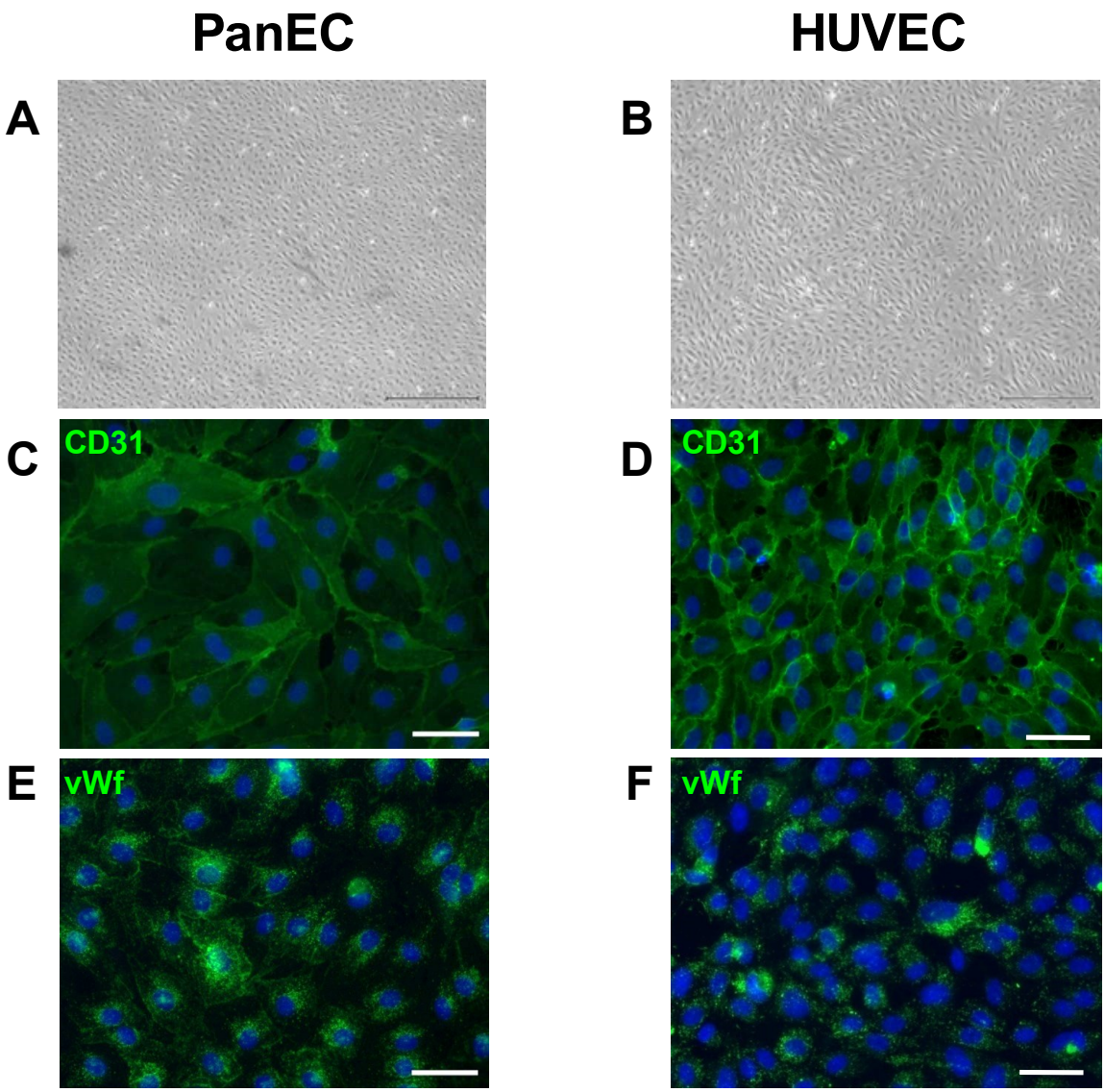


Figura 15. Morphology and Immunocytochemistry of Endhotelial Cells. Human pancreas-derived endothelial cells (PanECs) and human umbilical vein endothelial cells (HUVECs) were cultured and expanded in EGM-MV plus 5% FBS. Confluent monolayer with cobblestone-like morphology (**A** and **B**) suggest the purity of primary culture according to morphologic criteria and ensured by immunocytochemical detection of the pan-endothelial marker CD31 (green) (**C** and **D**) and von Willebrand factor (green) (**E** and **F**). Nuclear counterstaining by DAPI (blue).
(Scale bars, A and B= 500μm; C – F= 50μm).

Human Pancreatic Endothelial Cells (PanEC)

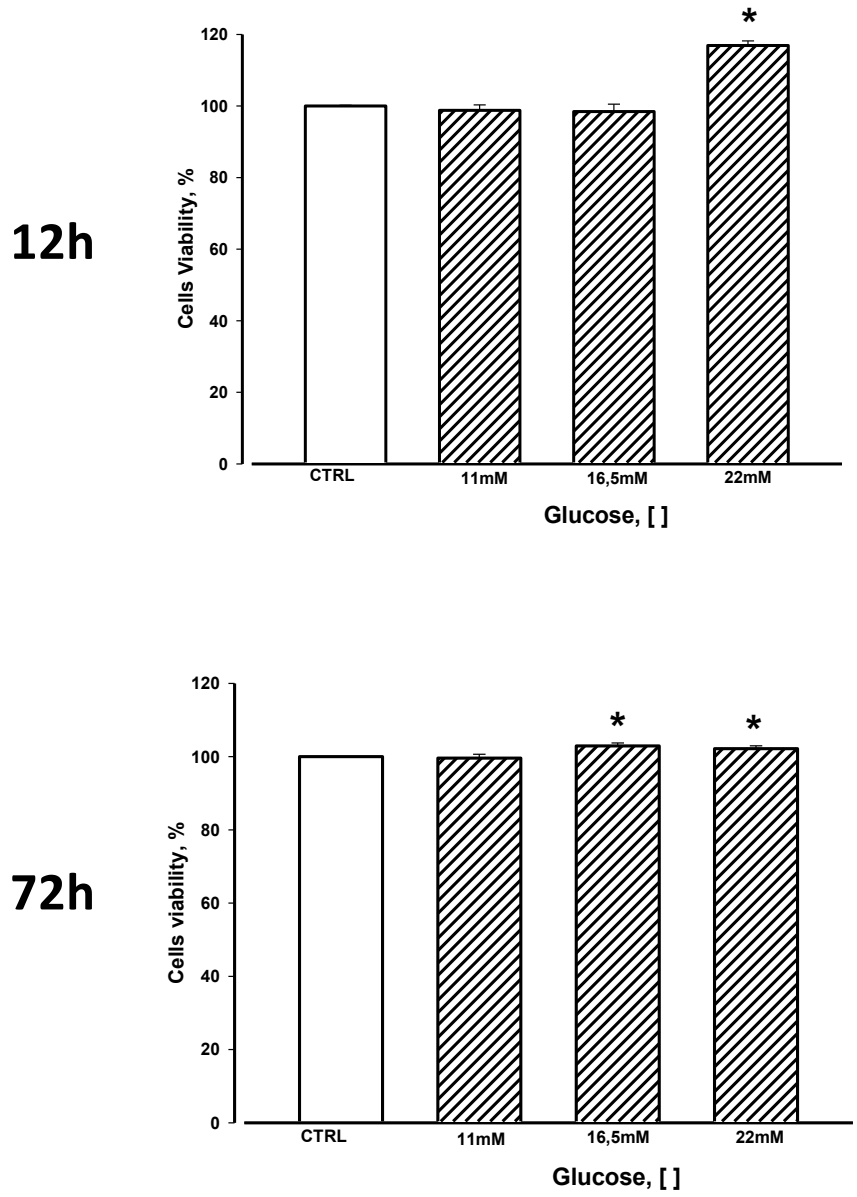
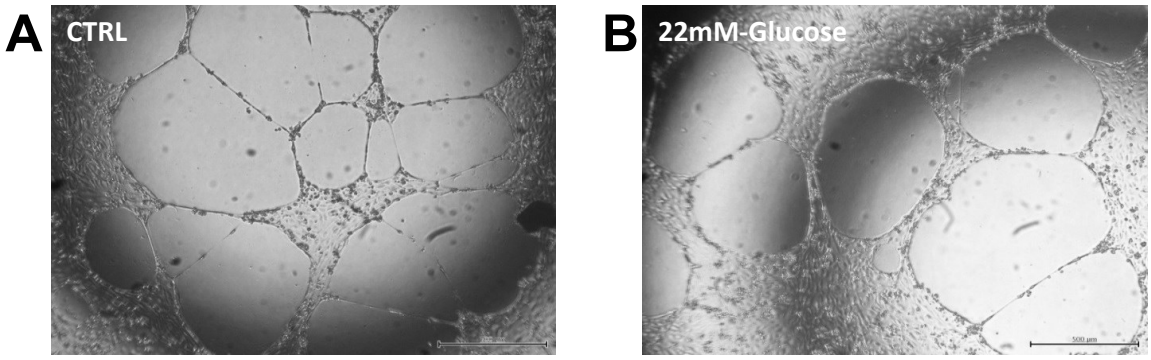


Figure 16. Cell Viability Assay. Representative histograms from MTT assays on PanECs treated with 11, 16.5 and 22mM glucose for 12 and 72 hours**.** CTRL correspond to normoglycemic condition (5.5mM-Glucose). As shown, PanECs increased proliferation in response to hyperglycemia, and to a more extent at early time point.
(* $p<0.05$ vs CTRL)



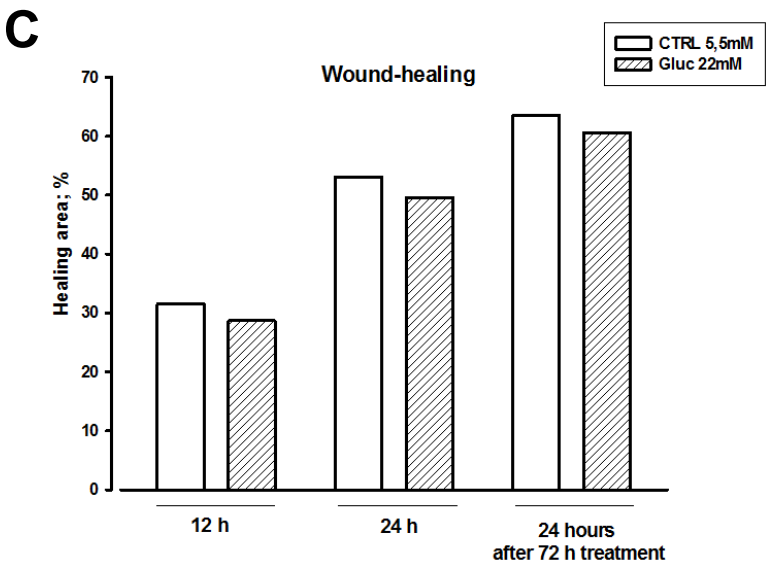


Figure 17. Angiogenesis Assay. A: The ability to organize into tubule-like networks on Matrigel was evaluated after 24 hours. Inhibition of tubular formation by EC lines was more evident following 22mM glucose concentration (**B**). Representative images of three independent experiments.
(Scale bars, A and B= 500µm).

Wound-healing Assay. C: Capacity of migration was evaluated on confluent monolayer of PanECs after 22mM glucose treatment for 12, 24, and 72 hours. A cell-based scratch assay followed by the quantification of the wounded area was performed. Results represent three independent experiments.

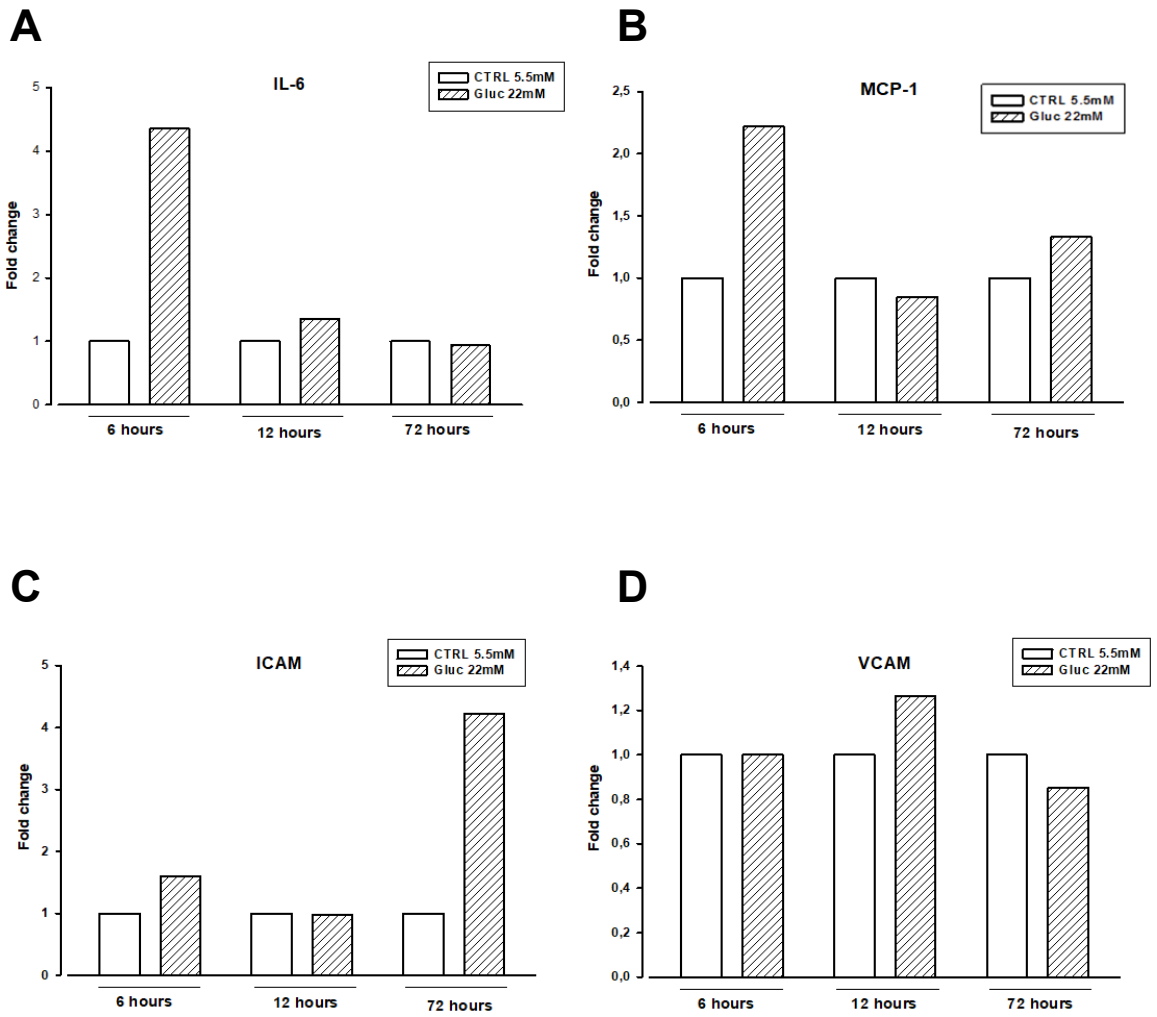


Figure 18. Real Time PCR Analysis. Gene expression of pro-inflammatory markers after 6 and 12h of hyperglycemia was performed, in order to quantify IL-6, MCP-1 and adhesion molecules ICAM and VCAM. Preliminary data refer to a single analysis. Data were expressed as fold-change vs CTRL values. At early time, PanECs showed an increased expression of IL-6 and MCP-1 in 22mM glucose concentration compared to normoglycemic condition (Panel **A** and **B**). Conversely, the expression of ICAM and VCAM showed an opposite trend. (Panel **C** and **D**).

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