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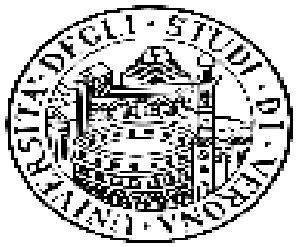

Doctoral program in Medical, Clinical and Experimental Science
XXIV COURSE

Pancreatic beta cells turnover: effects of endogenous hyperinsulinemia and amyloid deposits

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ABSTRACT

OBJECTIVES: The research activity focused on two research projects.

In the first project, we investigated the effects of high local insulin levels on beta cell turnover in a model of endogenous hyperinsulinism.

In the second project, we investigated the presence and quantity of amyloid deposits and their role on beta cell death in type 2 diabetes and diabetes secondary to pancreatic diseases.

BACKGROUND:

Insulin therapy has been suggested to preserve beta-cell mass in patients with diabetes through the mechanisms of beta-cell rest as well as direct effects on beta-cell proliferation. However, data about the effects of hyperinsulinism on beta-cell mass and turnover in humans are sparse.

Amyloid aggregates have been suggested to be involved in the pathogenesis of type 2 diabetes, causing beta cell death. Data present in literature make IAPP role in type 2 diabetes controversial.

PATIENTS AND METHODS:

First research project: pancreatic tissue specimens from 5 patients with pancreatic insulinomas and 10 non diabetic control subjects were examined. Pancreatic sections were stained for insulin, Ki67 (replication) and TUNEL (apoptosis), and quantitative morphometric analyses were performed.

Second research project: pancreatic tissues from 7 patients with type 2 diabetes, 10 patients with diabetes secondary to chronic

pancreatitis or pancreatic carcinoma and 11 non diabetic control subjects were examined. Pancreatic sections were stained for insulin, TUNEL (apoptosis) and Thioflavin S (amyloid) and morphometric analysis were performed.

RESULTS:

First research project: fractional beta-cell area was 1.11%±0.67% in the tumor-free pancreatic tissue of the insulinoma patients and 0.78% ±0.26% in the control group (p=0.19). There also were no differences in islet size (p=0.62) or beta-cell nuclear diameter (p=0.20). Beta-cell replication and apoptosis were infrequently detected, without any measurable differences between the groups. There were also no differences in percentage of duct cells expressing insulin (p=0.47), a surrogate marker for islet neogenesis.

Second research project: fractional beta cell area was 0.33% ± 0.07% in the pancreatic tissue of patients with diabetes secondary to pancreatic diseases, 0.57% ± 0.09% in patients with type 2 diabetes and 0.76% ± 0.13% in the control group (p=0.021). There were no differences in beta cell apoptosis (p=0.10) and extent of islet amyloid (p=0.13) or islet amyloid area (p=0.24)

CONCLUSIONS:

Beta-cell area and turnover are not significantly altered in the proximity of intra-pancreatic insulinomas. Future in vivo studies, ideally employing larger animal models, are warranted to further evaluate the impact of exogenous insulin on beta-cell turnover.

A significant difference in beta cell area but no differences in beta

cell apoptosis and amyloid deposition were found between type 2 diabetic patients, patients with diabetes secondary to chronic pancreatitis or pancreatic carcinoma and control subjects. Amyloid deposition is not a cause of beta cell death in type 2 diabetes. Future larger studies, involving also appropriate animal models and sophisticated techniques, could be useful to better understand the aggregation mechanisms of IAPP and its role in beta cell death.

1. INTRODUCTION

1.1 Pancreatic beta cells

Beta cells are the most diffuse endocrine cells in the Langerhans islets, roundly-oval shaped cell aggregates, highly vascularized, spread in the pancreas. Islet cells are organized in cords among a network of blood vessels, in which blood flows from the core to the islet's periphery (1). In an adult, total Langerhans islets weight is about 1-1,5 g and the size of every islet can range from less than 100 cells or 50 μm in diameter to more than 5.000 cells or 500 μm in diameter (1).

The pancreatic islets consist of four endocrine cell types:

-beta cells: with a multifaceted shape and clustered all around the blood vessels mainly in the core of the islet; they represent approximately 60-80% of the endocrine cells and secrete insulin.

-alfa cells: with a cylindrical shape and dispersed throughout the islet; they are smaller than beta cells, represent approximately 10-15% of the endocrine cells and secrete glucagon.

-delta cells: dispersed in the islet like alfa cells; they represent approximately 5-10% of the endocrine cells and secrete somatostatin.

-pp cells: localized in the periphery of the islet; they represent approximately 3-5% of the endocrine cells and secrete pancreatic polypeptide.

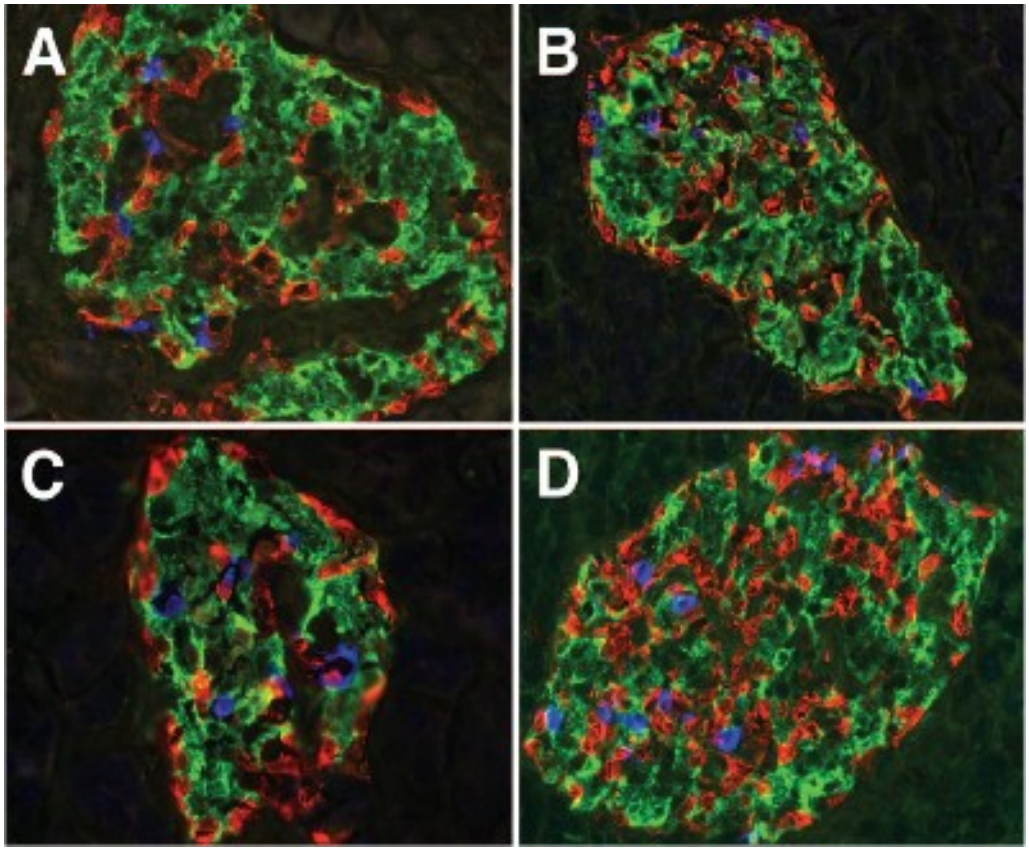


Figure 1: Histology of human pancreas. Histological sections of human pancreas are stained for islet hormones. Micrographs of four different human islets show the intermingling of β and non-β cells. (A–D) Magnification X40; β cells, green; α cells, red; δ cells, blue. *(Brissova M et al. J Histochem Cytochem 2005; 53, 9: 1087-1097)*.

In a mean size islet (150 μm in diameter) there are 1.000-1.500 beta cells and each of them has more than 10.000 granules of insulin (2).

The granules have a diameter of about 0,25-0,40 μm and can be of two different types: those mature, uniformly distributed in the cytoplasm with a rectangular crystalline electrondense midpoint and those immature, more clear and close to the Golgi apparatus forming a roundish mass sorrounded by a clathrin membrane. Pro-insulin is synthesized in the rough endoplasmic reticulum (RER) and packaged into immature secretory granules in the trans-Golgi network, a tubular-reticular structure

associated with the trans face of the Golgi, together with proinsulin conversion enzymes. When granules mature, the clathrin coat is lost and the intragranular environment becomes acid and proinsulin is converted to insulin and C-peptide. The insulin is stored into the granules as crystals with Zn^{2+} (3).

In addition to the genes for insulin (insulin-1 and insulin-2), there are other two markers characteristic of beta cells: IAPP (islet amyloid polipeptide) gene, encoding a peptidic hormone secreted together with insulin and GLUT-2 gene, encoding the glucose transporter of beta cell.

Glucose is the most important stimulus for insulin secretion; other stimuli are represented by nutrients, hormones, neurotransmitters and drugs (4, 5).

After entry into the beta cell through the uniporter GLUT 2, glucose is phosphorylated by the glucokinase enzyme and goes into the glycolysis reaction. Pyruvate, the end product of glycolysis, enters the mitochondria and goes into the citric acid cycle and, through the following steps in the mitochondrial respiratory chain, there is production of ATP. The increase in the cellular ATP/ADP ratio induces the closure of the ATP-dependent K^+ channels on the cell membrane, then, leading to membrane depolarization and activation of voltage-dependent Ca^{2+} channels that makes cytosolic [Ca^{2+}] increase (6-8). Elevated cytosolic [Ca^{2+}] leads to the stimulation of insulin exocytosis (9, 10).

But the Ca^{2+} signal alone is not sufficient for a sustained secretion because under clamped cytosolic [Ca^{2+}], glucose can still stimulate insulin secretion (11), suggesting the existance of mitochondrial messengers,

different from ATP, involved in this mechanism (12). These messengers can be glutamate, malonyl CoA, long-chain acyl CoAs (LC-CoA), and NADPH.

Glutamate is formed from α-ketoglutarate, an intermediate in the citric acid cycle, by mithocondrial enzyme glutamate dehydrogenase. It has been shown that glutamate directly stimulates insulin exocytosis in permeabilized cells, under conditions of fixed cytosolic [Ca^{2+}] in a mechanism, prabably, involving the reduction of granular membrane potential or the Ca^{2+} uptake into the granules (13, 14) or the granules swelling and their fusion with the plasma membrane (15).

Long-chain acyl CoAs (LC-acyl CoAs) are involved in the insulin secretion through the link between glucose stimulated insulin secretion (GSIS) and lipid synthesis (16, 17). After exposure of pancreatic beta cells to glucose, malonyl CoA content rises, leading to an inhibition of fatty acids oxidation and increase in lipid synthesis. Elevated levels of LC-acyl CoAs, derived from glucose metabolism as well as endogenous or exogenous lipids, can stimulate insulin secretion through an increased fusion of secretory granules with the beta cell plasma membrane in a mechanism involving the acylation or binding to specific proteins like SNARE or VAMP, important for insulin exocytosis (18).

NADPH can be generated in several reactions in the cell and also from pyruvate in the pyruvate-malate shuttle system across the mitochondrial inner membrane. Increased intracellular NADPH/$NADP^+$ ratio extends the inactivation of voltage dependent K^+ current by ATP-dependent K^+ channels on the cell membrane, then, inhibiting the

repolarisation of beta cell (19).

In addition to these mitochondrial molecules, there are also other substances affecting insulin secretion.

The sulfonylureas bind to a specific receptor SUR1 on the ATP-dependent K^+ channels and, directly, cause their closure with depolarization of cell membrane and insulin release from beta cell granules through an increase of intracellular [Ca^{2+}] (8).

Aminoacid arginine enters the beta cell and, because of its negative charge, causes cell membrane depolarization and, then, activation of voltage-dependent Ca^{2+} channels with the consequent increase in cytosolic [Ca^{2+}] and insulin secretion (20).

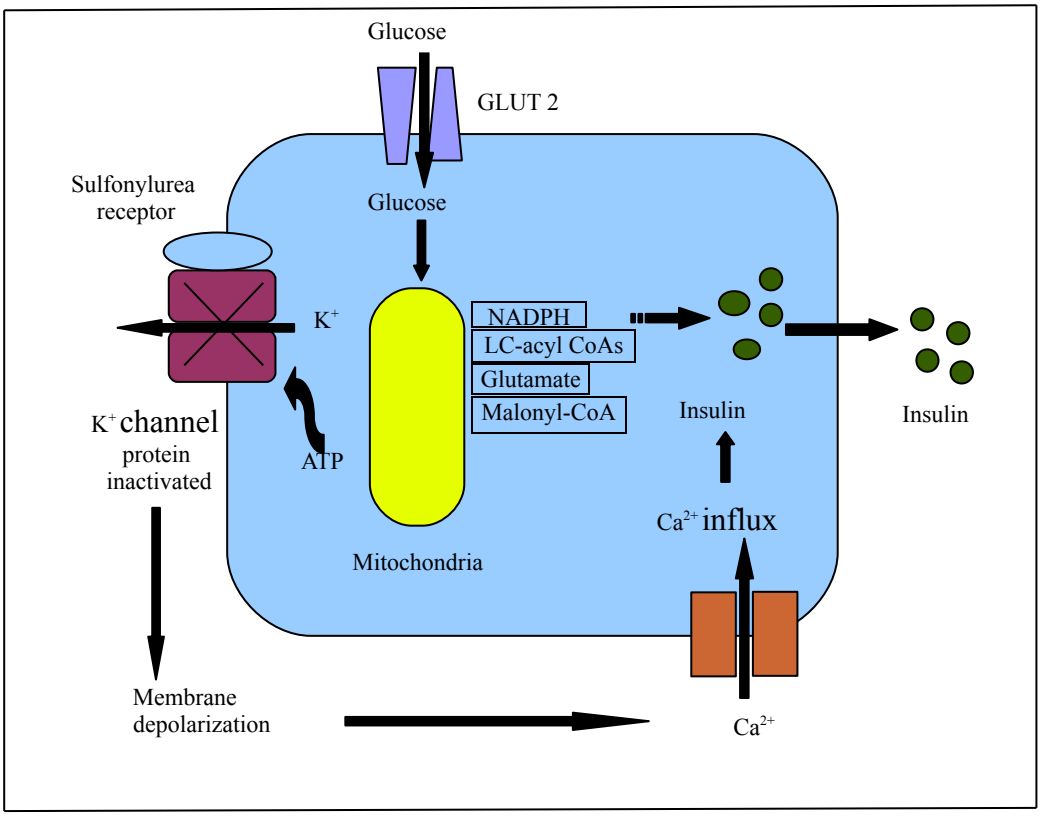


Figure 2: Beta cell insulin secretion.

The insulin secretion has a biphasic nature: the first phase is rapid, transient and lasts about 5-10 minutes while the second one is slow and sustained. The rate of insulin secretion (expressed as percentage of islet insulin content released per minute) is 0,14%/min in the first phase while it is 0,05%/min in the second phase (21). These rates correspond to about 18 granules released per min per beta cell in the first phase and 6 granules per min per beta cell in the second phase. Then, the total number of granules secreting insulin in the first phase is about 100 per beta cell, meaning that only 10% of the total granule number in a beta cell is responsable for the first insulin release. These granules are called readily releasable pool (RRP) and are associated to the plasma membrane. In contrast, the second phase is dependent from granules that form the, so called, reserved pool that release insulin after being transported to the plasma membrane (22).

The insulin exocytosis is highly regulated by a fusion machinery composed of SNARE proteins. This machinery is formed by two groups of SNARE proteins: those associated with plasma membrane, syntaxin1 and SNAP25, and that associated with secretory granules membrane, VAMP2. VAMP2 binds to syntaxin1 and SNAP25, forming a complex that forces the two plasma and secretory granule membranes together and with the Ca^{2+} channels on the beta cell surface. When Ca^{2+} channels open, intracellular ion content increases, activating a Ca^{2+}-sensitive protein in the SNARE complex, called synaptotagmin, that leads to the fusion of the two membranes (23, 24). The intragranular pH becomes less acid triggering the dissolution of crystal insulin and its release from the beta cell.

Then, insulin secretion is a finely regulated mechanism in order to keep plasmatic glucose concentrations constant either in fast time and after meals. Anyway, an adequate insulin secretion depends not only on a functional beta cell but also on a sufficient number of cells or beta cell mass that can assure a physiological amount of plasmatic insulin.

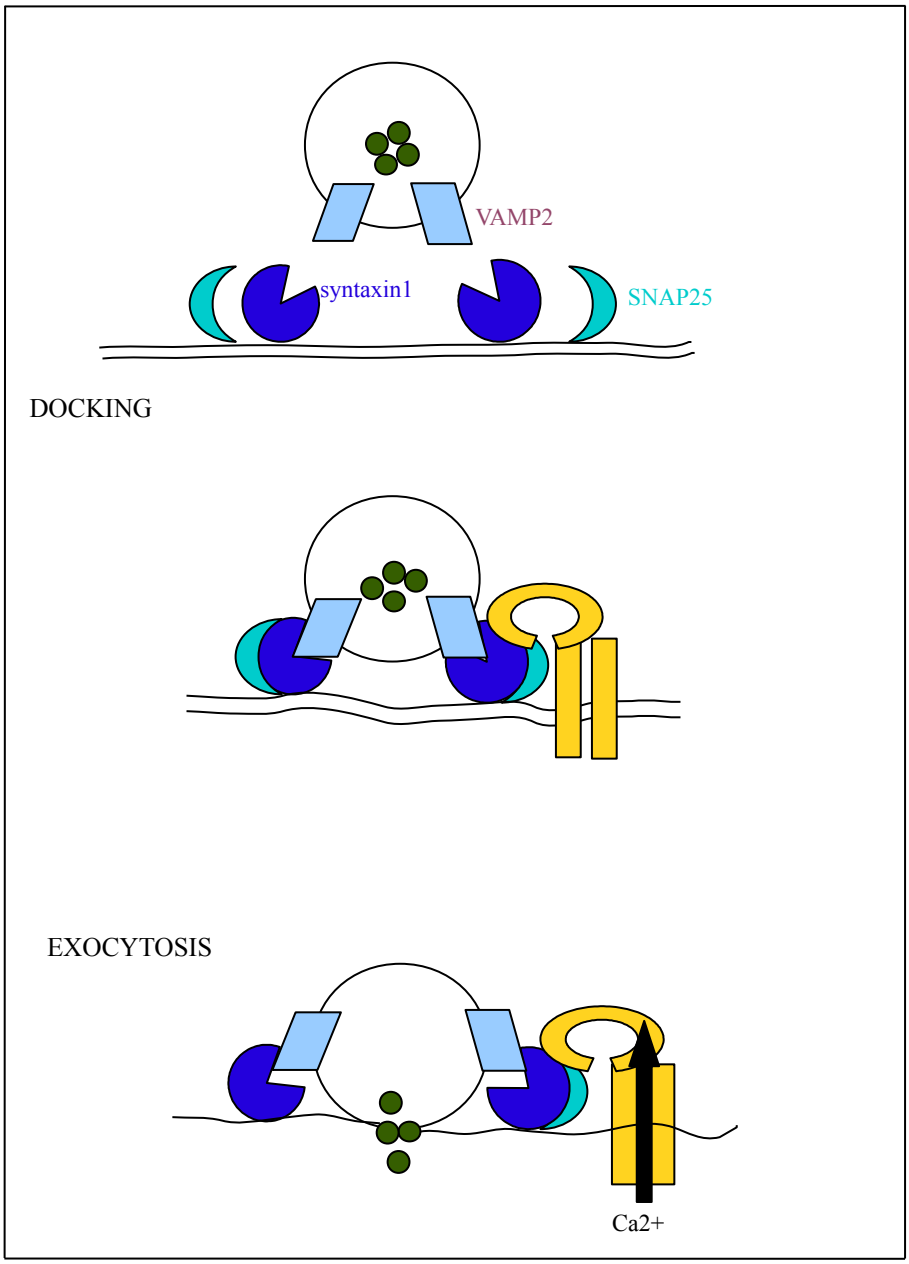


Figure 3: Insulin exocytosis involving SNARE and VAMP proteins. *(Rorsman P et al. Diabetologia 2003; 46, 8: 1029-1045).*

1.1.1 Beta cell mass

Beta cell mass is regulated by four mechanisms: beta cell size, beta cell replication, beta cell neogenesis or differentiation of precursor cells in pancreatic duct epithelium and beta cell apoptosis. While the first three processes preserve and expand the beta cell mass, the last one provokes its reduction.

The contribution of each of these mechanisms to the manteinance of beta cell mass differs in a species specific way (25). For example, it seems that beta cell replication rather than neogenesis plays a crucial role to produce new endocrine cells in young adult mice (26, 27), instead, both the processes appear to be important to increase the beta cell mass in humans (28, 29). It is plausible to think that the difference observed in beta cell replication and neogenesis can be ascribed to a different telomere lenght regulation and biology in these two species. The telomere shortening limits the replicative capacity of cells during aging in humans, while this mechanism normally lacks and is not related to replicative senescence in mice (29, 30). This phenomenon can hide the real contribution of beta cell neogenesis in forming new beta cells in mice.

Anyway, beta cell mass is a dynamic entity that can change at different stages of life and under different stimuli.

During prenatal life, pancreatic beta cells are detected in humans from gestational week 9 arising from possible precursor cells in ductal epithelium and beta cell replication occurs from that time during all stages of prenatal development. Beta cell apoptosis is also detectable in all these ages, suggesting an high level of beta cell turnover (31).

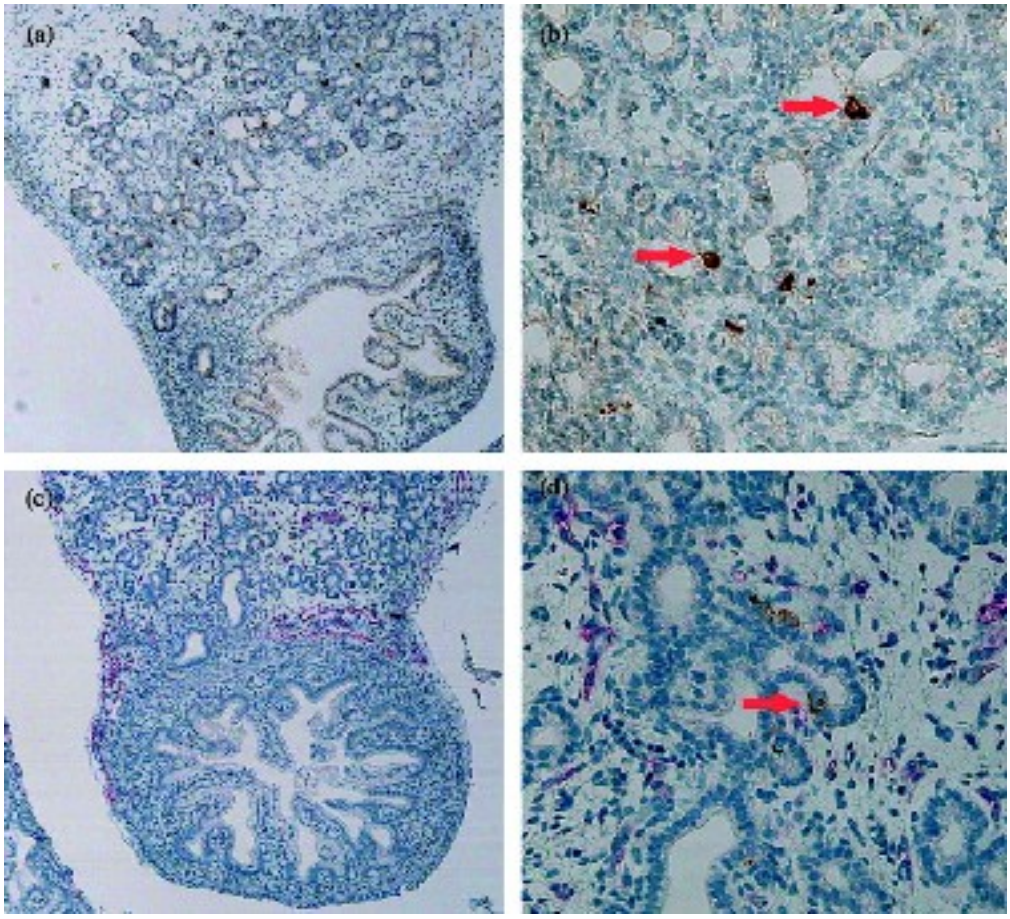


Figure 4: Images show the dorsal pancreatic evaginations from gut in two foetuses at week 8 (a and b) and at week 9 (c and d) p.c. Images were acquired at 100x (a and c) and at 400x magnification (b and d). Section were stained for glucagon (brown) (a and b) and for insulin (brown) and CD31 (red) (c and d). Red arrows point to endocrine cells next to exocrine ducts**.** *(Meier JJ et al. Eur J Endocrinol. 2010; 162, 3: 559-68).*

Based on rodent studies, it is supposed that beta cell turnover still takes place just after birth with high peaks of beta cell replication and neogenesis and a small increase in beta cell apoptosis (32).

During childhood and adult life, beta cell replication declines (33) as a result of different expression and activity of specific cell cycle regulators. Infact, cell cycle inhibitors, as the tumor suppressor protein p16 and the cyclin dependent kinase inhibitor p27, are much higher expressed in the beta cells of human adult rather than in prenatal beta cells (34). Besides

beta cell replication, also beta cell neogenesis and apoptosis decline with aging, though apoptosis can slightly increase in old years. Then, by balancing of these beta cell turnover events, beta cell mass reaches a kind of physiological level that is maintained during all adult life.

Changes from this level can be noted in some physiological and pathological conditions as pregnancy, obesity and type 2 diabetes.

During pregnancy there are complex endocrine and metabolic long term modifications in the mother body in order to respond to an increased metabolic load, then, to provide nutrients to the fetus and prepare the maternal organism to childbirth and lactation. In particular, the main adaptive changes in response to a growing need for insulin are: an increased glucose-stimulated insulin secretion with a lowered threshold for glucose-stimulated insulin secretion, an increased beta cell activity with an increased insulin synthesis and an increased glucose metabolism. Besides these changes, it is evident an expansion of beta cell mass mainly due to the effects of maternal hormones, prolactin and placental lactogen, on the islets (35) that stimulate not only insulin secretion but also beta cell replication as seen in rat, mouse and human islets in vitro. In particular, activation of the prolactin receptor stimulates the phosphorylation and nuclear accumulation of signal transducer and activator of transcription 5 (STAT5), which, then, represses menin through Bcl6 expression, allowing the beta cells to proliferate (36).

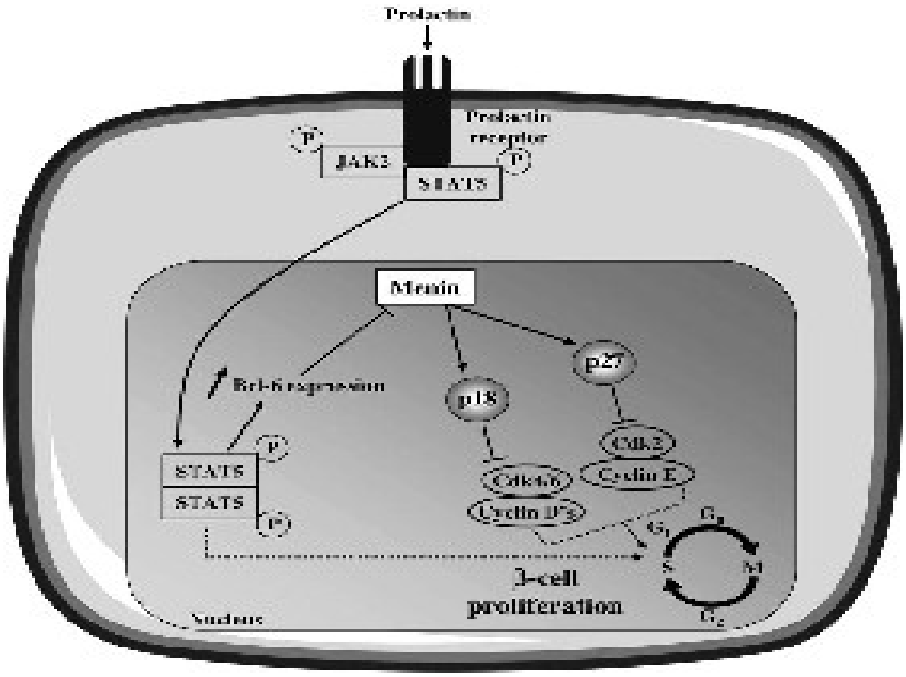


Figure 5: Regulation of beta cell proliferation via the prolactin receptor signalling *(Kargar C. et al. Diabetes, obesity and metabolism 2008 Nov; 10, Suppl 4: 43-53.).*

After partum, the beta cell mass reaches again a physiological level through a reduction of beta cell replication and an increase of beta cell apoptosis (37, 38).

Obesity is a multifactorial disease characterized by an excessive adipose tissue mass. It is often associated with insulin resistance, a reduced ability of insulin to promote glucose uptake by peripheral tissues. Obesity and insulin resistance bring to an increased metabolic load causing a beta cell mass growth (28, 39), sustained by an high rate of beta cell replication, neogenesis and a rise in cell volume as seen in experiments with obese rats and in human autopsy studies (28, 40). In this obese condition, when the beta cells loose the ability to compensate for the insulin resistance, diabetes comes.

Type 2 diabetes is a disease in which, contrary to what happen in pregnancy and obesity, the beta cell mass appears to be reduced.

1.2 Beta cell mass in type 2 diabetes

Diabetes is a disease characterized by multiple metabolic changes caused by various etiopathogenetic mechanisms. Type 2 diabetes is the most common form of diabetes and is caused by disorders of insulin biological action and deficit of insulin secretion, due to not well understood mechanisms. In this condition the beta cell mass shows both functional and quantitative changes.

From a functional point of view, beta cells gradually reduce and loose the first phase of insulin secretion with deficit also in the second release phase as diabetes progresses. Glucose-stimulated insulin secretion is reduced but the hormon secretion stimulated by other substances, as arginine, is slightly affected showing the presence of alterations in the glucose signaling pathway inside the beta cell (41).

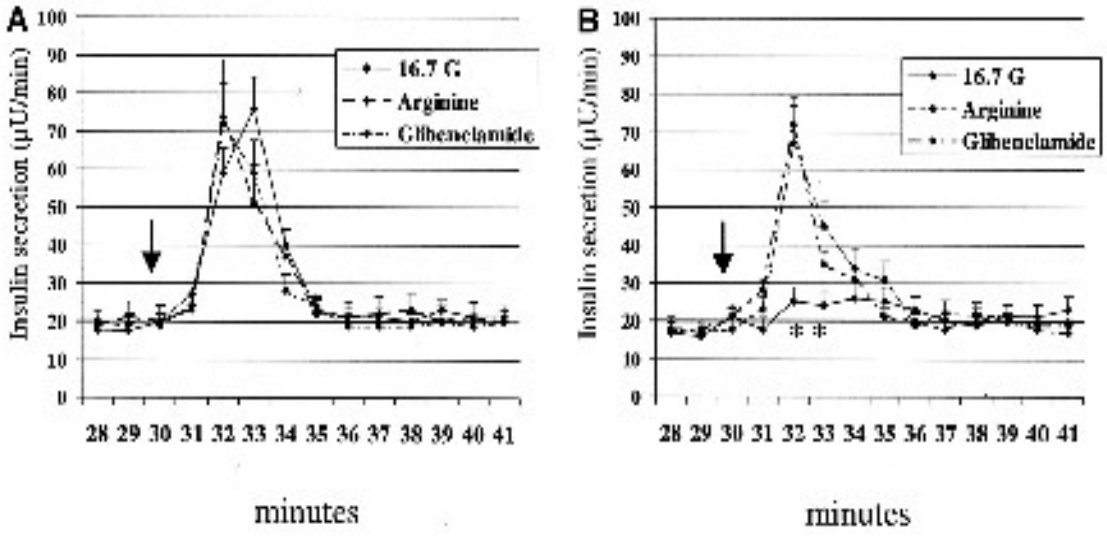


Figure 6: Early phase of insulin secretion from control and type 2 diabetic islets during perifusion experiments. Cell preparations are perifused with 3.3 mmol/l glucose solution for 30 min, and then with either 16.7 mmol/l glucose, 3.3 mmol/l glucose plus 20 mmol/l arginine, or 3.3 mmol/l glucose plus 100 µmol/l glibenclamide. Type 2 diabetic islets (b) do not show increase in insulin secretion during 16.7 mmol/l glucose compared with control islets (a). **P* < 0.05 of 16.7 mmol/l glucose vs. the other experimental conditions. *(Del Guerra S. et al. Diabetes 2005; 54, 3:727-35).*

Another defect is the increased ratio proinsulin/insulin, that results from an altered conversion of proinsulin in insulin and C-peptide in the mature beta cell granules (42).

From a quantitative point of view, the beta cell mass is reduced in type 2 diabetes both in humans and animal models and the magnitude of decrease correlates with the disease duration (43). The reduction of the beta cell mass in humans depends on reduction of islets quantity (43), reduction of beta cells in the islets for an increased apoptosis, quite evident in larger islets (43, 44), and reduction of beta cell mature granules (45).

1.2.1 Beta cell mass in animal models of type 2 diabetes

Many animal models of type 2 diabetes show a decrease in the beta cell mass.

Goto-Kakizaki (GK) rat is one of these; a non-obese Wistar substrain that, spontaneusly, develop type 2 diabetes mellitus early in life. GK rat model has the same defects related to functional and morphological characteristics of beta cells that are also present in the islets of type 2 diabetic patients, then, proving to be a useful model to understand the basis of diabetes in humans. The GK rats show fasting hyperglycemia soon after the birth, glucose intolerance and impaired glucose-induced insulin secretion due to an impaired function of beta cells, further worsed by chronic exposure to hyperglycemia (46), and a reduction of the beta cell mass to 50% of control Wistar rats. A reduced beta cell mass, due to problems during development, is mainly considered to be the primary defect leading to hyperglycemia and overt diabetes in this model. Infact, treatment with GLP-1 and its long-acting analog exendin-4 improves the glycemic control and severity of type 2 diabetes through a stimulation of beta cell replication and neogenesis (47).

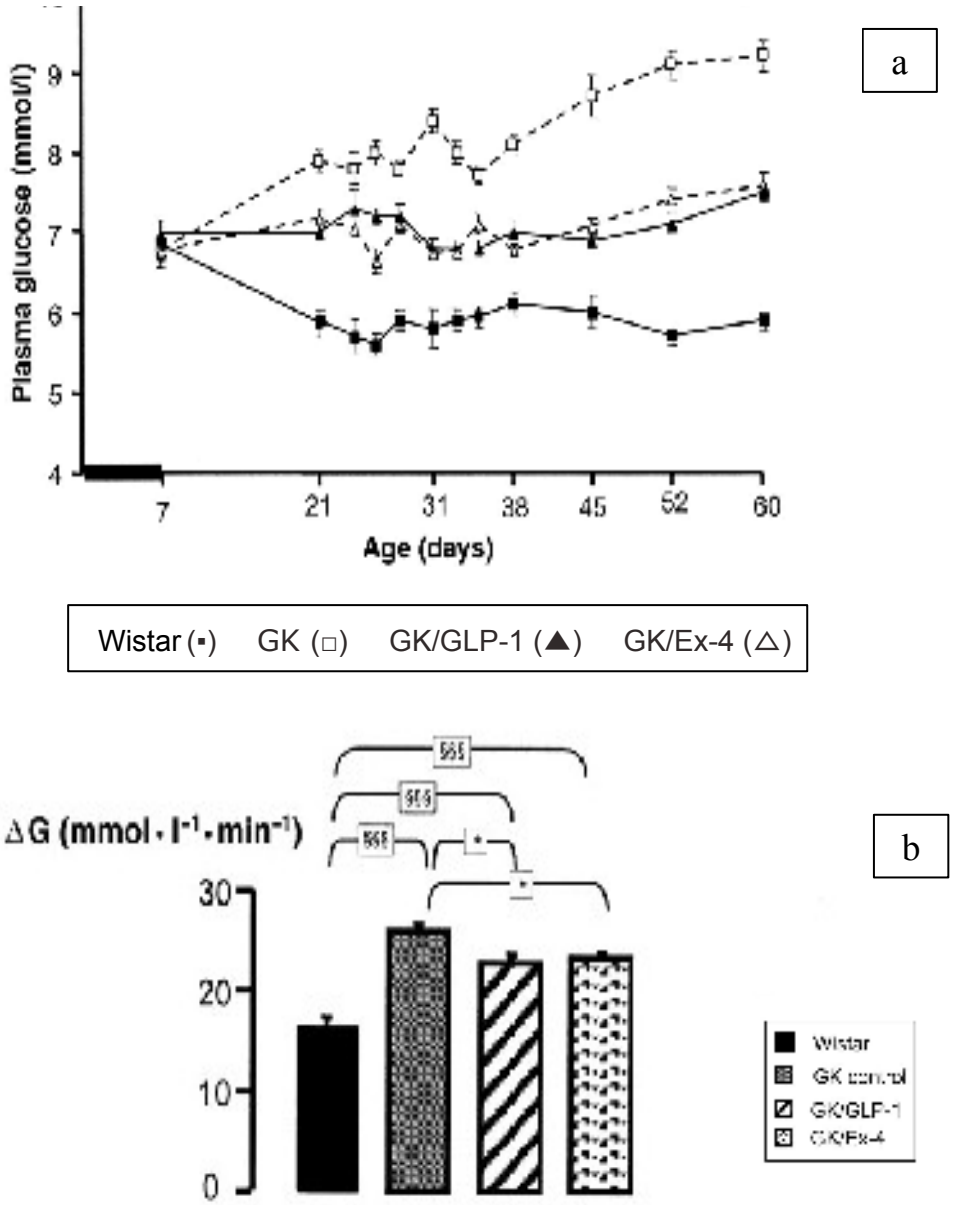


Figure 7. Basal plasma glucose and insulin levels of Wistar (▪), GK (□),GK/GLP-1 (▲) and GK/Ex-4 (△) from ages 7 days to 2 months. The increase in basal plasma glucose seen in GK group is blunted in the GK/GLP-1 and GK/Ex-4 rats (a). Tolerance to glucose measured as ΔG in the four groups. Tolerance to glucose is improved in the GK/GLP-1 and GK/Ex-4 rats even if it does not reach the normal value in Wistar group (b). §§§$P < 0.001$, §$P < 0.05$ vs. Wistar rats; *$P < 0.05$ vs. untreated GK rats. *(Tourrel C et al. Diabetes 2002; 51, 5: 1443-52)*.

Another model of experimental type 2 diabetes is represented by the Zucker diabetic fatty rats that lack the leptin receptor, and, then, inherit obesity. Soon after birth, after a compensatory hyperinsulinemic phase,

they loose the glycemic control and hyperglycemia comes followed by a reduction of plasma insulin levels. During this period, the beta cell mass decreases till to be almost 50% less compared to control non diabetic obese rats, showing that anomalies related to beta cell mass expansion contribute to hyperglycemia and are associated to a decrease of beta cell replication (40) and beta cell apoptosis (48).

Like Zucker diabetic fatty rats, db/db mice have a mutation in the leptin receptor and, spontaneously, become obese and hyperinsulinaemic. After 1 month from the birth, they develop hyperlipidemia, hyperglycemia, glucose intolerance and show a reduced beta cell mass and an altered beta cell function associated with anomalies in beta cell differentiation (49). The expression of genes involved in the maturation and maintenance of cellular differentiation, like PDX-1, Beta2/NeuroD, Nkx6.1 and Pax6, is reduced as well as the expression of genes involved in the beta cell glucose metabolism like the glucose transporter of beta cells, GLUT2, and the enzyme glucokinase important for the glucose phosphorylation and metabolism. Also genes, encoding ion channels taking part in the stimulation of insulin secretion, like kir6.2, a subunit of the ATP-sensitive K^+ channel, and SERCA2b and SERCA3, a sarco/endoplasmic reticulum Ca^{2+}-ATPase pump show an altered expression pattern (50). The reduction of beta cell mass in db/db mice can result also from incresead apoptotic events as proved by the activation, in the islets isolated from these mice, of X-box binding protein 1 (Xbp1) mRNA, a transcription factor partecipating in the unfolded protein response (UPR), a stress-signaling pathway active during the accumulation of unfolded proteins in the

endoplasmic reticulum (ER). Hyperglycaemia and high circulating levels of lipids in db/db mice can cause ER stress, eventually, culminating to beta cells apoptosis when UPR fails (51).

While the association between an altered functional beta cell mass and the onset or progression of diabetes is clear in these type 2 diabetes animal models, it is less evident or absent in others.

Psammomys obesus is a diurnal gerbil living in North African and Eastern Mediterranean semi-desert regions. In its habitat, with a low-calorie diet, it is neither obese nor hyperglycemic but few days of high calorie diet are enough to make this animal obese and diabetic. After 1 day of high calorie food, the hyperglycemia is associated with a reduction of 80-90% in pancreatic insulin reserve without changes in beta cell mass. On the following day, beta cell mass decreases of 40-50% to, spontaneously, return at the prediabetic level thanks to an increase in beta cell proliferation and neogenesis. But the pancreatic insulin content remains low, meaning. probably, that the new formed beta cells are not functionally mature and not able to secrete enough insulin to stop the hyperglycemia. A poor calorie diet reverses the diabetes status bringing to an increase in pancreatic insulin reserve without any changes in beta cell mass, showing that not beta cell mass but insulin content and secretory capacity determines the progression of diabetes in this animal model (52) .

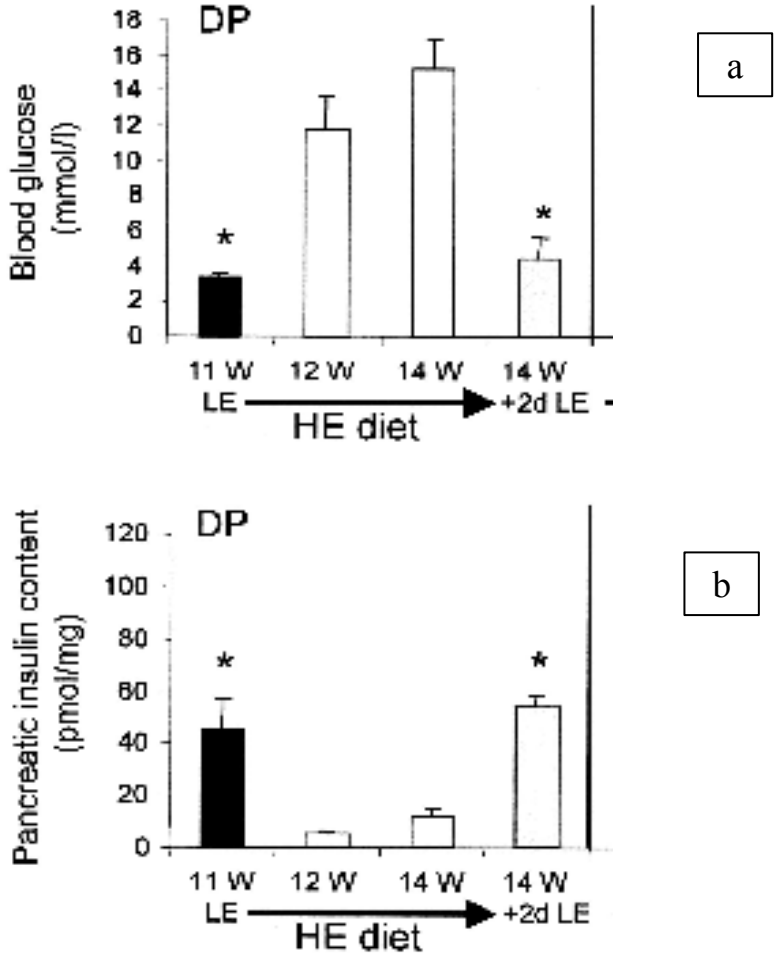


Figure 8: Blood glucose (a), pancreatic insulin content (b) in diabetic prone Psammomys obesus animals analysed at the ages of 11, 12 and 14 weeks on the HE (high calorie) diet and 14 weeks of HE diet followed by 2 days of LE (light calorie) diet. *P < 0.05 relative to animals in the 12 W and 14 W groups. *(Kaiser N et al. Diabetes 2005; 54, 2: 137-44).*

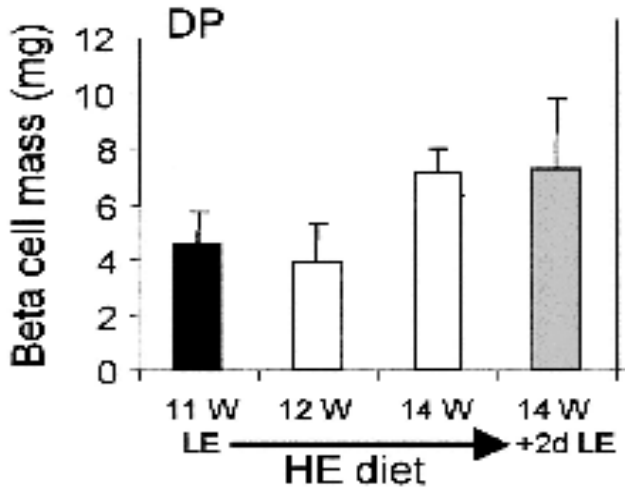


Figure 9: Beta cell mass in diabetic prone Psammomys obesus animals analysed at the ages of 11, 12 and 14 weeks on the HE diet and 14 weeks of HE diet followed by 2 days of LE. *(Kaiser N et al. Diabetes 2005; 54, 2: 137-44).*

Also partial pancreatectomy animal models contribute to investigate the role that beta cell mass has in the onset of hyperglycemia and diabetes. Experiments in rats show that 40% pancreatectomy has no effect on plasma glucose levels or insulin secretion in vivo and in vitro and the regrowth of beta cells, as showed by measurements of beta cell mass 19 days after surgery, is responsible for the maintenance of endocrine pancreatic normal function (53). But, while changes in plasma glycemic control are not associated to 40% reduction of beta cell mass, they are in the case of subtotal 90% pancreatectomy. 8-10 weeks after this kind of surgery, the remnant pancreas is analyzed showing an increase of both beta cell mass and not beta cell mass as well as pancreatic content of insulin, glucagon and somatostatin. Anyway, these improvements in beta cell mass are not associated with a parallel improvement in the islet function as the beta cell response to glucose results impaired (54).

Another model of experimentally induced type 2 diabetes is the streptozotocin (STZ) treated rats. STZ is a naturally occurring chemical toxic to the beta cells and a single injection of low-dose STZ causes moderate diabetes in adult rats. Two months after the injection, the beta cell mass is restored but insulin secretory defects are still present and 48-h glucose infusion to stimulate beta cell growth does not have effects on cell function despite a great increase in beta cell mass (55).

In conclusion, the data from these animal models show as the reduction of beta cell mass is associated to a decrease in beta cell function. It is the case of spontaneous animal models of type 2 diabetes: GK rat, ZDF rat and db/db mouse. Instead, the correlations between

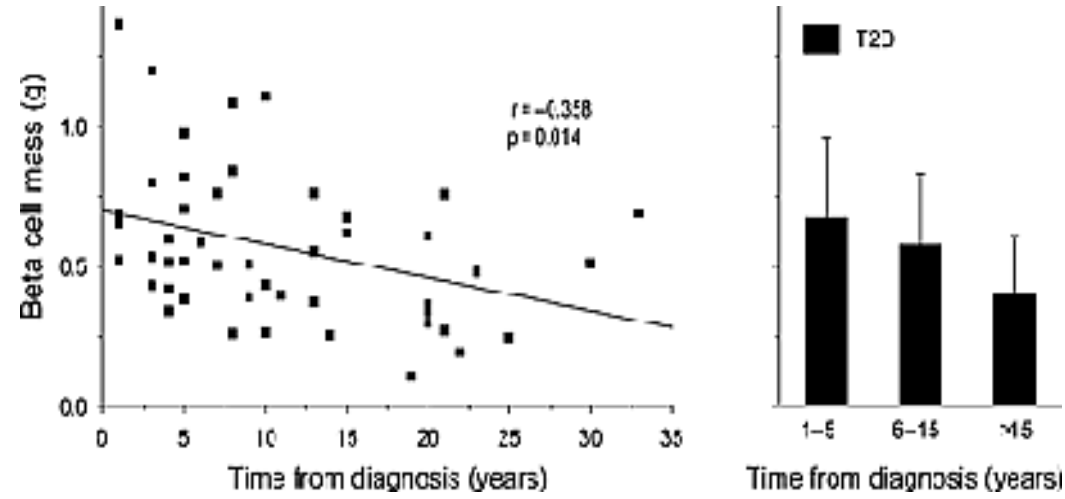
changes in beta cell mass and beta cell function lack in other conditions. It is the case of experimental animal models of type 2 diabetes: partial pancreatectomy, psammomys obesus and STZ injection where variations in the beta cell mass are not associated to changes in the beta cell activity. The difference between these two kinds of type 2 animal models can lay in their different metabolic environment. It is possible, infact, that states of prolonged hyperglycemia and hyperlipidemia, such as those present in pancreatectomized rats, STZ injected rats and psammomys obesus, can interfere with the growth of fully functional beta cells and normal control of plasma glucose levels.

The observations coming from these models, anyway, are useful to understand the mechanisms and the role of beta cell mass in the human type 2 diabetes despite the genetic differences of these species.

1.2.2 Beta cell mass in human type 2 diabetes

The difficulties in studying the beta cell mass in humans are related to the lack of instruments for in vivo measurements. Then, most of the data come not only from animal models but also from human autopsy studies.

In this sense, Rahier et al. are the first to perform their experiments in cadaveric donors. While at beginning, the scientists are not able to find any differences in the beta cell mass between type 2 diabetic and non diabetic control subjects (56), after following experiments, they show a reduction in beta cell mass of 41% and 38% in lean and obese diabetic patients, respectively, compared with controls. Also pancreatic insulin concentration results to be decreased in diabetics and while the beta cell mass is not correlated with age at the diagnosis, it decreases with duration of clinical diabetes. Infact, it is 24% and 54% lower than controls in subjects with <5 and >15 years of diabetes, respectively, showing that diabetes itself contributes to beta cell dysfunction (57).



a

b

Figure 10: Inverse correlation between beta cell mass and the duration of diabetes (a). Beta cell mass in diabetic patients subdivided in 3 categories of duration of clinical diabetes (b). *(Rahier J et al. Diabetes, Obesity and Metabolism 2008; 10, 4: 32-42).*

Also Butler et al. suggests that a decrease in beta cell mass is involved in an impaired insulin secretion. Obese subjects with impaired fasting glucose (IFG) and type 2 diabetes have a 40% and 63% reduction in relative beta cell volume compared with non diabetic obese cases while lean patients with type 2 diabetes show a 41% decrease compared with non diabetic lean cases. Beta cell replication and neogenesis rates are not different among groups unlike apoptosis frequency that is increased in type 2 diabetes cases compared with non diabetic controls, underlying that cellular death is the main mechanism responsible for the reduction of beta cells (28). The Langerhans islets isolated from type 2 diabetic cadaveric donors are smaller than islets isolated from controls with a reduced proportion of beta cells and an increased proportion of alfa cells. In some cases, beta cells are not present and alfa cells are more diffuse throughout the islet while the proportion of other endocrine cells is unchanged (43).

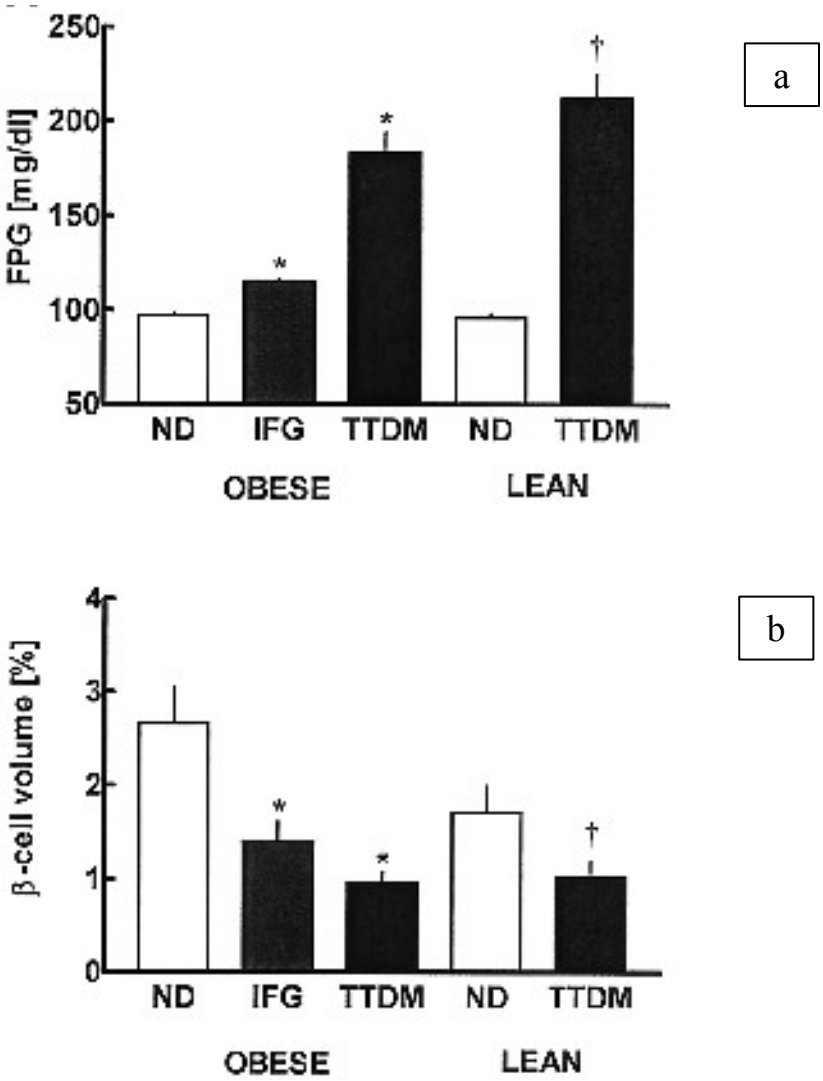


Figure 11: Fasting plasma glucose (a) and beta cell volume (b) in obese (non diabetic, IFG and diabetic) and lean (non diabetic and diabetic) cases. *P<0.05 compared with non diabetic obese subjects. †P<0.05 compared with non diabetic lean subjects. *(Butler AE et al. Diabetes 2003; 52, 1: 102-10).*

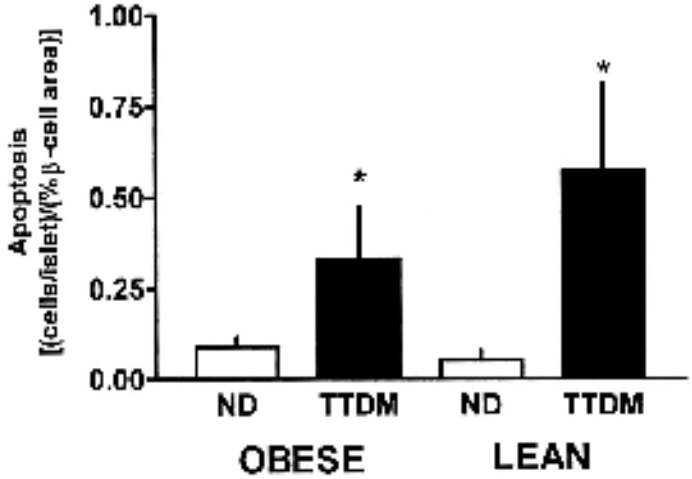


Figure 12: Frequency of beta cell apoptosis in obese (non diabetic and diabetic) and lean (non diabetic and diabetic) cases. *P<0.05 compared with non diabetic subjects. *(Butler AE et al. Diabetes 2003; 52, 1: 102-10).*

Islets from diabetic patients are not only morfologically and quantitatively altered but show also functional anomalies. They secrete less total insulin and exhibit a more elevated threshold for the glucose stimulated insulin response in in vitro islet perifusion assay and when transplanted to immunodeficient diabetic mice, they are not able to restore euglycemia contrary to normal control islets (43).

If on the one hand the studies on cadaveric donors are useful for quantitative analysis of pancreatic tissue, on the other hand they can present some limits: postmortem autolysis in the pancreas (58) and lack of clinical data of subjects with unrecognized diabetes (59). Keeping in mind those possible problems, Yoon et al. perform their experiments in live people with and without type 2 diabetes undergone to pancreatectomy for removal of benign or malignant pancreatic tumors. As in studies involving cadaveric donors, these diabetic patients have a reduction of relative beta cell volume and beta cell mass and an increase of relative alfa cell volume and alfa cell mass together with a high insular alfa/beta cell ratio compared with control patients (44).

The importance to have a proper quantity of functional beta cells to keep plasma glucose levels within normal values range in humans is evident in the curvilinear relationship existing between beta cell mass and fasting glucose concentration whereby at values upper than a certain pancreatic endocrine mass (1.1 ± 0.1%), great changes correspond to small changes in plasma glucose levels, whereas, at values lower than that threshold endocrine mass, small changes correspond to great changes in plasma glucose levels (60).

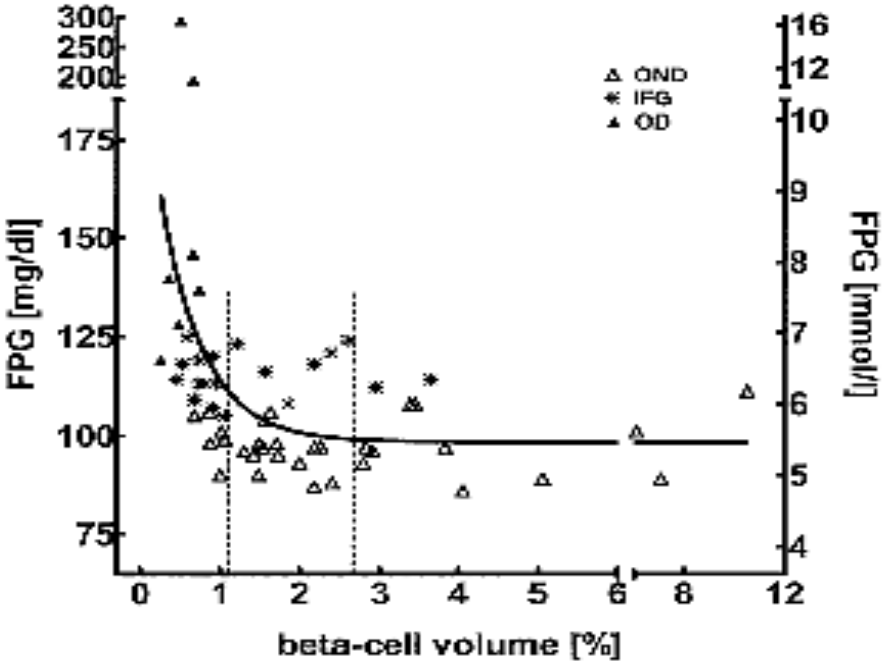


Figure 13: Relationship between percentage of beta cell volume in pancreas and plasma glucose in obese humans without insulin or oral antidiabetic treatment. *(Ritzel RA. et al. Diabetes care 2006 March; 29, 3: 717-718.).*

As the reduction of beta cell mass contributes to the loss of glycemic control and development of hyperglycemia in type 2 diabetes (28, 60), strategies to promote beta cell regeneration and lower plasma glucose levels can reverse or prevent the onset of the disease (60).

1.3 Beta cells regeneration as a cure for type 2 diabetes

The idea of enhancing beta cell regeneration as a potential future treatment approach for patients with diabetes has been prompted by the significant deficit in pancreatic beta cell mass in patients with type 1 and type 2 diabetes and the beta cell ability to regenerate (61, 62). We have seen that beta cell mass is a dinamic entity that increases in some conditions, as pregnancy and obesity (63), through replication of preexisting beta cells and transdifferentiation of endocrine progenitor cells residing inside the ductal epithelium (28, 35). Then, appropriate stimuli can induce the formation of new beta cells. Several experimental studies have demonstrated some potential for beta cell regeneration in rats and mice even if in dependence of age. Young mice increase beta cell replication and, then, beta cell mass, maintaining normoglycemia, in response to high fat diet, a single dose of STZ and after short-term treatment with exendin-4, glucagon-like peptide 1 (GLP-1) agonist in contrast to old mice (64, 65).

Among various pharmacological agents able to enhance new beta cell formation in vitro and in animal models, the gut hormones glucagon like peptide 1 (GLP-1), gastric inhibitory polypeptide (GIP) and gastrin have recently gained widespread attention.

GLP-1 is encoded in the proglucagon gene and results from a post-translational cleavage of the product of this gene by the prohormone convertase PC1/3. It is a hormone mainly produced in enteroendocrine L cells of the gut and secreted into the blood stream when food containing fat, protein hydrolysate and/or glucose enters the duodenum. It has been seen that GLP-1 stimulates the glucose-induced insulin secretion in

subjects with an impaired glucose tolerance and type 2 diabetes (66) and has an insulinotropic effect as it enhances insulin gene transcription, that in rats is mediated by the activation of cAMP response element (CRE) of the insulin 1 gene promoter (RIP1) (67), and insulin biosynthesis (68). It, also, regulates the expression of genes encoding proteins implicated in the glucose transport, like GLUT2, and metabolism, like glucokinase, through the PI 3-kinase/PDX-1 transduction signalling pathway in INS-1 cells (69) and enhances beta cell proliferation via transactivation of the epidermal growth factor receptor (EGFR). The hormone binds its receptor GLP-1R on the surface of beta cell membrane and activates the tyrosine kinase c-Src that is responsible for the proteolytic processing of membrane-anchored beta cellulin (BTC) or another EGF-like ligands, that binds to EGFR causing the activation of PI 3-kinase pathway (70).

Like GLP-1, also glucose-dependent insulinotropic polypeptide (GIP) stimulates insulin secretion through the amplification of exocytosis, as shown in mouse pancreatic beta cells, and preserves beta cell mass, inhibiting cellular apoptosis. GIP activates the PI-3k/PkB pathway leading to phosphorylation of Foxo1 and down regulation of bax, a pro-apoptotic member of the Bcl-2 family (71). As a result of the insulinotropic effects, the incretin hormones, GLP-1 and GIP, are used in type 2 diabetes therapy with two approaches: extending the half-lives inhibiting their degradative enzyme, dipeptidyl peptidase IV (72), and producing long acting incretin analogs (73).

Regarding gastrin, it has been shown that the hormone promotes beta cell replication in patients with pancreatic gastrinomas (76) and

induces, in combination with the epidermal growth factor (EGF), islet beta cell neogenesis in rodents (75) and adult human islets (74). Then, in addition to GLP-1 and GIP therapies, complementary approaches to regeneration of beta cell mass in type 2 diabetes can be represented by the combination of agents, such as EGF and gastrin.

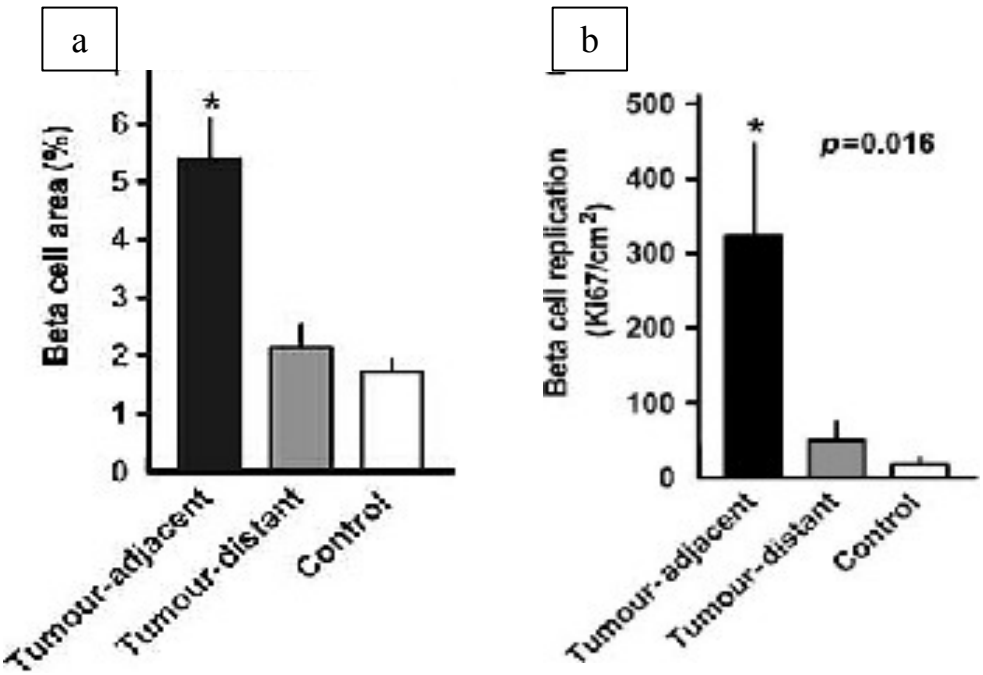


Figure 14: Fractional beta cell area (a) and frequency of beta cell replication (b) in tumour-adjacent and tumour-distant pancreatic sections from patients with gastrinomas and control subjects. *P<0.001 compared with control subjects. *(Meier JJ et al. Diabetologia 2006; 49, 11: 2689-96).*

1.3.1 Effects of insulin on beta cells turnover

Insulin belongs to a family of correlated proteins called insulin like growth factors, IGF, and is involved in the energy metabolism regulation. Its primary function is to lower the plasma glucose levels, promoting the glucose uptake into the muscles and adipose tissue by glucose transporter 4 (GLUT4) and into the liver through an increase of glucose phosphorylation and glucose utilization for the glycogen synthesis and metabolism. Then, insulin increases the synthesis of proteins and glycogen into the muscles and triglycerides into the adipose tissue. Beside this function, insulin has also some beneficial effects on islet cell turnover: it can stimulate the beta cell replication through PI 3-kinase and Raf-1 kinase pathways activation in mouse islets (78) and prevents the cell apoptosis induced by serum withdrawal in primary human and mouse islets in vitro. This last effect is associated with an increase of Pdx1 translocation to the nucleus and is absent in islets 50% deficient in the transcription factor, showing that it is an important target of insulin (77). In addition, treatment with insulin in patients soon after the diagnosis of type 2 diabetes has beneficial effects more than treatment with sulphonylurea agent. Infact, It has been shown that, after 2 years of therapy, glucagon-stimulated C-peptide response and fasting insulin levels are more increased and HbA1c levels are better in patients treated with insulin versus the glibenclamide group (79). This shows that insulin improves the beta cell function and, then, the glycemic control. Similar results can also be seen in a chinese study involving newly diagnosed type 2 diabetes people in which patients treated with an early intensive insulin therapy

obtain glycemic control in less time compared to patients treated with oral hypoglicemic agents. After normoglycemia is achieved and maintained for 2 weeks in both groups, the drug therapy is suspended and the patients undergo only a diet and physical exercise. After 1 year follow up the remission rate results to be higher in the insulin group than in the oral agent group, as well as the beta cell function estimated by the homeostasis model assessment (HOMA) (80).

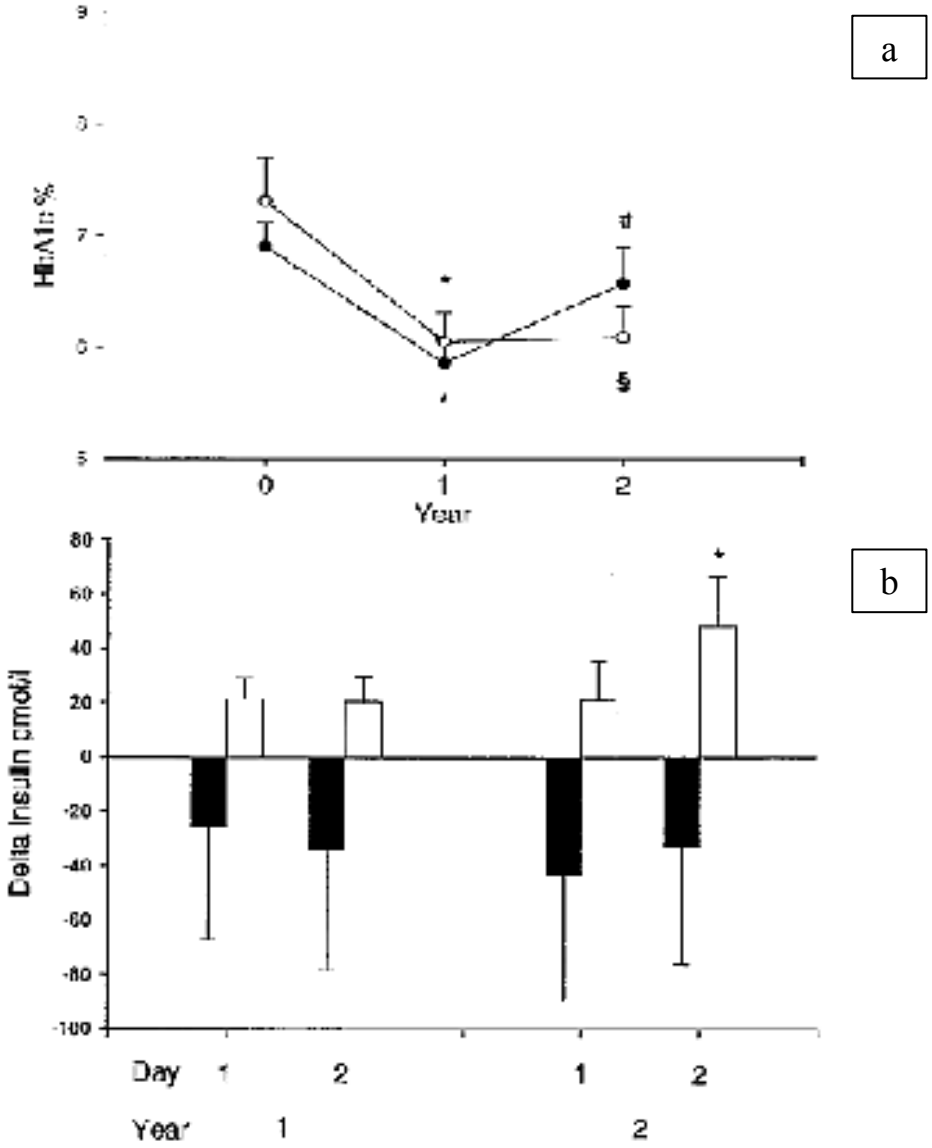


Figure 15: HbA1c levels during the study (a) **P* < 0.01 year 0 vs. 1, §*P* < 0.005 year 0 vs. 2, #*P* < 0.01 year 1 vs. 2. Incremental or decremental values of fasting levels of insulin versus baseline (b). *P=0.02 glibenclamide vs. insulin. ▪, glibenclamide; □, insulin. *(Alvarsson M et al. Diabetes Care 2003; 26, 8: 2231-7).*

1.4 ISLET AMYLOID POLYPEPTIDE (IAPP): General characteristics

As mentioned above, the Langerhans islets of type 2 diabetes patients have morphological features related not only to changes in beta and alfa cells quantity but also to amyloid deposition and exocrine fibrosis.

Amyloid is a specific protein aggregation in which molecules are in beta sheet conformation and bound to each other mainly by hydrogen bonds. This state of aggregation creates thin fibrils that appear as unbranching structures of 5-10 nm in diameter and indeterminate lenght at electron microscopy (81). The fibrillogenic peptide is the Islet Amyloid Polypeptide (IAPP), normally produced in the beta cell and stored in the secretory granules and cosecreted with insulin in response to different stimuli.

IAPP is a 37 aminoacids peptide derived from a precursor, preproIAPP, a 89 aminoacids peptide encoded by a gene on the chromosome 12 (82). PreproIAPP contains a signal peptide that is removed in the endoplasmic reticulum (ER); then, proIAPP and proinsulin are cleaved by two endoproteases, the prohormone convertase 2 (PC2) and the prohormone convertase 1/3 (PC1/3) in the late Golgi and secretory granules.

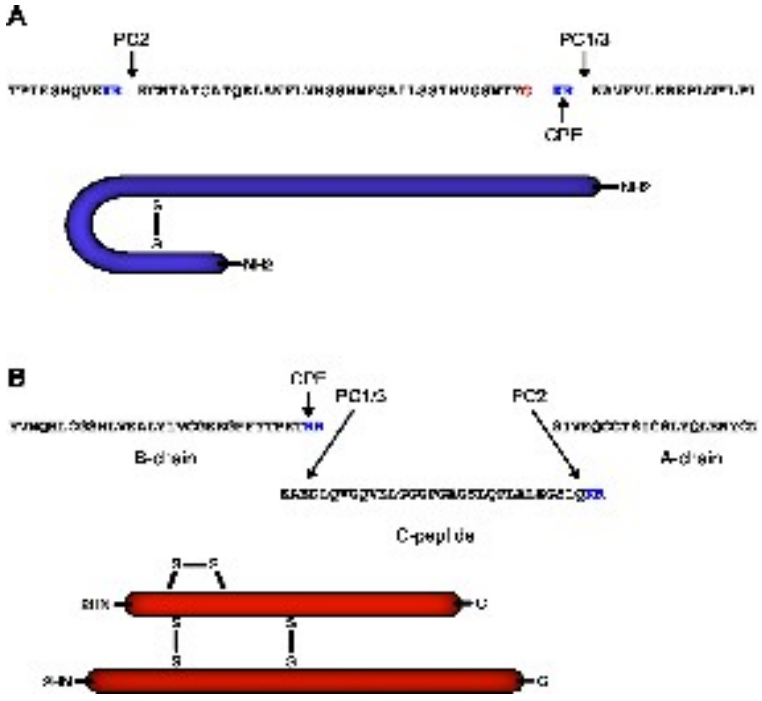


Figure 16: The aminoacid sequence of human proIAPP (a) and the aminoacid sequence of human proinsulin (b) with the cleavage sites for PC2 and PC1/3. *(Westermark P et al. Physiol Rev 2011; 91, 3: 795-826).*

IAPP is a fibrill-prone protein and is protected from aggregation in the granules, where it's highly concentrated, by the interaction with other molecules or ions, some of which can be the insulin, that strongly inhibits IAPP fibrils formation in vitro, pH and calcium concentration (83). Then, it is possible that changes in the secretory vesicles environment can induce the initial aggregation. IAPP is a hormone and exerts its activity binding to receptors, some of which have been identified in the brain (84) and renal cortex of rats (85). Its functions are not fully understood although some physiological effects on Langerhans islets and central nervous system are clear. In the first case, IAPP is involved in the regulation of insulin and glucagon secretion: the basal glucose stimulated insulin secretion is

enhanced when IAPP is at low concentrations, while it is inhibited when the hormone is at high concentrations (86). Although, IAPP inhibits also the glucagon release in hypoglycemic conditions or when stimulated by different secretagogues, like L-arginine, in isolated rat islets (86), the effect is not seen in all experiments, making IAPP role in this mechanism a bit controversial (87). In the second case, it has been shown that IAPP slows the gastric emptying (90) and reduces the food intake in rats when administered either chronically (88) and at low doses (89) and in humans only when administered at low concentrations (91). Other effects of IAPP include vasodilatation (92), regulation of renal filtration (93) and calcium homeostasis (94).

1.4.1 IAPP amyloidogenicity and type 2 diabetes in animal models

In the past, the role of IAPP in the pathogenesis of type 2 diabetes has not been extensively examined because of the lack of amyloidosis naturally occurring in the Langerhans islets of laboratory rodents. The aminoacid sequence omology of IAPP is high, about 80%, between different mammalian species; in particular, -NH_2 and -COOH terminal parts are conserved through the evolution, probably, because of their important biological activity. Anyway, only humans, non human primates and cats express a form of IAPP able to form amyloid fibrils that, instead, lacks in rodents. In vitro experiments show that peptides corrisponding to IAPP aminoacid region 20-29 of human and cat species form amyloid like fibrils, while the corresponding IAPP segment from rodent species is inert. Aminoacid substitutions have proved that the sequence at positions 25-29 of human IAPP is highly amyloidogenic and the proline residue, a beta-sheet breaker, present at position 28 in the molecule of many rodents, inhibits the fibrils formation (95). Then, the protein primary structure is fundamental to determine the correct protein folding that, anyway, is also influenced by other factors like pH, temperature and ion concentration. Keeping in mind these differences in IAPP structure between species, most informations related to islet amyloidosis associated with type 2 diabetes come from experiments with domestic cats, monkeys, as well as transgenic rodents for human IAPP.

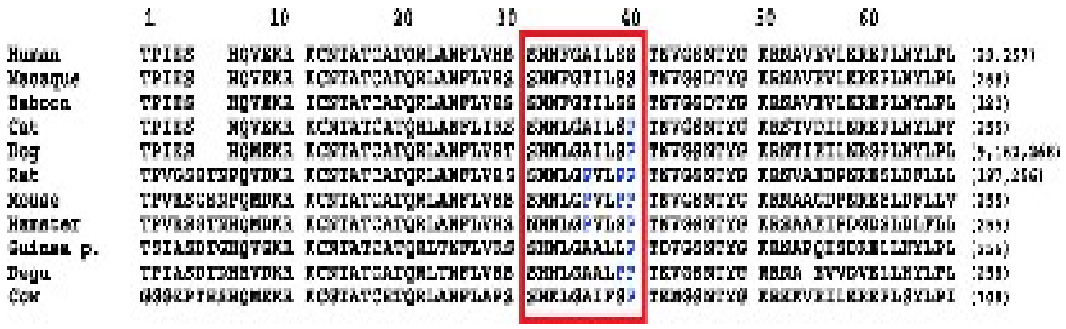


Figure 17: The aminoacid sequence of proIAPP of some mammalian species. IAPP is strongly conserved but shows variation in the 20-29 region of IAPP. This corresponds to residues 31-40 of proIAPP, evident in the red box. Proline residues 36 and 39 are essential for aggregation inhibition. *(Westermark P et al. Physiological reviews 2011; 91, 3: 795-826).*

Some studies performed in monkeys show that amyloid deposits are present not only in the islets of diabetic animals, with an occupation of 37-81% of the insular area, but also in normoglycemic and hyperinsulinemic monkeys, where amyloid occupies 0.03%-45% of the islet space, suggesting that fibril aggregates are formed prior to the development of overt diabetes, possibly being a factor in the destruction of beta cells (96). Amyloid is more prevalent in aging monkeys and increases with severity of diabetes mellitus and its quantity correlates significantly with serum glucose, tryglicerides and insulin (97).

In diabetic cats the islet amyloid is associated with a significant decrease in alfa and beta cell volume fractions but the volume of endocrine cells in normoglycemic glucose-intolerant cats is not different from controls, showing that the reduction in beta cell mass is not the only factor leading to diabetes and the replacement of beta cells with amyloid is not the cause of the disease although it can contribute to the progression of diabetes (98, 99).

Further experiments come from baboons, in which islet amyloidosis

severity, meant as increase in the percentage of insular area occupied, correlates with fasting plasma glucose, HbA1c, glucagon values and beta cell function parameters and furthermore with a decreased relative beta cell and increased relative alfa cell volumes. These results point out that probably the Langerhans islet dysfunction, a fundamental aspect of type 2 diabetes, depends on beta cell death, alfa cell proliferation and islet amyloidosis (100) that, then, can be not only a result of the pathology but possibly also a causative factor.

Other aspects of this complex relation between amyloid deposits and type 2 diabetes come up from studies in transgenic rodents expressing human IAPP gene, mainly, under control of the rat insulin promoter (101). Some of these mice develop diabetes mellitus spontaneously (102) or under a diet high in fat (103) or induction of insulin insensitivity (104).

In addition to amyloid deposits in the islets of animals with type 2 diabetes, there are also small intra- and extracellular amorphous IAPP aggregates visible prior to the onset of the disease and that can be important in affecting the beta cell survival (102).

1.4.2 IAPP amyloidosis and type 2 diabetes in humans

Islet amyloidosis is described for the first time by Opie that observes scattered groups of irregular, rounded, globular masses of hyaline material located immediately outside the capillary walls and larger deposits replacing the cells and occupying most of the insular area (105). Those aggregates have been found to be comprised of a major component, protein IAPP, and minor components, apolipoprotein E, the heparan sulfate proteoglycan perlecan and serum amyloid P component (SAP) (109-111).

Since then, the amyloid deposits have been discovered in the islets of type 2 diabetic subjects at post-mortem with a percentage of patients varied from almost 100% to 40% or less (28, 106-108) and a main spread in the pancreas corpus, body, tail and anterior part of the head, but not in the caput, the 'pancreatic polypeptide rich' part of the head, underlining that amyloid occurs only in the islets containing beta cells (106). The extent of islet amyloid deposition in diabetic patients is really variable; the number of islets affected is very heterogeneous ranging from < 1% to 80%, as well as the amount of amyloid per islet ranging from <0.5% to up to 80% (106, 112-114). The frequency and intensity of islet amyloidosis is also related to the age of patients (107, 108), being uncommon in individuals younger than 40 years and found in about 10% of the diabetics between 50 and 60 years of age and in over 50% of the diabetics over 70 years (115).

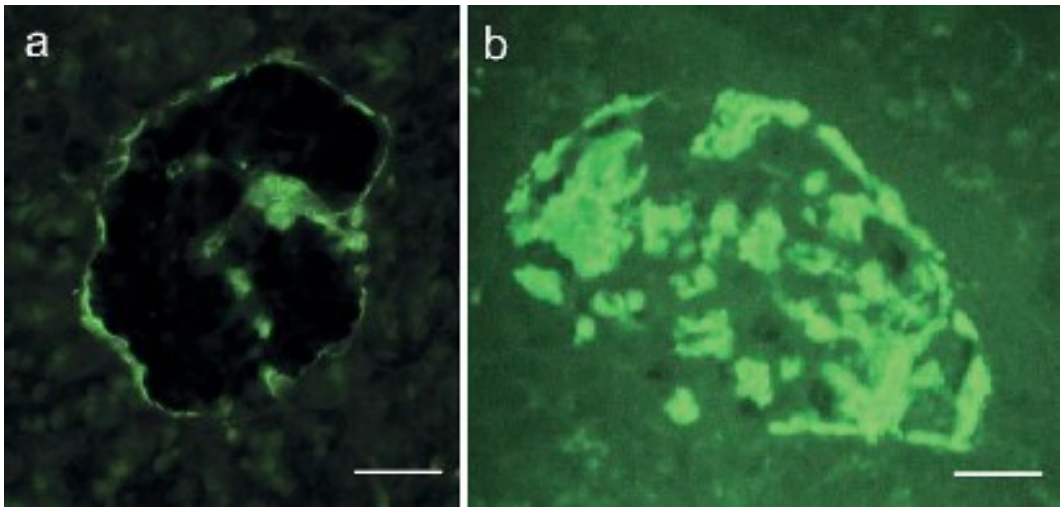


Figure 18: Islet amyloid deposits labelled with thioflavin S in 2 subjects with type 2 diabetes. a) Islet from a 86 years old subject treated with oral agents. A mean of 3% islet area was occupied by amyloid in this subject b) Islet from a 76 years old subject treated with oral hypoglycemic drugs. A mean of 20% islet area was occupied by amyloid in this patient. *(Clark A et al. Diabetologia 2004; 47, 2: 157-169).*

Islet amyloid is present also in non diabetic subjects but is much less common than in diabetics, affecting fewer islets with a less extent, in relation to age of patients (108, 112, 114). The presence of amyloid deposits in some subjects without type 2 diabetes and not in all type 2 diabetes patients makes the role of amyloid deposition in the onset and progression of this kind of disease quite complex in humans. Several studies have reported that islet amyloidosis is associated with reduced islet volume and beta cell area in diabetics (106, 116) and a clear connection exists between the stage of type 2 diabetes and the extent of islet amyloid (117), suggesting that the role amyloid seems to have in the disease is related to beta cell dysfunction and death.

Based on the fact that amyloid fibrils of Aβ protein are toxic to neurons in Alzheimer's disease, different studies have been performed to evaluate if also amyloid fibrils of IAPP have the same toxic effects on the

cells. It has been shown that IAPP fibrils provoke beta cell apoptosis through a direct contact with the beta cell surface in rat and adult human islet cells (118) while small IAPP aggregates, called oligomers, trigger beta cell death through the formation of abnormal vesicle-like membrane structures that destabilize the cellular ionic homeostasis (119). They insert into the cell membrane and produce these ion channel-like structures, mainly as trimers or octamers (122), in a mechanism dependent on lipid membrane composition, ion strenght and membrane potential (120, 121). IAPP oligomers and their cytotoxic properties have been studied in vitro mostly using high resolution microscopy techniques, like time-lapse atomic-force microscopy, but, lately they have been observed also in vivo, in transgenic mice, where they form intracellularly within the secretory pathway and appear to disrupt the membranes of endoplasmic reticulum, Golgi, secretory granules and mitochondria (123). One study has reported that there is also an extensive oligomer deposition in islets of human pancreatic tissue where apoptotic beta cells are more diffuse (114). Despite these results, the role of amyloid oligomers in the pathogenesis of type 2 diabetes is still controversial because of the lack of methods able to clearly detect IAPP oligomers in vivo. Infact, the antibody anti oligomers, A11, used in the experiments with human and mouse pancreases, appears to bind, besides amyloidogenic species, also other natively folded proteins, such as heat shock proteins (124) and alfa-synuclein protein (125), proving to be less specific. Furthermore, most post-mortem and in vitro studies have shown the presence of amyloid fibrils in type 2 diabetes patients (28, 106-108) in a strict contact with apoptotic beta cells (118),

suggesting their implication in membrane damage through the interaction with specific sensitive calcium channels on the cell surface, involved in the cell survival (126). In addition, others studies have shown how the inhibition of amyloid fibrils reduces the apoptosis of beta cells in culture (127, 128). Then, the role of IAPP aggregates in the pathogenesis of type 2 diabetes is still debatable as it is not clear if IAPP aggregates are a cause or consequence of beta cell dysfunction or death and if they are cause, which structures, fibrils or oligomers, are really cytotoxic. The development of methods able to detect in a specific way the different forms of human IAPP and more in vivo studies could elucidate their mechanisms of action in type 2 diabetes and eventually lead to pharmacological treatment to prevent or stop the decline of beta cell function.

2. AIM OF THE STUDY

The research activity we carried on is related to 2 research projects focusing on the beta cell turnover and morphological alterations of Langerhans islets in a condition of high insulin secretion and a reduced insulin secretion because of faulty beta cells or because of pancreatic diseases.

Then, the aim of the first project was to evaluate the effects of high local insulin levels on beta cell turnover in vivo, in a model of endogenous hyperinsulinism. Then, we examined the tumor-adjacent tissue of patients with intra-pancreatic insulinomas and pancreatic tissue of control subjects to evaluate possible differences in:

-Beta cell area ⟹ Fractional beta cell area, islet size and nuclear diameter of beta cells

-Beta cell turnover ⟹ Replication, apoptosis and neogenesis of beta cells

The aim of the second project, that is still in progress, was to examine the presence and quantity of amyloid deposits and their role on beta cell death in type 2 diabetes and diabetes secondary to pancreatic diseases. Then, we examined the pancreatic tissue of patients with type 2 diabetes, patients with diabetes secondary to pancreatitis or pancreatic carcinoma and control subjects to evaluate possible differences in:

-Beta cell area ⟹ Fractional beta cell area

-Beta cell death ⟹ Beta cell apoptosis

-Amyloid ⟹ Extent of islet amyloid, Islet amyloid area

3. MATERIALS AND METHODS (I)

3.1 Pancreatic tissue samples

3.1.1 Cases

Human pancreatic tissue was obtained at surgery from 5 patients, who underwent surgery for the removal of insulinomas (Table 1 and 2). The diagnosis of insulinoma was made on the basis of clinical symptoms (i.e. repeated fasting hypoglycaemia, resolution of symptoms by glucose administration, etc.), as well as prolonged fasting tests. In all cases, the clinical diagnosis was confirmed by a trained pathologist based on the detection of insulin-positive pancreatic tumors. Tumor-free tissue was available from all cases.

3.1.2 Control subjects

10 control subjects of similar age and BMI were chosen as controls (Table 1). All control patients had undergone pancreatic surgery for the removal of intra or extra pancreatic tumours not influencing the beta cell mass and function: benign pancreatic adenomas (n = 8), tumors of the choledochus (n = 1), and an intraperitoneal metastasis from breast carcinoma (n = 1). In all tissue samples, significant alterations of the overall pancreatic integrity were excluded by careful histological examination. None of the control subjects had a history of diabetes, but fasting glucose concentrations were in the diabetic range in four of these patients.

Table 1. *Tumour characteristics of cases.*

Case n.	**Intrapancreatic tumour localisation**	**Tumour dignity**
1	Tail	Benign
2	Tail	Benign
3	Tail	Malignant
4	Head	Malignant
5	Body	Benign

Table 2. *Characteristics of cases and control subjects.*

	Control subjects	***Cases***
SEX (F+M)	8+2	3+2
AGE (years)	60.3 ± 21.3	54.0 ± 22.3
BMI (kg/m²)	24.5 ± 3.2	24.6 ± 3.1
FASTING BLOOD GLUCOSE (mg/dl)	117.8 ± 28.49	86.8 ± 25.97
HbA1c (%)	5.56 ± 0.45	4.9 ± 0.54

Data are means ± SD.

3.2 Fluorescence and immunohistochemical tissue staining

Pancreatic tissue was fixed in formaldehyde and embedded in paraffin. Sequential 5 µm sections were stained as follows:

3.2.1 FRACTIONAL BETA CELL AREA, ISLET SIZE, BETA CELL NUCLEAR DIAMETER AND BETA CELL NEOGENESIS

To assess fractional beta-cell area, islet size, the percentage of insulin-positive duct cells, and the nuclear diameter of beta-cells, all tissue sections were immunohistochemically stained for insulin and DNA as follows: After heating at 37°C over night or at 58°C for 30 minutes, pancreatic tissue sections were deparaffinised in two subsequent 10-

minute xylene-incubations and subsequently hydrated through graded alcohol-series (100%, 95%, 70%, water). To block endogeneous peroxidase-activity, 70% alcohol contained 3% hydrogen peroxide. Antigen-retrieval was performed in DakoCytomation Target Retrieval Solution pH 9 (Dako, Glostrup, Denmark Code S 2367) by heating for 20 minutes. After cooling of the samples to room temperature and a brief rinse in distilled water, sections were subsequently blocked for unspecific protein binding with TBS containing 3% BSA and 0,2% Triton X100 for 10 minutes and for endogeneous biotin by use of the Dako Cytomation Biotin Blocking System (Dako, Code X0590). Then, a guinea-pig-derived antiserum against pig insulin was applied for 1 hour at a dilution of 1:400 (Dako, Glostrup, Denmark Code A0564). Subsequently, insulin was stained red by the use of the Dako-LSAB Real Detection System (Glostrup, Denmark, Code K5005) according to the manufacturer`s recommendations with 5-minute-washes in TBS between the incubations. For a blue nuclear counterstaining, specimens were incubated in hematoxylin (Dako, Glostrup, Denmark, Code S3301) for 5 minutes followed by a 5-minute-incubation in tap water. Finally, samples went through a series of ascending alcohol concentrations (70%, 96%, 100%) and were mounted with Entellan (Merck, Darmstadt, Germany, Code 1.07961.0/00). Under omission of the primary antibody, no staining could be observed.

3.2.2 BETA CELL REPLICATION

Ki67-expression was visualised by the HRP-catalysed generation of a brown chromogen using the Dako-Envision Dual Link System (Glostrup, Denmark Code K4065) according to the instructions of the manufacturer. Insulin was stained red by the use of the Dako-LSAB Real Detection System (Glostrup, Denmark, Code K5005). After heating at 37°C over night or at 58°C for 30 minutes, pancreatic tissue sections were deparaffinised in two subsequent 10-minute xylene-incubations and then hydrated through graded alcohol-series (100%, 95%, 70%, water). To block endogeneous peroxidase-activity, 70% alcohol contained 3% hydrogen peroxide. Antigen-retrieval was performed in DakoCytomation Target Retrieval Solution pH 9 (Dako, Glostrup, Denmark Code S 2367) by heating for 20 minutes. After cooling of the samples to room temperature and a brief rinse in distilled water, sections were subsequently blocked for unspecific protein binding with TBS containing 3% BSA and 0,2% Triton X100 for 10 minutes and for endogeneous biotin by use of the Dako Cytomation Biotin Blocking System (Dako, Code X0590). Then, the tissues were incubated in mouse primary antibody against human Ki67 (Dako, Glostrup, Denmark Code M7240 1:50) for 1 hour. Subsequently, the antigen was stained brown by the use of the Dako-Envision-Kit (Dako, Glostrup, Denmark, Code K4065) according to the instructions of the manufacturer. To detect insulin, slides were incubated in a guinea-pig-antiserum against pig insulin (Dako, Code A0564, 1:400) for 30 minutes. Then, LSAB staining was done according to the manufacturer`s recommendations with 5-minute-washes in TBS between the incubations.

For a blue nuclear counterstaining, specimens were incubated in hematoxylin (Dako, Glostrup, Denmark, Code S3301) for 5 minutes followed by a 5-minute-incubation in tap water. Finally, samples went through a series of ascending alcohol concentrations (70%, 96%, 100%) and were mounted with Entellan (Merck, Darmstadt, Germany, Code 1.07961.0/00). Under omission of the primary antibodies, no staining could be observed. Tonsil, known to strongly express Ki67 at the protein level {e.g. \Troncone, 2005 #795;Korbonits, 2002 #802} was used as a positive control.

3.2.3 *BETA CELL APOPTOSIS*

To investigate the turnover of beta-cells, pancreatic tissue sections were stained simultaneously for DNA strand breaks and insulin as follows: Antigen retrieval was performed in pH 6 citrate buffer (Dako, Glostrup, Denmark Code S 2369) for 30 minutes. Then, TUNEL staining was performed by using the in situ cell death detection kit from Roche (Roche, Code 11 684 795 910), according to the manufacturer's recommendations. Prior to the immunostaining for insulin, samples were blocked for unspecific binding of the antibodies with TBS containing 3% BSA and 0,2% Triton X100 for 15 minutes. For the detection of insulin, the guinea-pig-derived antiserum against pig insulin described above was applied for 30 min at a dilution of 1:100 followed by the incubation in Cy3 (red)-labelled secondary antibody developed in goat (Jackson Immuno Research, Code 106-165-603) that was applied in a dilution of 1:800 for 15 minutes. All antibodies were diluted in Antibody Diluent from Dako

(Glostrup, Denmark, Code S0809). Finally, the nuclei were stained with DAPI (blue) by incubation in a 0,33 μg/ml DAPI (Invitrogen, Paisley, UK,Code SKU#1306]) solution in PBS for 5 minutes. Following 5-minute-incubations in 3 changes of distilled water, the tissue sections were mounted with Dako Fluorescence Mounting Medium (Glostrup, Denmark, Code S-3023) and stored at 4-8°C in the dark. Positive and negative controls were run with each batch of samples. DNAse-treated tissue served as a positive control for the TUNEL reaction. As a negative control, primary antibodies and terminal transferase were omitted.

3.3 Morphometric analysis

For all morphometric analysis, pancreatic tissue specimens stained immunohistochemically as above described were used. All measurements were performed by applying specific tools of the software Axiovision (version 4.5) (Zeiss, Oberkochen, Germany) to images of the tissue samples that were generated by an Axioplan 2-microscope (Zeiss, Oberkochen, Germany) at appropriate magnifications.

3.3.1 Fractional beta-cell area

The fractional beta-cell area of the control tissue sections and the healthy parts of the resected tissue from insulinoma patients was determined by imaging the entire pancreatic section using the Axioplan microscope equipped with a motorized-stage, at 100-fold magnification. The Mosaix tool of the software Axiovision was used to create a tile image. The pancreatic tissue was surrounded using the contour 'measurement'

tool of the software, excluding artefacts or other different kind of tissues, possibly present in the section, from the analysis. Then, we measured the red areas corresponding to the insulin-positive areas in the selected pancreatic tissue using the colour detection 'measurement' tool. We used the same tool also to measure the red and not red areas in the tissue corresponding to the entire pancreatic area. Then, the beta cell area was expressed as percentage of insulin-positive area to the entire pancreatic area.

3.3.2 Islet size

For the determination of islet size, at least 20 representative islets in the healthy part of the resected tissue were imaged in the insulinoma cases and control subjects, each at 20x magnification, using the Axioplan microscope. Only islets containing a minimum of 5 beta-cells were selected. The size of each islet, expressed as insular area, was determined individually using the contour “measurement” tool of the software Axiovision (Zeiss, Oberkochen, Germany).

3.3.3 Beta cell nuclear diameter

To asses the nuclear diameter of beta-cells, 5 randomly selected islets per section were photographed at 40x magnification using the Axioplan microscope. Five representative beta cell nuclei per islet were chosen if they had clear and definable nuclear boundaries within beta cells, circular shape and appeared as they were cut through their maximum diameter. Then, the nuclei were encircled by the contour

“measurement” tool of the software Axiovision and their area was expressed (Zeiss, Oberkochen, Germany). The respective diameter was then calculated from the area displayed.

3.3.4 Beta cell neogenesis

To examine the possible formation of new beta-cells from ductal progenitor cells, the percentage of insulin-positive duct cells was determined, as described (28). For these purposes, 10 randomly chosen visual fields containing pancreatic ducts were imaged at 20x magnification using the Axioplan microscope. Duct-cells were manually counted and the number of insulin-positive duct-cells per field was expressed in relation to the total number of duct cells.

3.3.5 Beta-cell replication

To determine the frequency of beta-cell replication, 10 random islets per slide stained for insulin and Ki67 were imaged at 20x objective magnification using the Axioplan microscope. The number of cells co-stained for insulin and Ki67 was counted manually and expressed in relation to the total number of insulin-positive cells.

3.3.6 Beta-cell apoptosis

To determine the frequency of beta-cell apoptosis, 10 random islets per slide stained for insulin and TUNEL were imaged at 20x objective magnification using the Axioplan microscope. The number of cells co-stained for insulin and TUNEL was counted manually and expressed in

relation to the total number of insulin-positive cells.

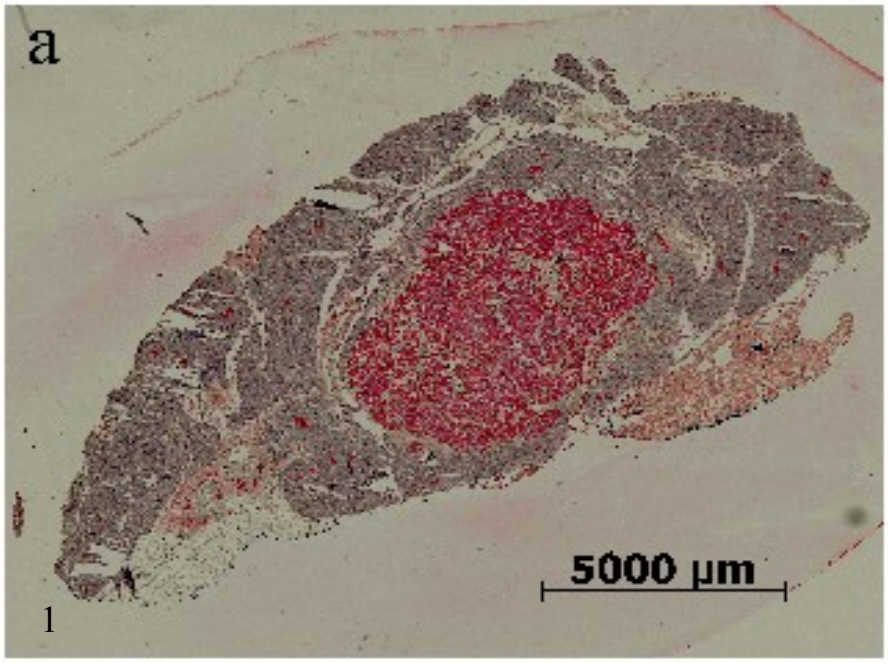
3.4 Statistical analysis

Subject characteristics are reported as means ± SD, results are presented as means ± SEM. Statistical calculations were carried out by one-way ANOVA and Student's t-test using Prism4 (Graph Pad software Inc., La Jolla, CA, USA).

4. RESULTS (I)

4.1 Pancreas morphology

The presence of insulinoma tissue was confirmed by careful examination of the tissue sections by a trained pathologist following specific insulin staining in all cases. A morphological evaluation of the tumor-adjacent pancreatic tissue did not reveal any major abnormalities in the insulinoma patients. Thus, islets of regular shape and number could be detected in the unaffected tissue in all cases. Examples of tissue-scans showing an insulin-producing tumor and healthy tissue from a control-tissue are given in Figures 19 a and b1 and b2, respectively.



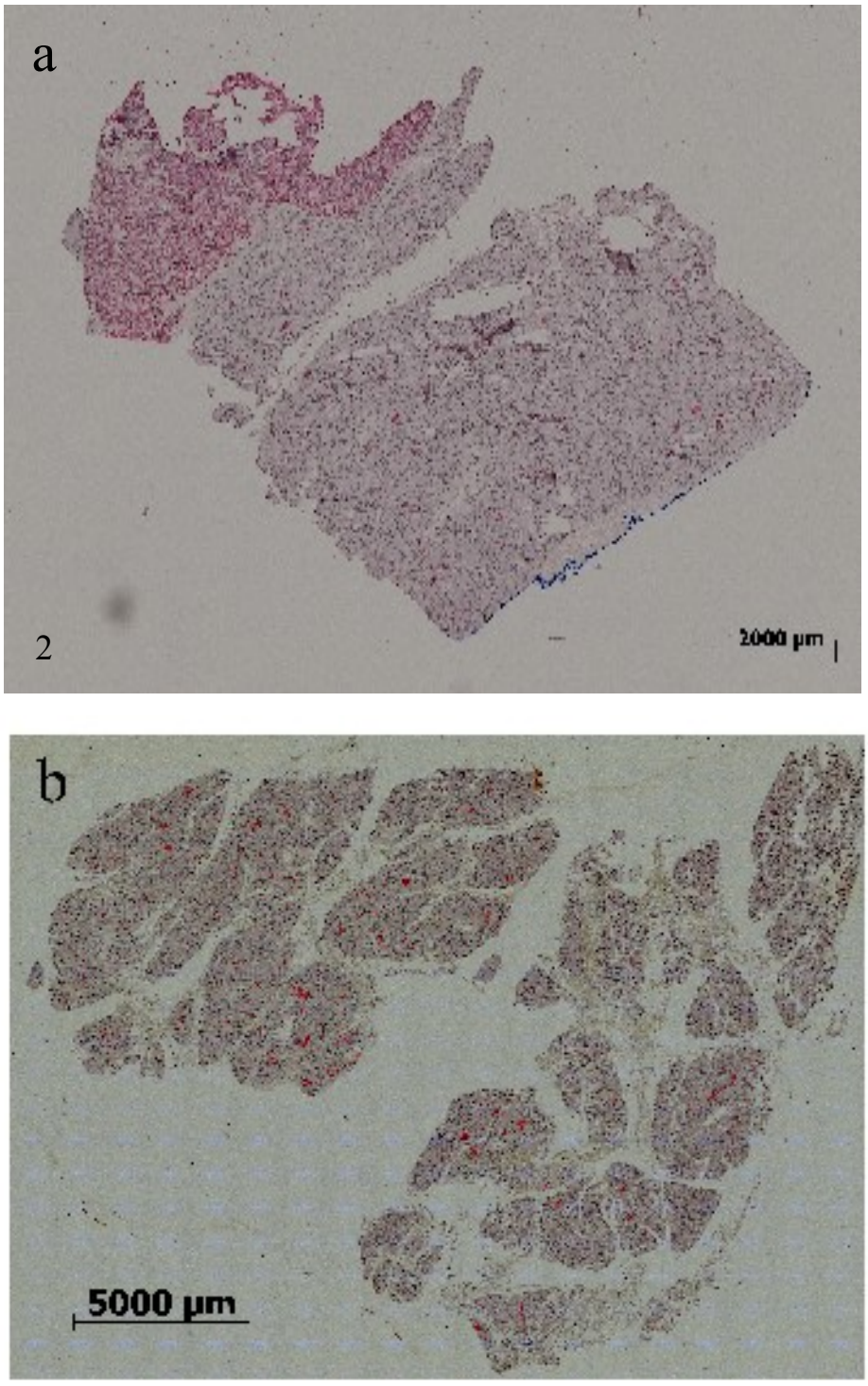


Figure 19: Representative tissue sections from two insulinoma patients (female, age 62 (a1); female, age 56 (a2)) and a control pancreas (female, age 69; B). The large insulin-producing tumor in panel (A) is evident in the centre of the section. The pancreatic sections were immunohistochemically stained for insulin and imaged by scanning the entire tissue section at 100x magnification. The final pictures were created by assembling the individual photos as tile image.

4.2 Quantitative morphometry

Fractional beta-cell area was 1.11% ± 0.30% in the tumor-free pancreatic tissue of the insulinoma patients and 0.78% ± 0.08% in the control group (p = 0,19) (Figure 20).

There also were no differences in either mean islet size (6121.9 ± 506.3 μm^2 vs. 6879.8 ± 992.8 μm^2, respectively, p = 0.62) (Figure 21) or the nuclear diameter of the beta-cells (6.48 ± 0.30 µm vs. 6.87 ± 0.15 µm, respectively, p = 0.20) (Figure 22) between both groups. Thus, the morphometric analysis of the tissue samples did not reveal any evidence of either beta-cell hypertrophy or beta-cell hyperplasia.

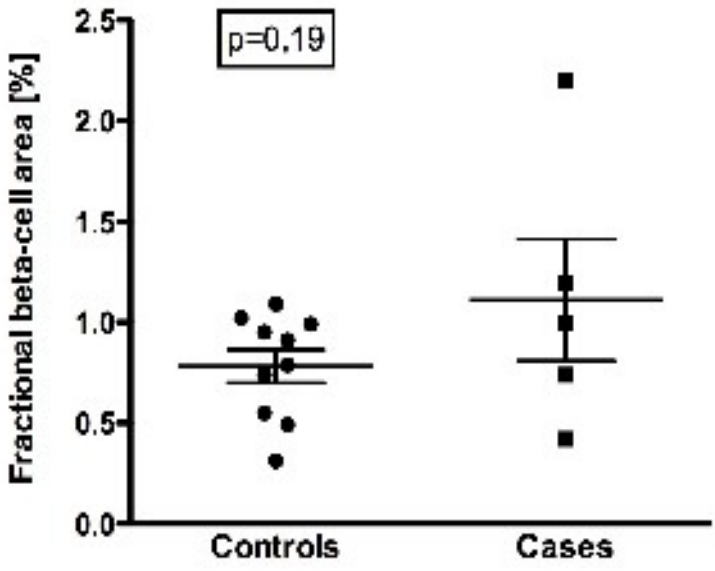


Figure 20: Fractional beta-cell area in the tumor-free pancreatic tissue of five insulinoma cases and ten control subjects. Results are presented as means ± SEM. Statistics were carried-out using Students' t-test.

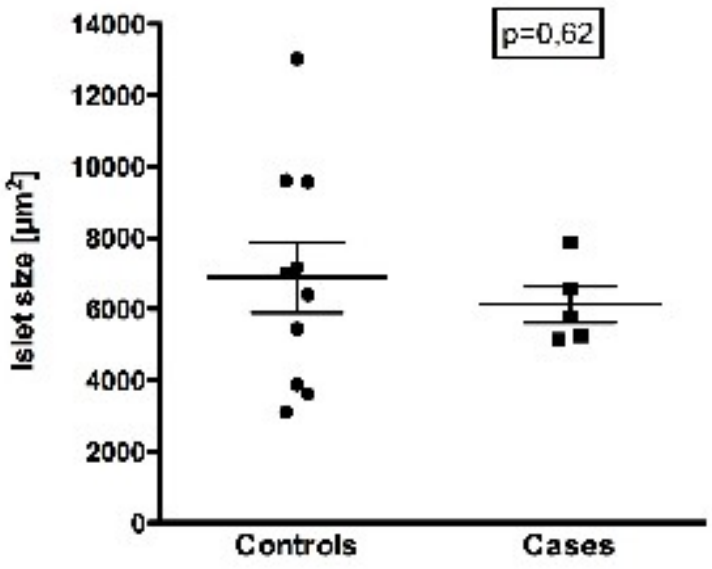


Figure 21: Islet size in the tumor-free pancreatic tissue of insulinoma cases and control subjects. Results are presented as means ± SEM. Statistics were carried-out using Students' t-test.

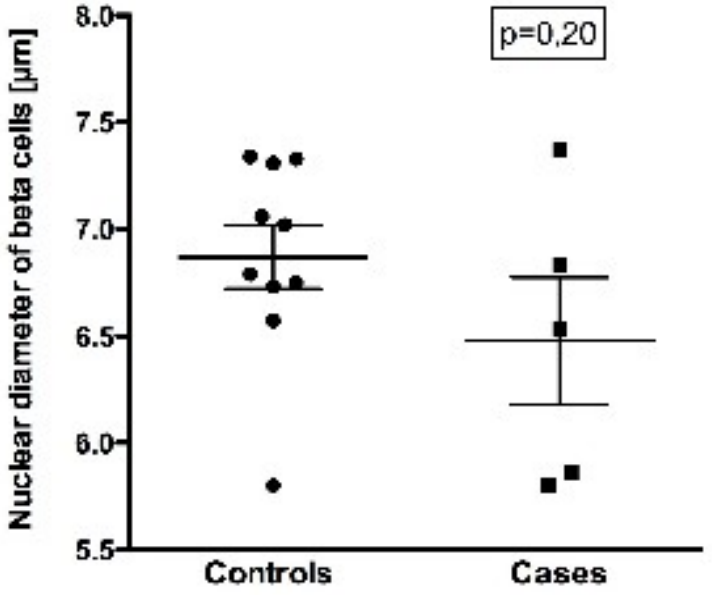


Figure 22: Nuclear diameter of beta cells in the tumor-free pancreatic tissue of insulinoma cases and control subjects. Results are presented as means ± SEM. Statistics were carried-out using Students' t-test.

4.3 Beta cell turnover

Beta-cell replication, as determined by specific Ki67 labelling, was observed infrequently in both the insulinoma patients and controls (0% vs 0.03% ± 0.02%, respectively) (Figure 23). There were no obvious differences in the frequency of beta-cell replication between the groups, although the overall number of replicating cells was too low to calculate a meaningful percentage. However, beta-cell replication was abundantly detected in the actual tumor tissue, thereby affirming the validity of the staining.

In order to estimate the potential contribution of ductal islet neogenesis, the percentage of ductal cells expressing insulin was quantified as well. However, there were also no significant differences in this surrogate parameter (0.41% ± 0.26% vs. 0.24% ± 0.10%, respectively; $p = 0.47$) (Figure 24).

Beta-cell apoptosis was determined by means of TUNEL labelling. There was a very low number of apoptotic beta-cells in both groups, with no detectable differences in the frequency of TUNEL staining between insulinoma patients and controls (0.04% ± 0.02% vs 0%). Examples of TUNEL-positive beta-cells and exocrine tissue are given in figure 25a and b, respectively.

When the four control subjects with increased fasting glucose concentrations were excluded from the analyses, the results remained similar, with no significant difference in any of the parameters being detectable between insulinoma patients and controls.

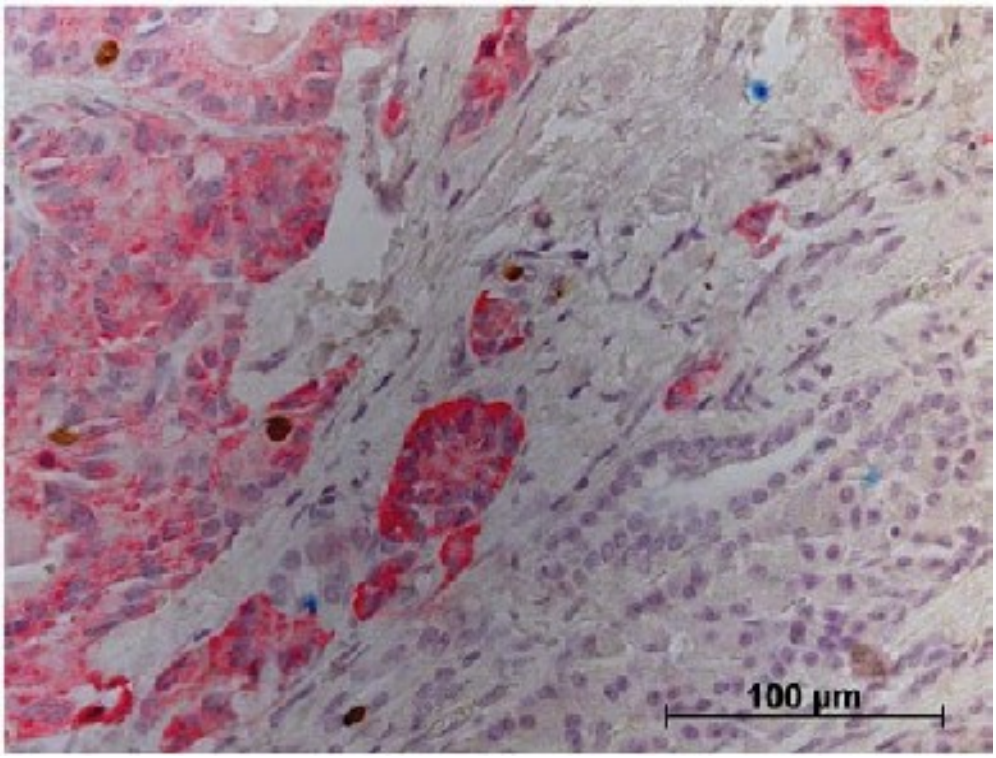
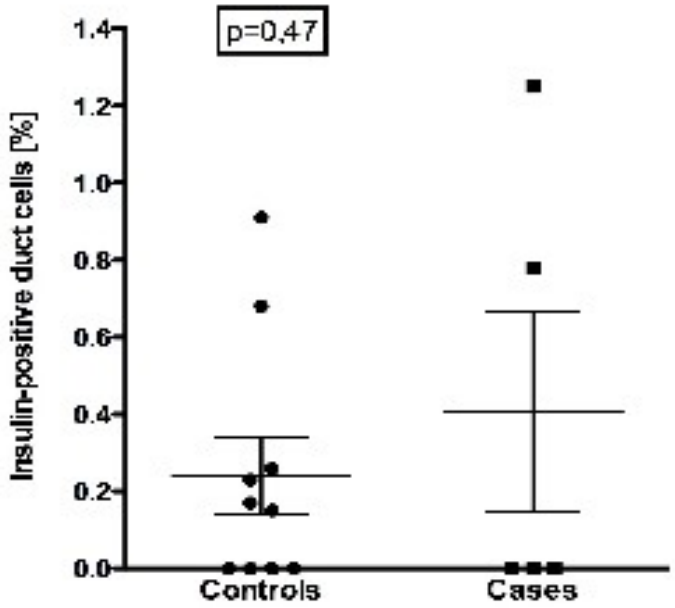
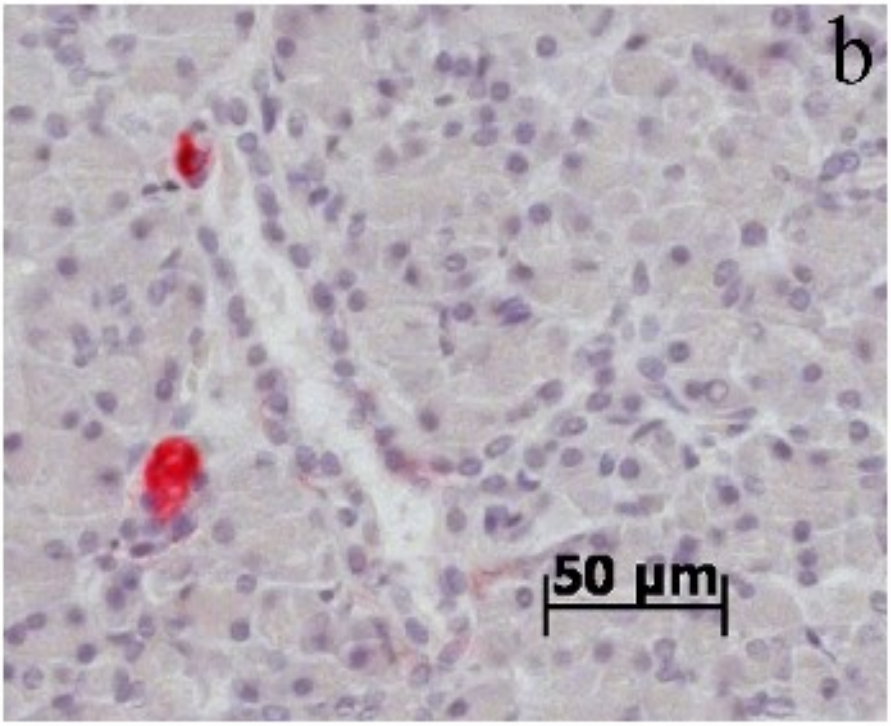


Figure 23: Representative tissue section from an insulinoma patient (female, age 62) stained for Ki67 (brown) and insulin (red). A number of Ki67-positive beta-cells is found within the tumour (upper left), whereas replication occurs only sporadically in the adjacent exocrine tissue. The picture was taken at 400Å~ magnification.


p=0,47
Insulin-positive duct cells [%]
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Controls
Cases

a


50 µm

b

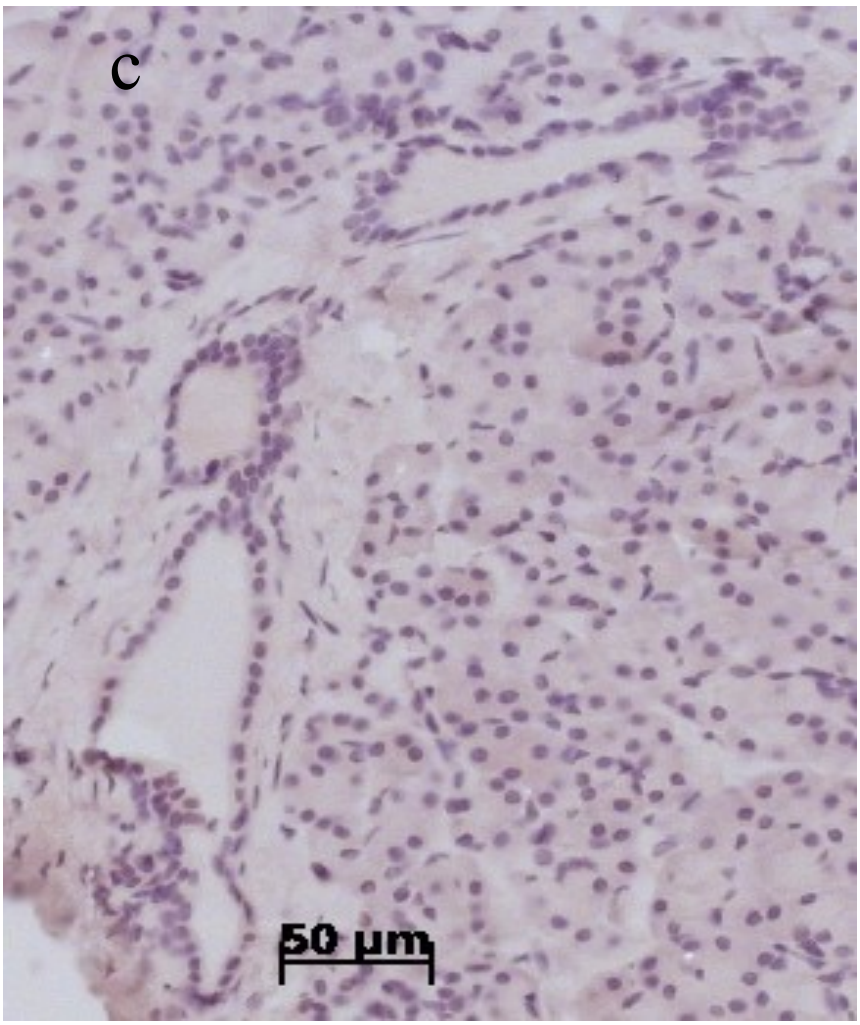


Figure 24: Frequency of insulin-positive duct cells in five insulinoma cases and ten control subjects. Results are presented as means ± SEM. Statistics were carried-out using Students' t-test (a). Representative picture of an insulin-positive pancreatic duct cell in an insulinoma case (male, age 51) (b) and a control subject (female, age 58) (c). The section was immunohistochemically stained for insulin and imaged at 200-fold magnification.

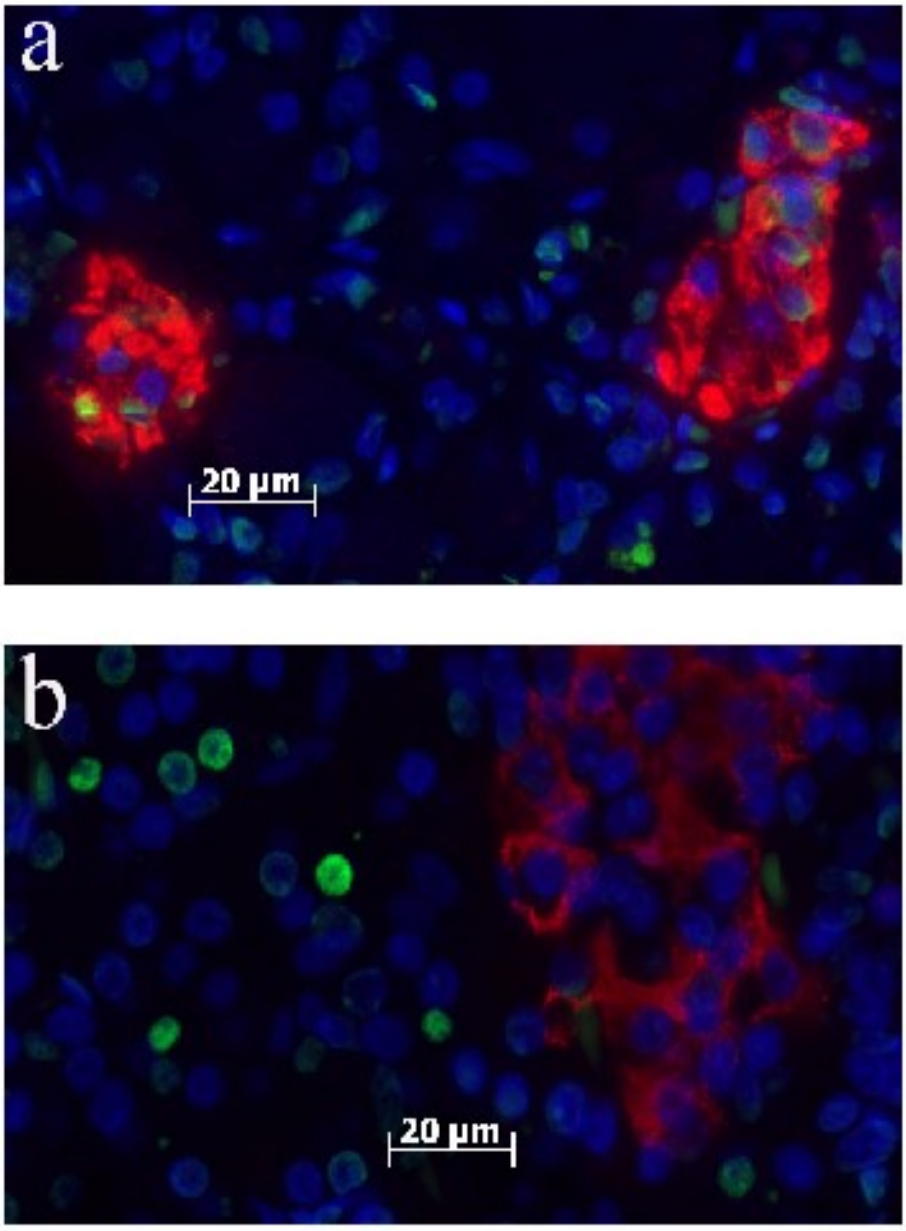


Figure 25: Representative figures showing a TUNEL-positive beta-cell in an insulinoma case (A; female, age 56) and in the exocrine pancreatic tissue of a control patient (B; female, age 69 years). The sections were stained for TUNEL (green) and insulin (red). The pictures were taken at 400x magnification.

5. DISCUSSION (I)

The present study was undertaken to examine, whether the unregulated insulin release from intra-pancreatic insulinomas would influence beta-cell mass and turnover in the surrounding pancreatic tissue. A detailed morphometric analysis of human pancreatic specimens from five patients with intra-pancreatic insulinomas and ten control subjects did not reveal any differences in either beta-cell area, beta-cell replication and islet neogenesis, or beta-cell apoptosis between the groups.

The results of the present study in insulinoma patients are at variance with a previous evaluation of human pancreatic tissue from patients with intra-pancreatic gastrinomas (76). Using a similar experimental approach, this previous study had revealed a marked increase in fractional beta-cell area and beta-cell replication in the pancreatic tissue immediately adjacent to the tumors. These findings have lent support to the concept of an induction of beta-cell replication by gastrin.

There has been a long-standing debate as to the potential preservation of beta-cell mass by insulin. Thus, in tissue culture studies high concentrations of insulin have been found to induce beta cell replication and reduce beta-cell apoptosis (77), and experiments in beta-cell specific insulin receptor knock-out (βIRKO) mice have revealed a significant reduction in beta-cell mass and age-dependent manifestation of diabetes in the affected animals, thereby supporting a role for endogenous insulin in the growth and maintenance of beta cell mass (129, 130).

Furthermore, clinical studies directly comparing endogenous insulin secretion after two years of glucose-lowering therapy with either pre-mixed insulin or glibenclamide have shown a significant preservation of beta-cell function by the insulin therapy approach (79). In a similar way, early initiation of insulin therapy in patients with newly-onset type 2 diabetes has led to a marked preservation of beta-cell function (80), a finding reminiscent of the “honeymoon period” often observed in patients with type 1 diabetes after the initiation of insulin therapy (131).

This potential preservation of beta-cell function by insulin could theoretically be mediated by two different mechanisms: Thus, a direct effect of insulin on beta-cell survival has been suggested in a recent series of elegant studies (77). These experiments have pointed towards an Akt-independent pathway, potentially involving the protein bridge-1, a Pdx1-binding partner and regulator of beta-cell survival. The second mechanism by which exogenous insulin could potentially reduce the extent of beta-cell apoptosis involves the mechanism of beta-cell rest. According to this concept, a chronically increased insulin secretory demand, induced by e.g. Hyperglycaemia, insulin resistance of beta-cell deficiency, leads to an over-stimulation of insulin secretion, which subsequently drives the beta-cells to increased insulin biosynthesis (132). The chronic up-regulation of insulin biosynthesis may then lead to protein misfolding, the induction of endoplasmatic reticulum (ER) stress, and ultimately beta-cell apoptosis (133-135). According to such reasoning, exogenous insulin treatment might lower the secretory demand on the beta-cells, thereby potentially protecting the cells from undergoing apoptosis (136, 137).

However, while local over-production of insulin from insulioma tissue may on the one hand protect beta-cells from apoptosis, an alternative scenario is also conceivable: Thus, it is a well-known phenomenon from other endocrine organs that local hyperplasia and excessive hormone secretion can lead to a compensatory regression of the non-affected cells. Such mechanism characterises for example the regulation of cortisol secretion from the contralateral adrenal gland in cases of unilateral adrenal adenomas (138, 139). Assuming, that such compensatory mechanism exists in the islets as well, one might even expect a reduction in beta-cell area as well as an increase rate of beta cell apoptosis in the tumor-free pancreas from insulinoma patients.

In the present experiments, neither pancreatic beta-cell area, nor beta-cell replication and islet neogenesis, nor beta-cell apoptosis were different between the tumor-free tissue from insulinoma patients and control subjects. While this may suggest that the effects of insulin on beta-cell proliferation in adult humans in vivo are less pronounced that one might have expected from rodent studies, it is also well possible that the protective effects of the locally elevated insulin concentrations were completely outbalanced by a compensatory regression of the insulinoma-free pancreatic tissue.

Some limitations need to be born in mind with respect to the present results: first, the control group was comprised of patients with benign pancreatic adenomas and extrapancreatic tumors. This was done by necessity, because patients without any pancreatic diseases are not readily subjected to pancreatic surgery. However, in the present cases,

any obvious alterations of the pancreatic tissue were excluded by a careful pathological examination of the tissue.

Secondly, the overall frequencies of beta-cell replication and apoptosis were relatively small, thereby not allowing for formal statistical comparisons. Therefore, it is still possible that minor differences in islet cell turnover were overlooked because of the small numbers. Along the same line, it is conceivable that significant differences between insulinoma patients and control subjects would become detectable if a larger number of cases were examined. However, in previous studies about the regulation of beta-cell area and turnover in humans, the differences in beta-cell replication between the groups were often several-fold (76, 140, 141), suggesting that if present at all, any difference between the insulinoma patients and control subjects would be minor at best.

While the present results therefore need to be interpreted with some caution, it should be noted that any direct attempt to examine beta-cell mass and turnover after exogenous insulin treatment is clearly limited by the obvious in-availability of human pancreatic specimens from healthy human subjects for study purposes. Also, examining the pancreas of surgical patients receiving pre-operative insulin treatment for clinical reasons bears the unavoidable risk of a strong selection bias, because insulin treatment is typically prescribed at more advanced stages of diabetes. Therefore, even though the present findings need to be carefully interpreted, they may be of some clinical relevance in order to judge, whether raising circulating insulin levels preserves of beta-cell mass and turnover in living humans.

Although the present study did not reveal any evidence of beta-cell protection by endogenous hyperinsulinaemia in the pancreas of patients without diabetes, this may well be different in a group of diabetic patients with overt hyperglycaemia. In fact, chronic hyperglycaemia has long been recognised as a key factor leading to the induction of beta-cell apoptosis (142, 143). It is therefore possible, that the local release of insulin from intra-pancreatic insulinomas has a much greater effect on beta-cell mass and turnover in patients with overt diabetes. However, because the coincidence of type 2 diabetes and insulinoma is quite rare, such pancreatic specimens were unfortunately not available for the present analyses.

6. MATERIALS AND METHODS (II)

6.1 Pancreatic tissue samples

6.1.1 Patients with type 2 diabetes

Human pancreatic tissue was obtained at surgery from 7 patients (Table 3). Of these patients, 4 underwent surgery for the removal of benign pancreatic adenomas, 1 for the removal of intraductal papillary mucinous tumor, 2 underwent pancreatectomy because of tumors of the ampulla or papilla of Vater. All the patients had a history of known diabetes and were taking antidiabetic medications (Table 4).

6.1.2 Patients with diabetes secondary to pancreatic disease

Human pancreatic tissue was obtained at surgery from 10 patients (Table 3), who underwent pancreatic resections. Of these patients, 7 had been diagnosed with chronic pancreatitis and 3 with pancreatic carcinoma. In all cases, the clinical diagnosis was confirmed by a trained pathologist. All the patients had a history of known diabetes and were under antidiabetic treatment (Table 4).

6.1.3 Control subjects

11 non diabetic patients were chosen as controls (Table 2) as they underwent surgery for the removal of tumours not influencing the beta cell mass and function: 7 had benign pancreatic adenomas, 2 had intraductal papillary mucinous tumor and 2 had tumors of the ampulla of Vater and of the ductus choledochus, respectively. Significant alterations of the overall pancreatic integrity were excluded by careful histological examination and none of the control subjects had a history of diabetes.

Table 3. *Characteristics of patients.*

	Control subjects	**Patients with type 2 diabetes**	**Patients with diabetes secondary to pancreatic disease**
SEX (F+M)	8+3	4+3	6+4
AGE (years)	59 ± 14.5	68.6 ± 8.1	63 ± 11.7
FASTING BLOOD GLUCOSE (mg/dl)	97.55 ± 12.4	128.3 ± 45.8	147.5 ± 19.3
HbA1c (%)	5.62 ± 0.33	7.5 ± 1.14	7.7 ± 1.17

Data are means ± SD.

Table 4. *Patients subdivision according to antidiabetic treatment.*

	Patients with type 2 diabetes		***Patients with diabetes secondary to pancreatic disease***			
	Insulin	***Oral***	***Insulin***	***Oral***	***Diet***	***None***
NUMBER	4	3	6	1	2	1
SEX (F+M)	2+2	2+1	3+3	0+1	2+0	1+0
AGE	71.8±3.5	64.3±11.5	58±10.7	62	79±1.4	62

Data are means ± SD.

6.2 Fluorescence and immunohistochemical tissue staining

Pancreatic tissue was fixed in formaldehyde and embedded in paraffin. Sequential 5 µm sections were stained as follows:

6.2.1 Fractional beta cell area

To assess the fractional beta-cell area, all tissue sections were immunohistochemically stained for insulin and DNA as follows: After heating at 37°C over night or at 58°C for 30 minutes, pancreatic tissue sections were deparaffinized in two subsequent 10-minutes xylene-incubations and subsequently hydrated through graded alcohol-series (100%, 95%, 70%, water). Antigen retrieval was performed in Dako Cytomation Target Retrieval Solution pH 6 (Dako, Glostrup, Denmark, Code S2369) by heating for 30 minutes. After cooling of the samples to room temperature and a brief rinse in distilled water, sections were subsequently blocked for unspecific protein binding with TBS containing 3% BSA and 0,2% Triton X100 for 10 minutes, for endogenous alkaline phosphatase causing unspecific staining by use of Levamisole from the Dako-Envision Dual Link System (Dako, Glostrup, Denmark, Code K4065) and for endogenous biotin by use of the Dako Cytomation Biotin Blocking System (Dako, Glostrup, Denmark, Code X0590). Then, a guinea pig antiserum against pig insulin was applied for 30 minutes at a dilution of 1:400 (Dako, Glostrup, Denmark, Code A0564). Subsequently, insulin was stained red by the use of the Dako-LSAB Real Detection System (Dako, Glostrup, Denmark, Code K5005) according to the manufacturer`s recommendations with 5-minutes TBS-washes between the incubations.

For a blue nuclear counterstaining, specimens were incubated in hematoxylin (Dako, Glostrup, Denmark, Code S3301) for 5 minutes followed by a 5-minutes tap water-incubation. Finally, samples went through a series of ascending alcohol concentrations (70%, 96%, 100%) and 5-minutes xylene-incubation and were mounted with Entellan (Merck, Darmstadt, Germany, Code 1.07961.0/00). Under omission of the primary antibodies, no staining could be observed.

6.2.2 Beta cell apoptosis and islet amyloid

To investigate the beta-cell apoptosis and the amount of amyloid in the islets, pancreatic tissue sections were stained simultaneously for DNA strand breaks, insulin, amyloid and DNA as follows: Pancreatic tissue sections went through heating and deparaffinizing procedures as above described. Antigen retrieval was performed in Dako Cytomation Target Retrieval Solution pH6 (Dako, Glostrup, Denmark, Code S 2369) for 30 minutes. For the detection of islet amyloid deposits, the sections were stained with Thioflavin S (Sigma, St Louis, USA, Code T1892-25G) for 8 minutes at room temperature. Then, TUNEL staining was performed by using the in situ cell death detection kit from Roche (Roche, Mannheim, Germany, Code 12 156 792 910), according to the recommendations of the manufacturer. Prior to the immunostaining for insulin, samples were blocked for unspecific binding of the antibodies with TBS containing 3% BSA and 0,2% Triton X100 for 15 minutes. For the detection of insulin, a guinea pig anti-insulin antibody was applied for 30 minutes in a dilution of 1:400 followed by the incubation in Dylight 649-labelled secondary

antibody developed in goat (Jackson Immuno Research, W. Baltimore Pike, USA, Code 706 495 148) that was applied in a dilution of 1:100 for 30 minutes. All antibodies were diluted in Antibody Diluent with Background Reducing Components (Dako, Glostrup, Denmark, Code S3022). Finally, the nuclei were stained with DAPI by incubation in a 0,33 µg/ml DAPI (Invitrogen, Paisley, UK,Code SKU#1306) solution in PBS for 5 minutes. Following 5-minute-incubations in 3 changes of distilled water, the tissue sections were mounted with Dako Fluorescence Mounting Medium (Dako, Glostrup, Denmark, Code S3023) and stored at 4-8°C in the dark. Positive and negative controls were run with each batch of samples. DNAse-treated and kidney tissues served as a positive control for the TUNEL reaction and the Thioflavin S staining, respectively. As a negative control, primary antibodies, terminal transferase and thioflavin S were omitted.

6.3 Morphometric analysis

For all morphometric analysis, pancreatic tissue specimens stained immunohistochemically as above described were used. All measurements were performed by applying specific tools of the software Axiovision (version 4.5) (Zeiss, Oberkochen, Germany) to images of the tissue samples that were generated by an Axioplan 2-microscope (Zeiss, Oberkochen, Germany) at appropriate magnifications.

6.3.1 Fractional beta-cell area

The fractional beta-cell area of all tissue sections was determined by imaging the entire pancreatic section using the Axioplan microscope equipped with a motorized-stage, at 100-fold magnification. The Mosaix tool of the software Axiovision was used to create a tile image. The pancreatic tissue was surrounded using the contour 'measurement' tool of the software, excluding artefacts or other different kind of tissues, possibly present in the section, from the analysis. Then, we measured the red areas corresponding to the insulin-positive areas in the selected pancreatic tissue using the colour detection 'measurement' tool. We used the same tool also to measure the red and not red areas in the tissue corresponding to the entire pancreatic area. Then, the beta cell area was expressed as percentage of insulin-positive area to the entire pancreatic area.

6.3.2 Beta-cell apoptosis

To determine the frequency of beta-cell apoptosis, 20 random islets

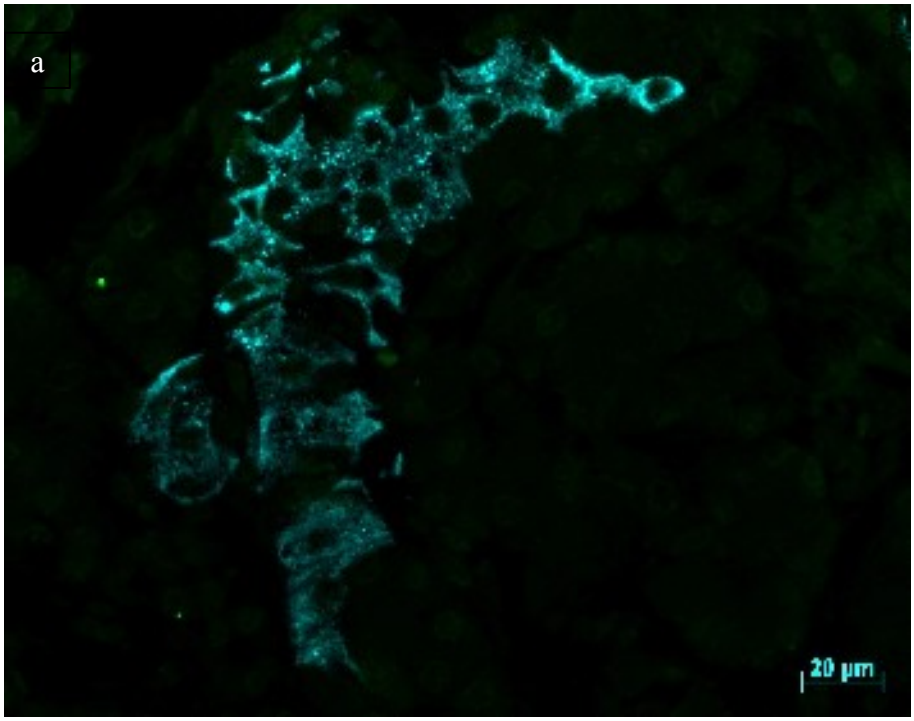
per slide stained for insulin, TUNEL, amyloid and DAPI were imaged at 200-fold magnification using the Axioplan microscope. The number of cells co-stained for insulin and TUNEL was counted manually and expressed in relation to the total number of insulin-positive cells.

6.3.3 Islet amyloid

To determine the islet amyloid, 20 random islets per slide stained for insulin, TUNEL, amyloid and DAPI were imaged at 200-fold magnification using the Axioplan microscope. The islets were scored for the extent of islet amyloid using a scale from 0 to 5, where 0 indicated that there were no amyloid deposits in the islets, 1 indicated the presence of small amyloid deposits (mainly spherical puncta and curvilinear structure) in the islets, 2 indicated the presence of bigger amyloid deposits (spherical puncta and larger curvilinear structures) in the islets, 3 indicated the presence of aggregates of amyloid fibrils of small extent in the islets, 4 indicated the presence of aggregates of amyloid fibrils of mid extent in the islets, 5 indicated the presence of aggregates of amyloid fibrils of wide extent in the islets. In the case of islets with score 4 and 5, the “measurement” tool of the software Axiovision (Zeiss, Oberkochen, Germany) was used to measure the amyloid positive area in the islet and the total insular area. The amyloid quantity in the islet was expressed as percentage of amyloid positive area to the total islet area. Examples of scores for islet amyloid are given in figures 26, 27, 28, 29 and 30.

6.4 Statistical analysis

Subject characteristics are reported as means ± SD, results are presented as means ± SEM. Statistical calculations were carried out by one-way ANOVA using Prism4 (Graph Pad software Inc., La Jolla, CA, USA).



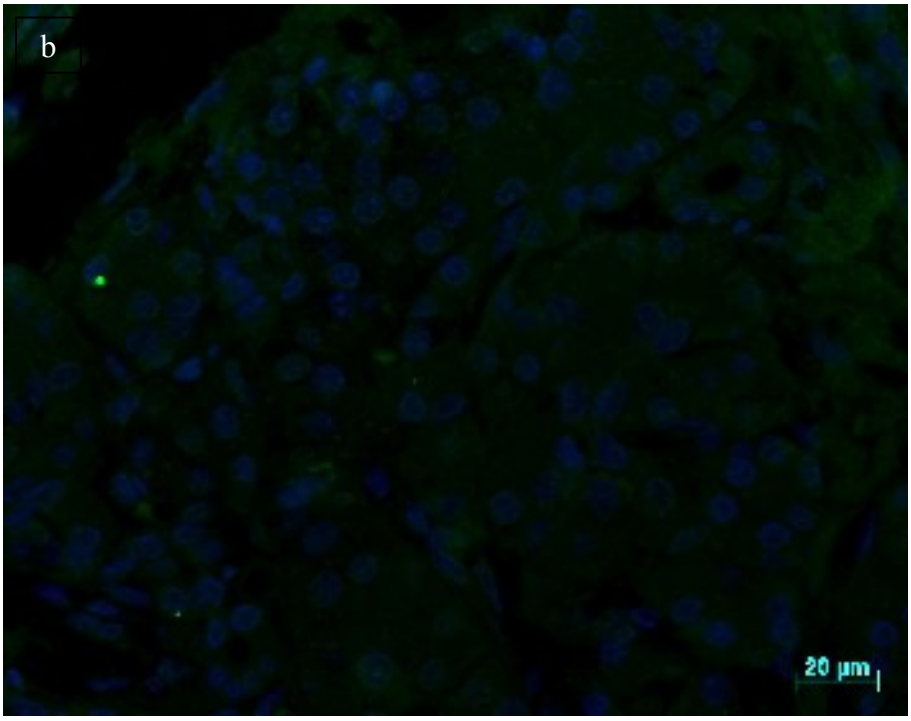


Figure 26: An example of an islet with score 1. Staining for amyloid with Thioflavin S (green) and insulin (cyan) (a). Staining for amyloid with Thioflavin S (green) and nuclei (blue) (b). The pictures were taken at 400-fold magnification.

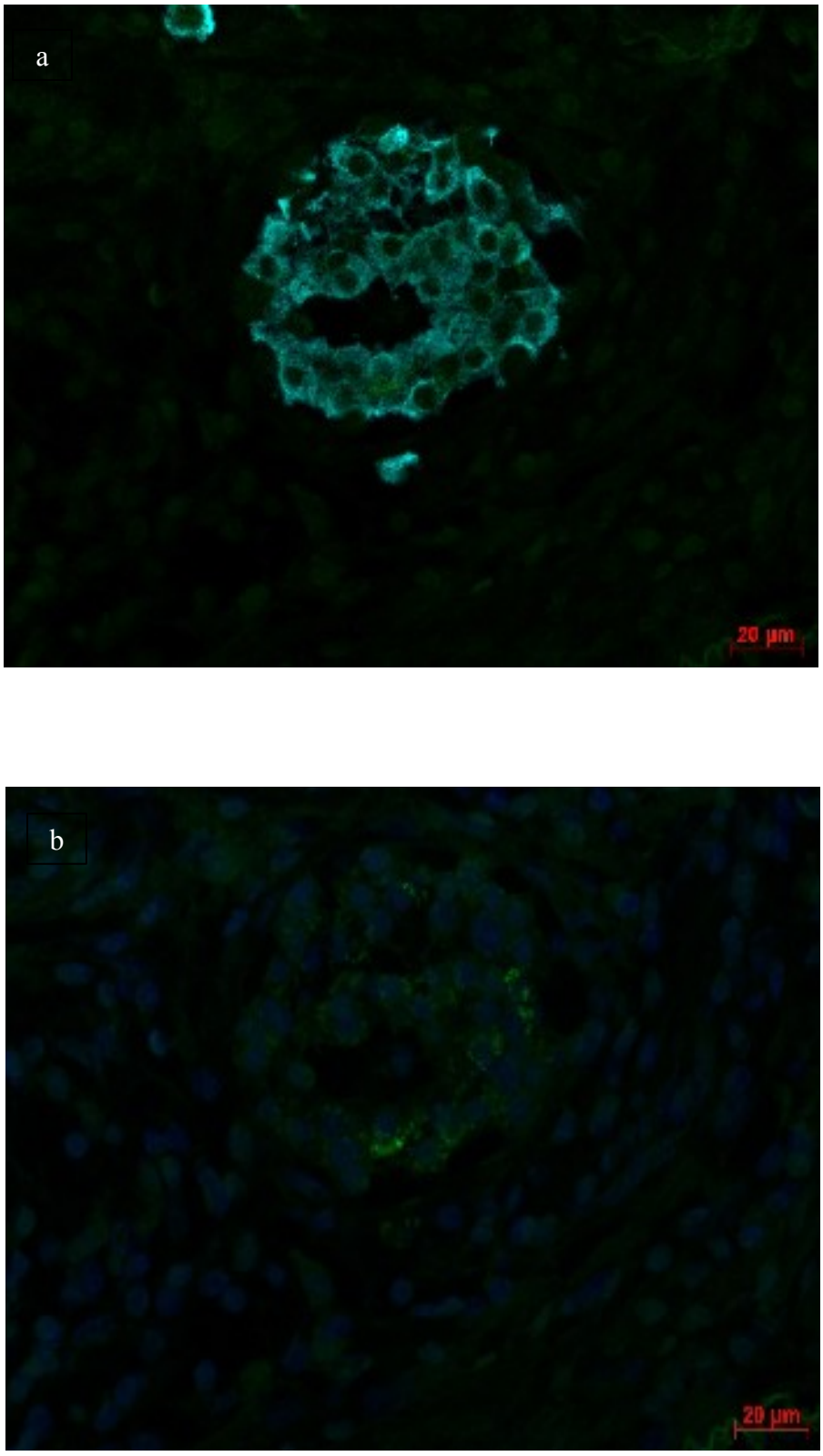


Figure 27: An example of an islet with score 2. Staining for amyloid with Thioflavin S (green) and insulin (cyan) (a). Staining for amyloid with Thioflavin S (green) and nuclei (blue) (b). The pictures were taken at 400-fold magnification.

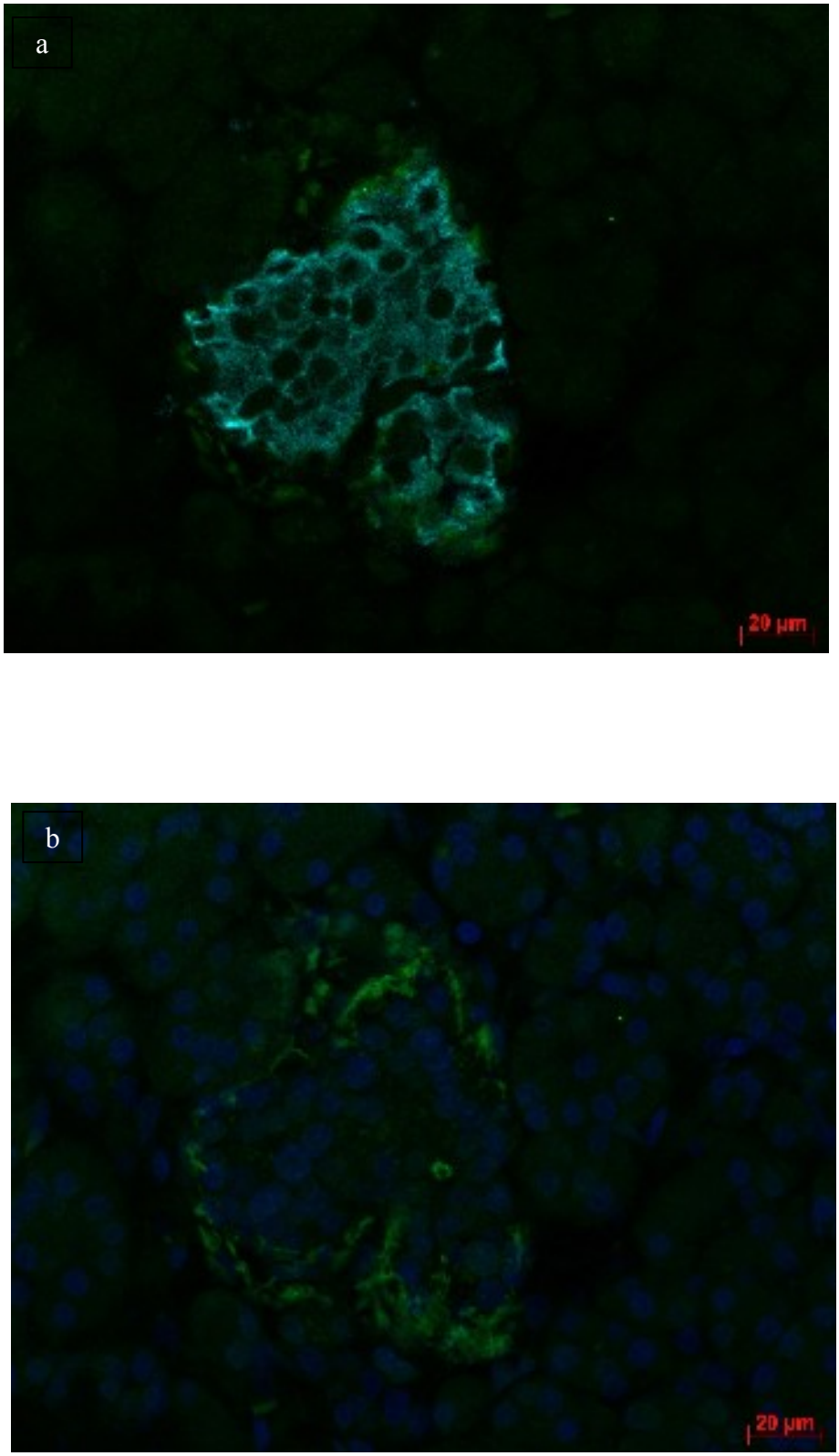


Figure 28: An example of an islet with score 3. Staining for amyloid with Thioflavin S (green) and insulin (cyan) (a). Staining for amyloid with Thioflavin S (green) and nuclei (blue) (b). The pictures were taken at 400-fold magnification.

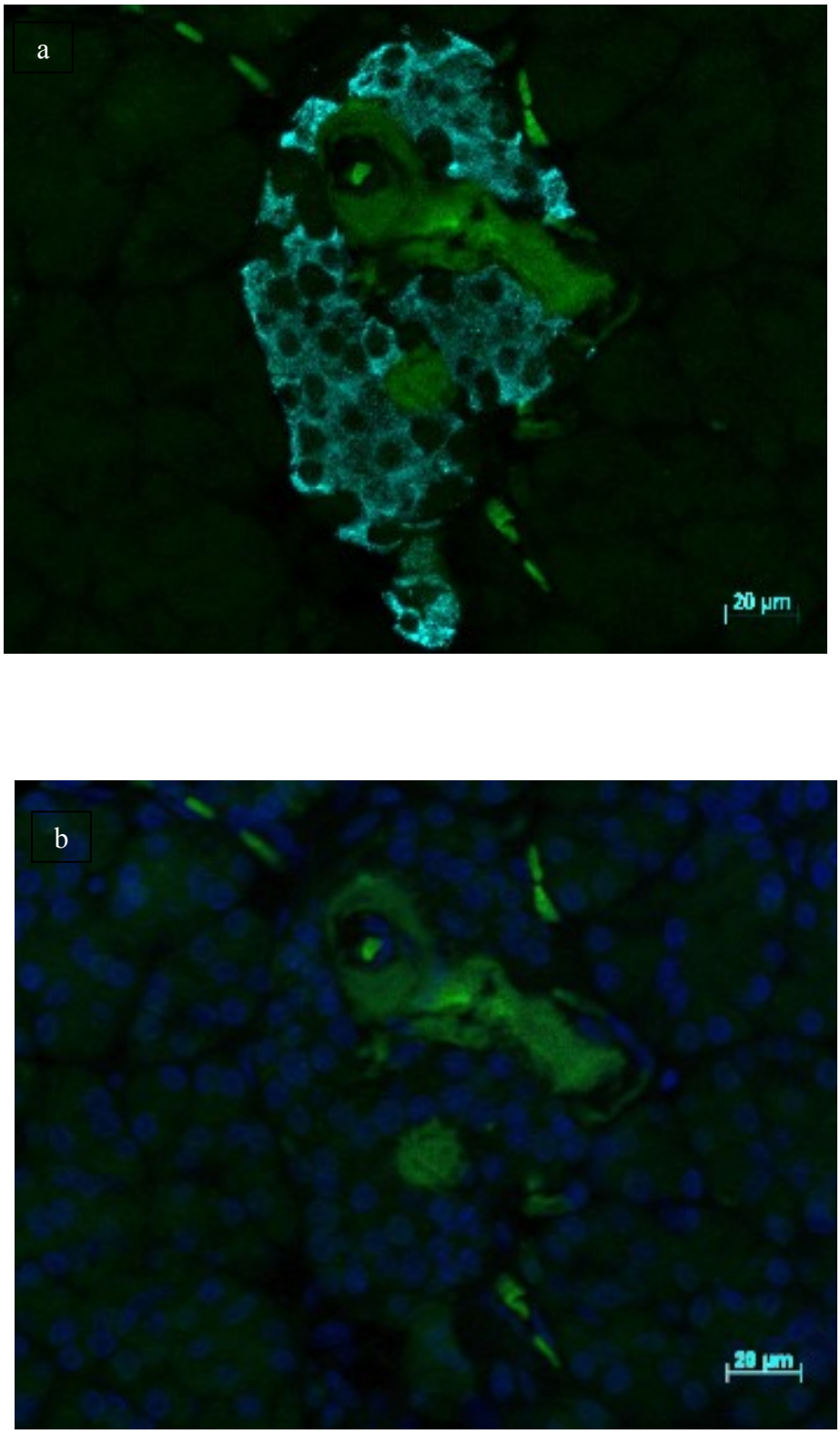


Figure 29: An example of an islet with score 4. Staining for amyloid with Thioflavin S (green) and insulin (cyan) (a). Staining for amyloid with Thioflavin S (green) and nuclei (blue) (b). The pictures were taken at 400-fold magnification.

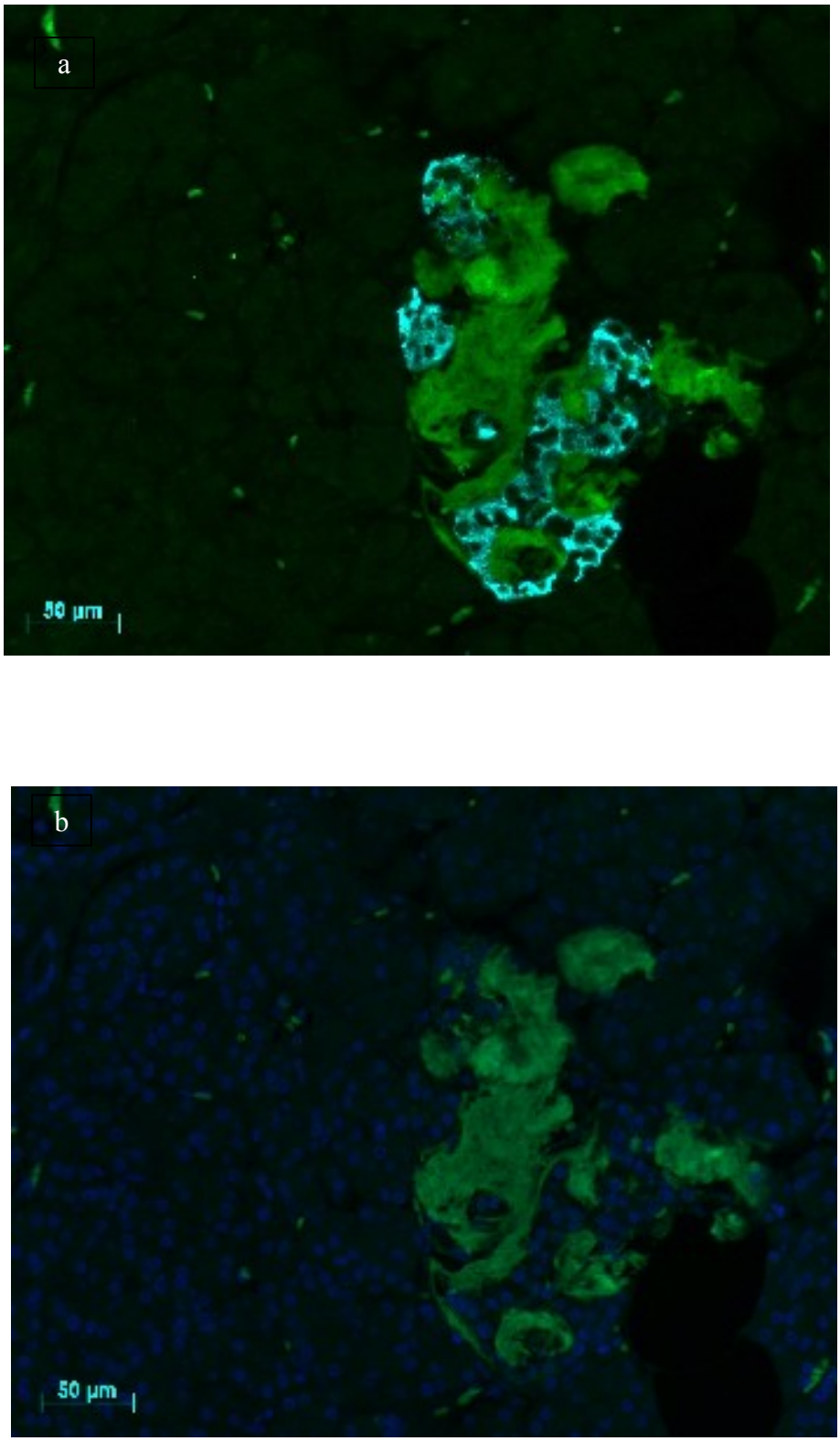


Figure 30: An example of an islet with score 5. Staining for amyloid with Thioflavin S (green) and insulin (cyan) (a). Staining for amyloid with Thioflavin S (green) and nuclei (blue) (b). The pictures were taken at 200-fold magnification.

7. RESULTS (II)

4.1 Quantitative morphometry

There was a significant difference in fractional beta-cell area between all three groups. Fractional beta-cell area was higher in the control subjects than in patients with type 2 diabetes and patients with diabetes secondary to pancreatic disease. Fractional beta-cell area was lower in patients with diabetes secondary to pancreatic disease than in patients with type 2 diabetes *(*0.76% ± 0.13%, 0.57% ± 0.09% and 0.33% ± 0.07%, respectively, p=0.021) (Figure 31). Examples of Islets of Langerhans in the three groups are given in Figure 32 a, b and c.

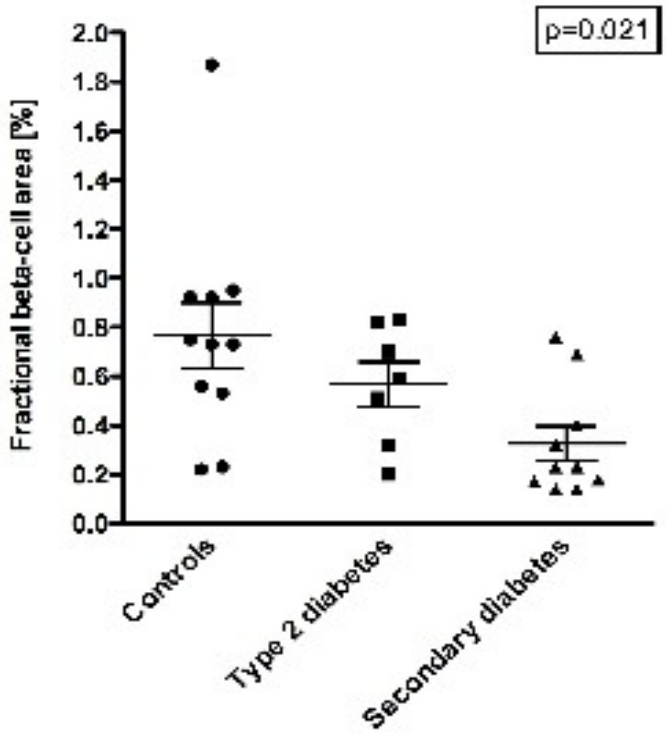
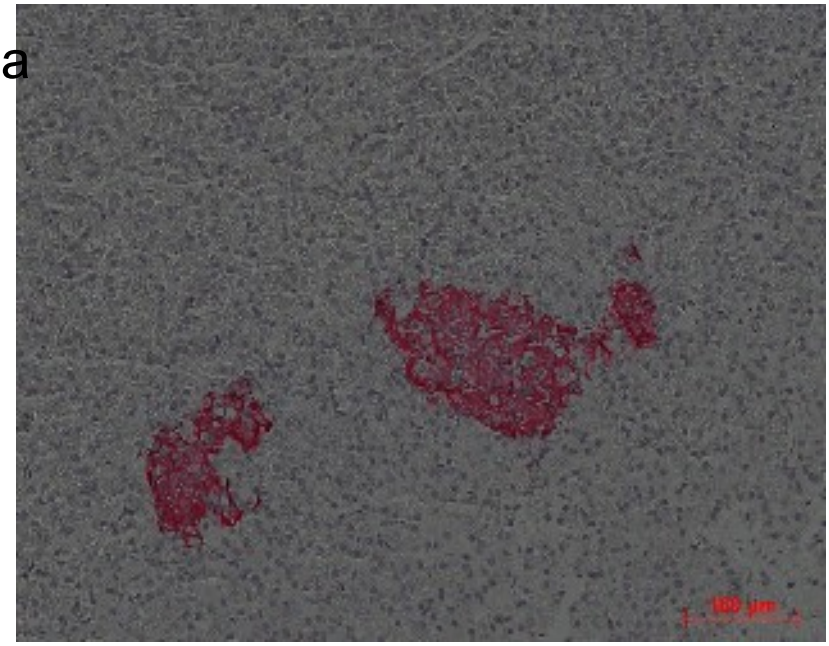
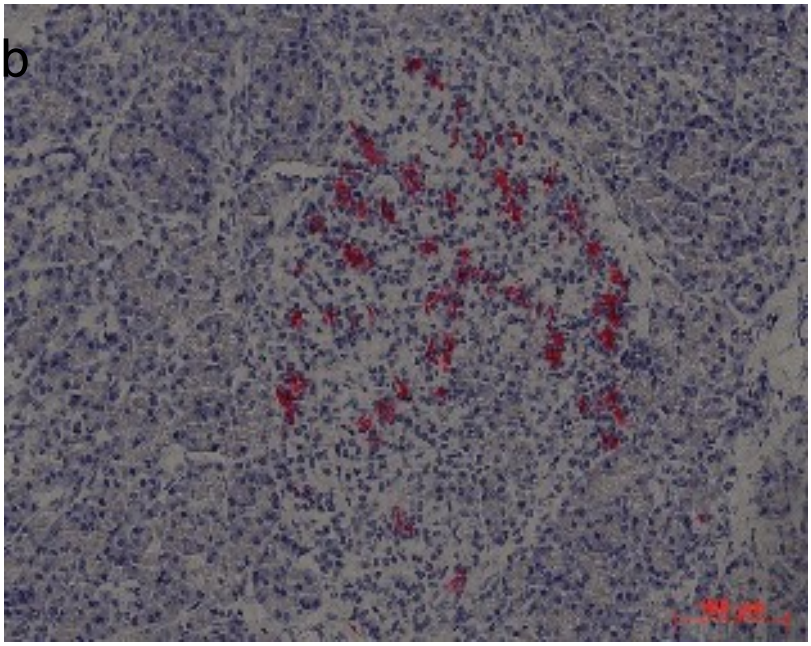


Figure 31: Fractional beta-cell area in patients with type 2 diabetes, patients with with diabetes secondary to pancreatic disease and patients without diabetes (*controls*). Results are presented as means ± SEM. Statistics were carried-out using one-way ANOVA. *P<0.05.

a



b



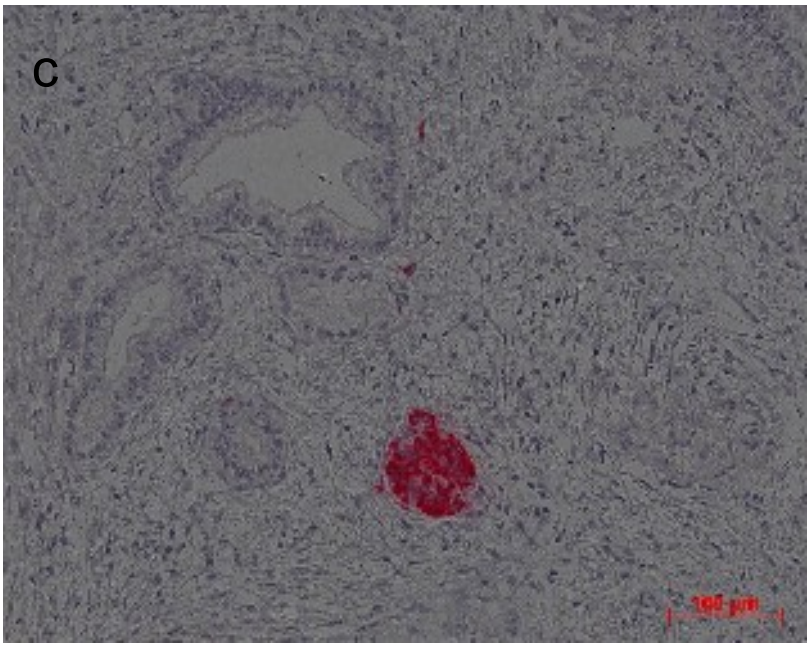


Figure 32: Representative tissue sections from a) control subject without diabetes (a; female, age 69), b) patient with type 2 diabetes (b; male, age 68) and c) patient with pancreatic carcinoma and type 2 diabetes (c; female, age 78). The pancreatic sections were immunohistochemically stained for insulin and imaged at 200-fold magnification.

4.2 Beta-cell apoptosis

The overall frequency of apoptosis was relatively small, thereby not allowing for formal statistical comparisons. Anyway, beta cell apoptosis was observed more frequently in patients with diabetes secondary to pancreatic diseases. Beta-cell apoptosis was 0.07 % ± 0.07 % in the control group, 0.05 % ± 0.05 % in patients with type 2 diabetes and 0.58 % ± 0.29 % in patients with diabetes secondary to pancreatic diseases (p=0.10) (Figure 33). Examples of TUNEL-positive beta cells and TUNEL-negative beta cells are given in Figure 34 a, b and c.

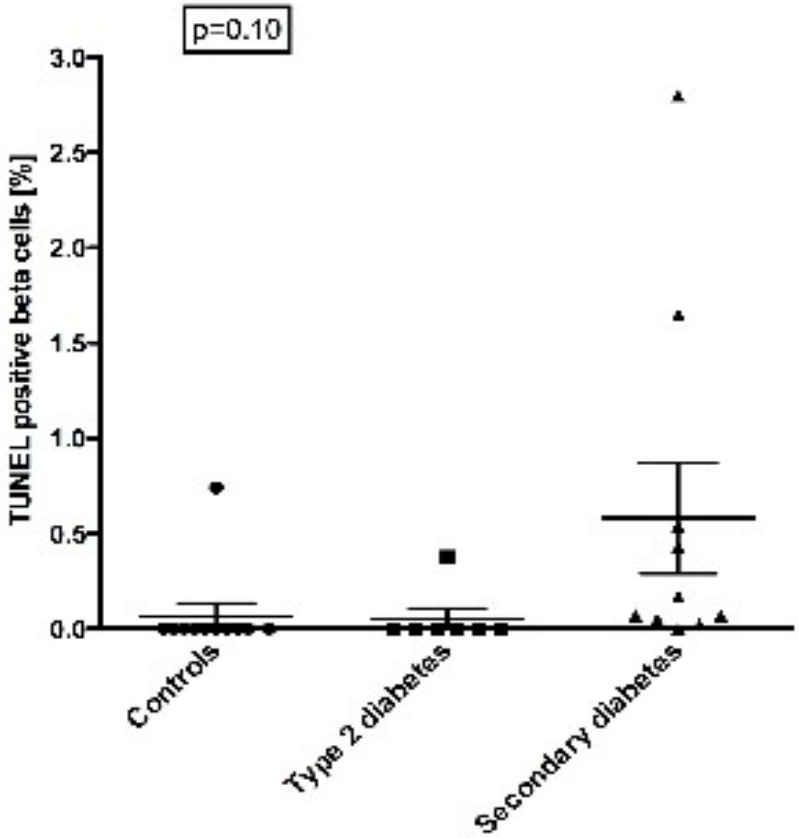
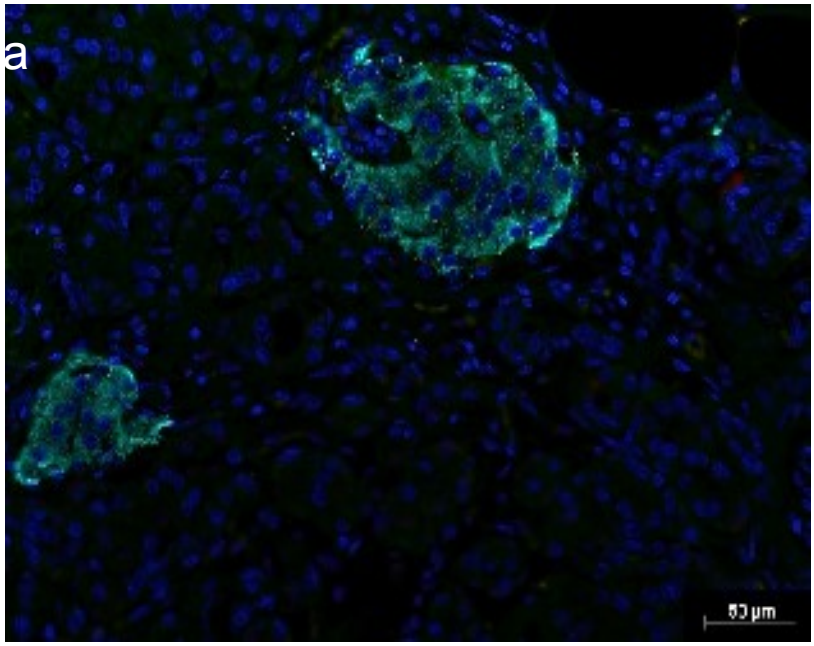
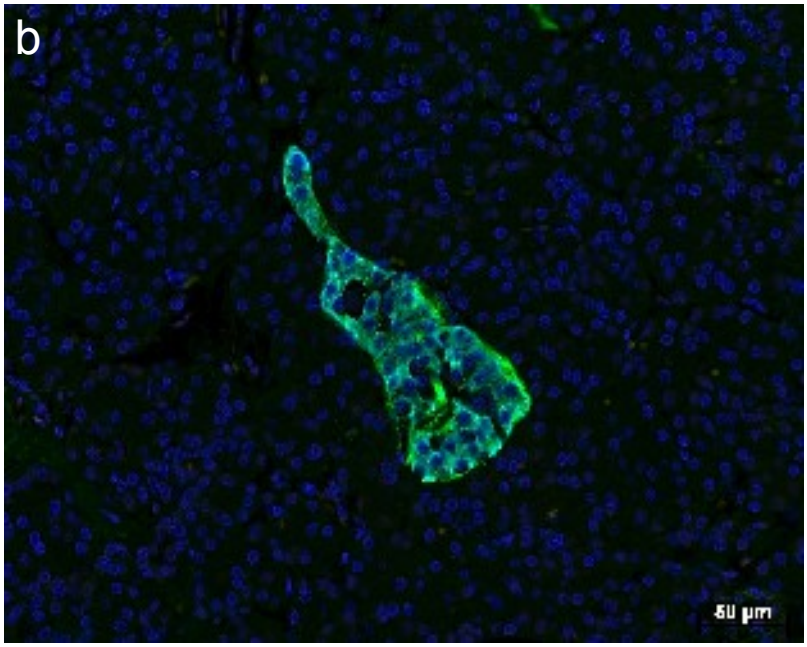


Figure 33: Frequency of TUNEL positive beta cells in patients with type 2 diabetes *(cases 1),* patients with chronic pancreatitis or pancreatic carcinoma and type 2 diabetes (*cases 2*) and patients without diabetes (*controls*). Results are presented as means ± SEM. Statistics were carried-out using one-way ANOVA.


a
50 µm


b
50 µm

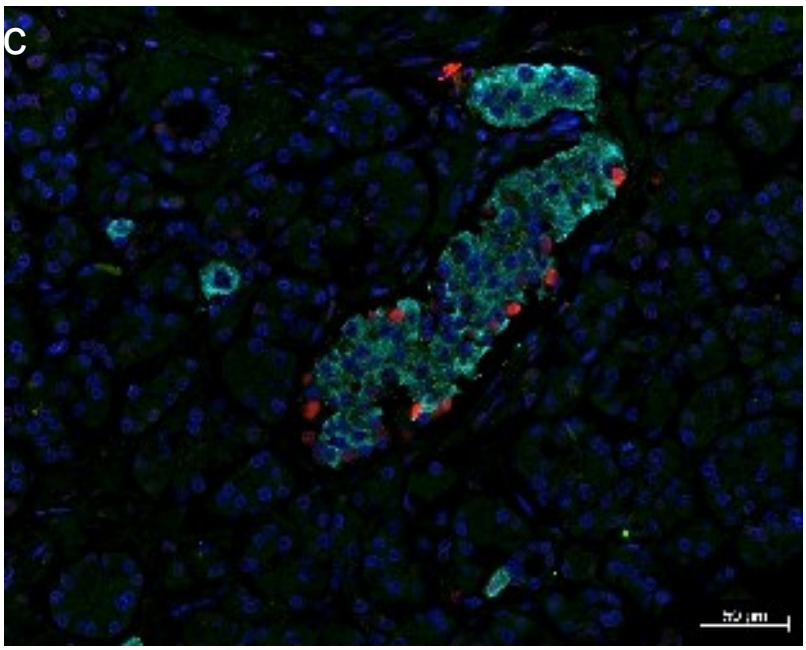


Figure 34: Representative tissue sections from a) control without diabetes (a; female, age 66), b) patient with type 2 diabetes (b; female, age 70) and c) patient with pancreatic carcinoma and type 2 diabetes (c; female, age 80). The pancreatic sections were stained for amyloid with Thioflavin S (green), insulin (cyan), cell apoptosis with TUNEL (red) and nuclei with DAPI (blue). The pictures were taken at 200-fold magnification.

4.3 Islet Amyloid

Islet amyloid was present in small amounts in most of the islets of all subjects in the three groups. There were no significant differences in the extent of islet amyloid between the groups. Only in one patient of patients with type 2 diabetes we observed big aggregates of amyloid fibrils in the majority of the islets. The extent of the islet amyloid was 0.99% ± 0.05% in the control group, 1.44% ± 0.33% in patients with type 2 diabetes and 1.04% ± 0.08% in patients with diabetes secondary to pancreatic disease (p=0.13) (Figure 35). Islet amyloid area was 0.35% ± 0.27% in the control group, 2.71% ± 2.35% in patients with type 2 diabetes and 0.27% ± 0.27% in patients with diabetes secondary to pancreatic disease (p=0.24) (Figure 36). Examples of amyloid deposits in the control group, in patients with diabetes secondary to pancreatic disease and in patients with type 2 diabetes are given in Figure 37 a and b, Figure 38 a and b and figure 39 a and b, respectively.

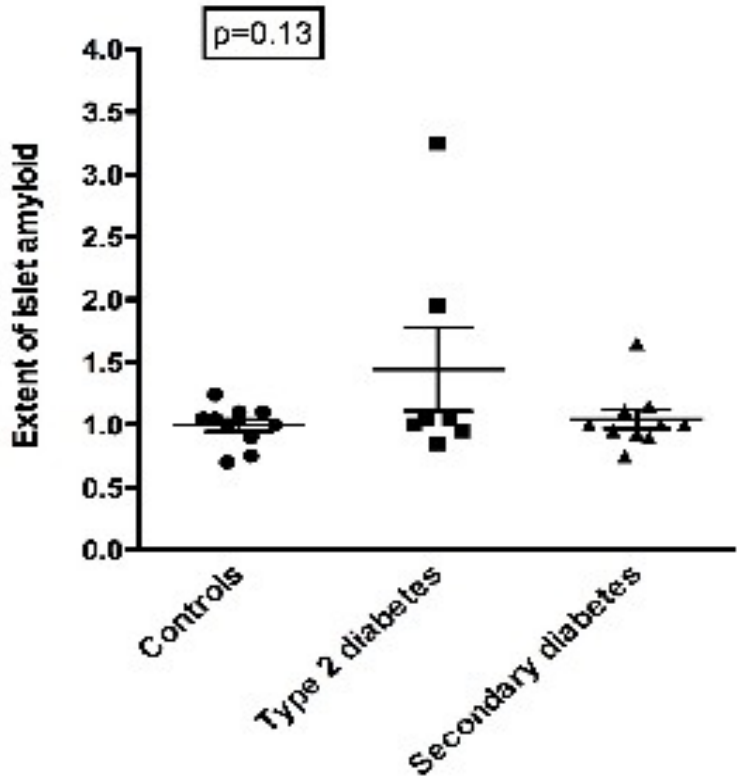


Figure 35: Extent of islet amyloid in patients with type 2 diabetes *(cases 1*), patients with chronic pancreatitis or pancreatic carcinoma and type 2 diabetes (*cases 2*) and patients without diabetes (*controls*). Results are presented as means ± SEM. Statistics were carried-out using one-way ANOVA.

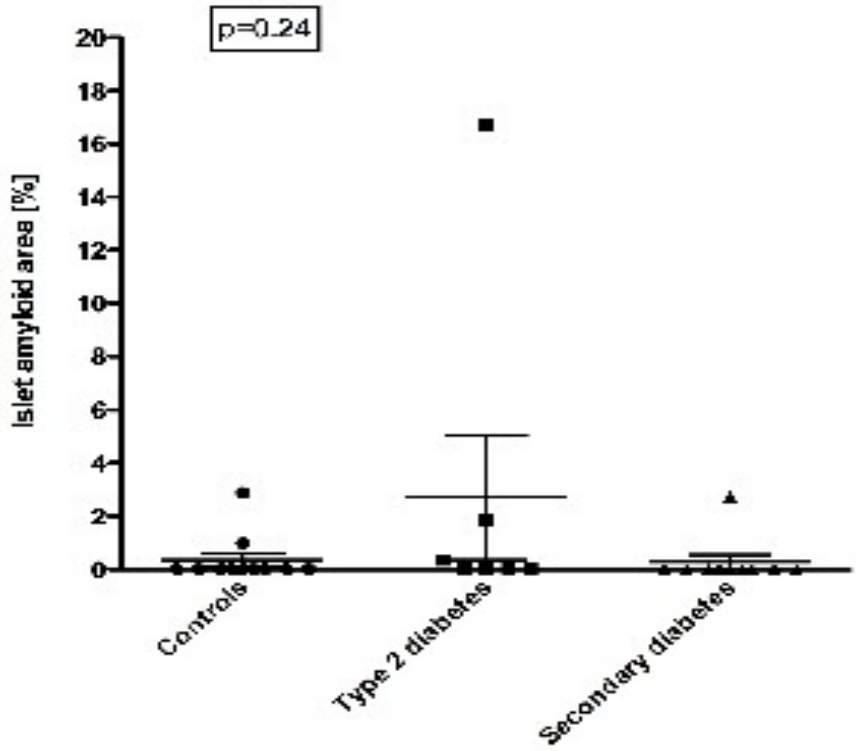


Figure 36: Percentage of amyloid positive area in islets of patients with type 2 diabetes (cases 1), patients with chronic pancreatitis or pancreatic carcinoma and type 2 diabetes (cases 2) and patients without diabetes (controls). Results are presented as means ± SEM. Statistics were carried-out using one-way ANOVA.

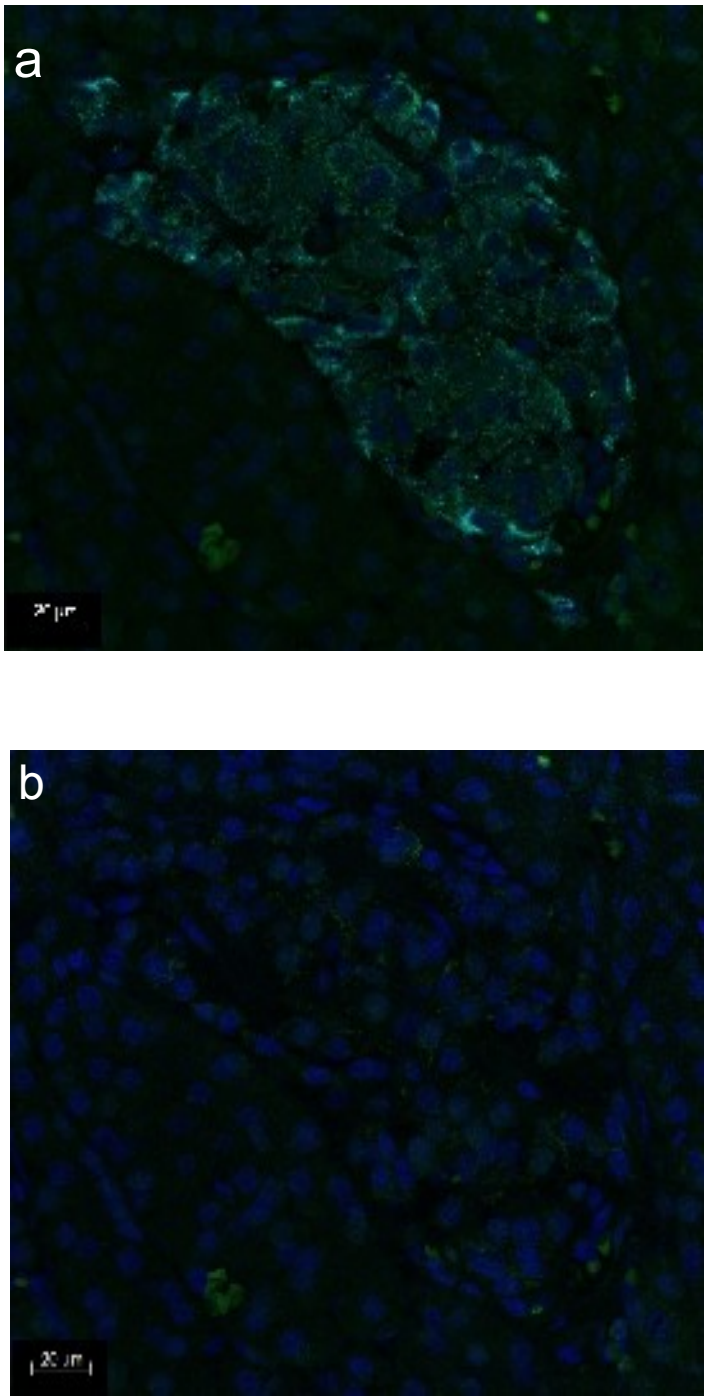


Figure 37: Pancreatic islet amyloid in a 66-year-old female without diabetes (control). The pancreatic sections were stained for amyloid with Thioflavin S (green), insulin (cyan) and nuclei with DAPI (blue). Small amyloid deposits, mainly spherical puncta and curvilinear structure, were found in islets containing several insulin producing cells. The pictures were taken at 400-fold magnification.

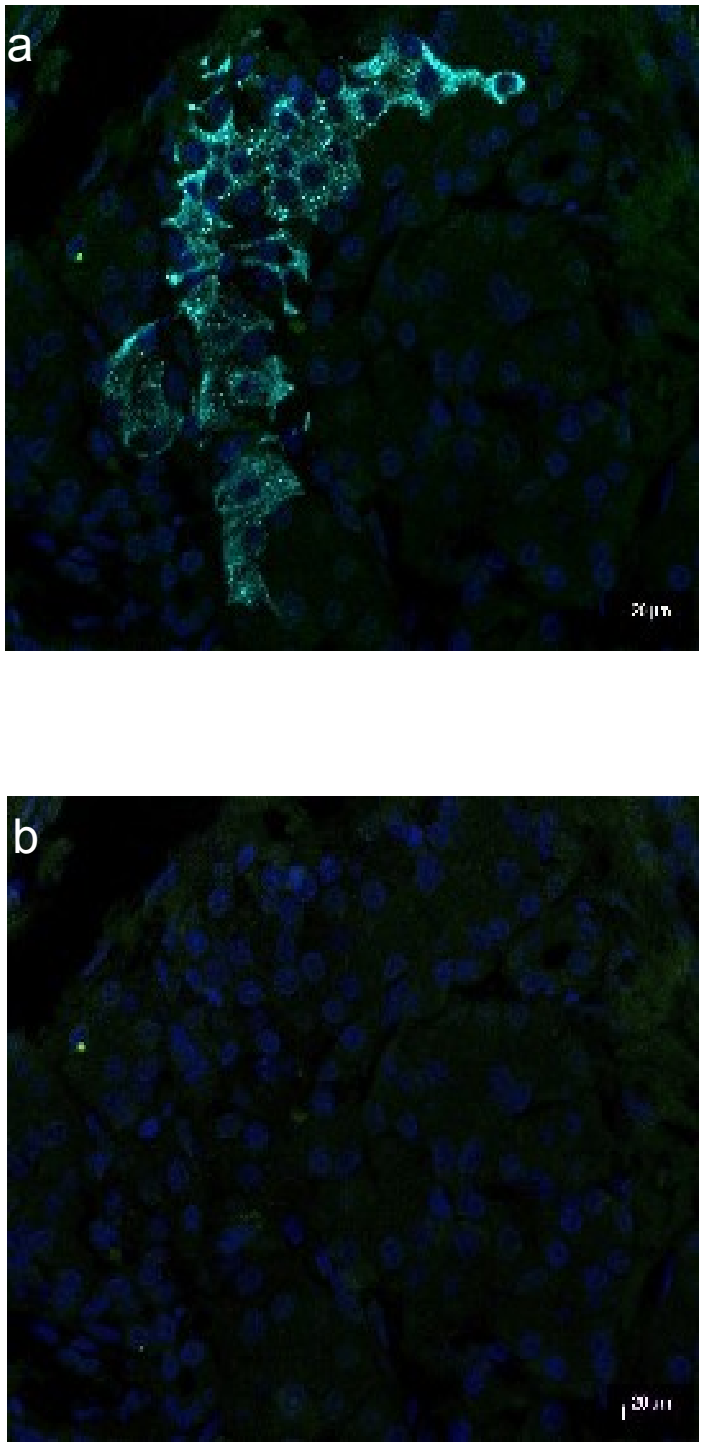
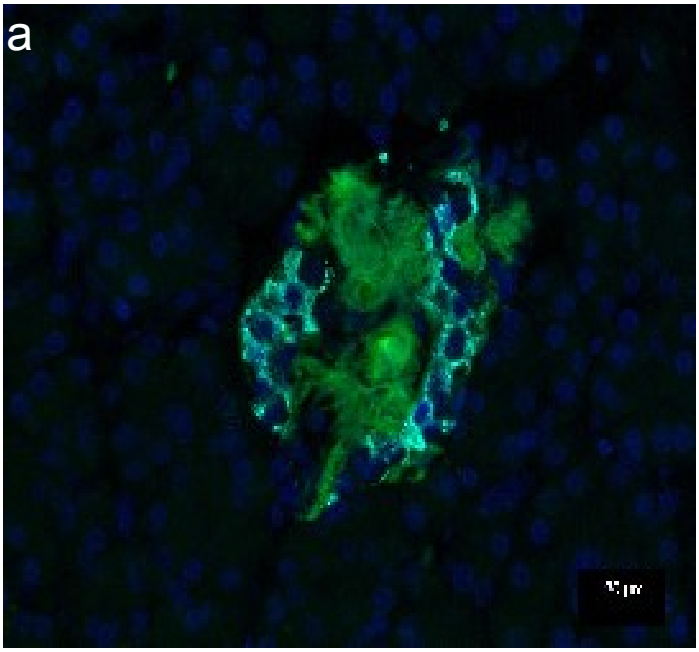


Figure 38: Pancreatic islet amyloid in a 80-year-old female with with pancreatic carcinoma and type 2 diabetes (case 2). The pancreatic sections were stained for amyloid with Thioflavin S (green), insulin (cyan) and nuclei with DAPI (blue). Small amyloid deposits as spherical puncta were found in islets containing many insulin producing cells. The pictures were taken at 400-fold magnification.



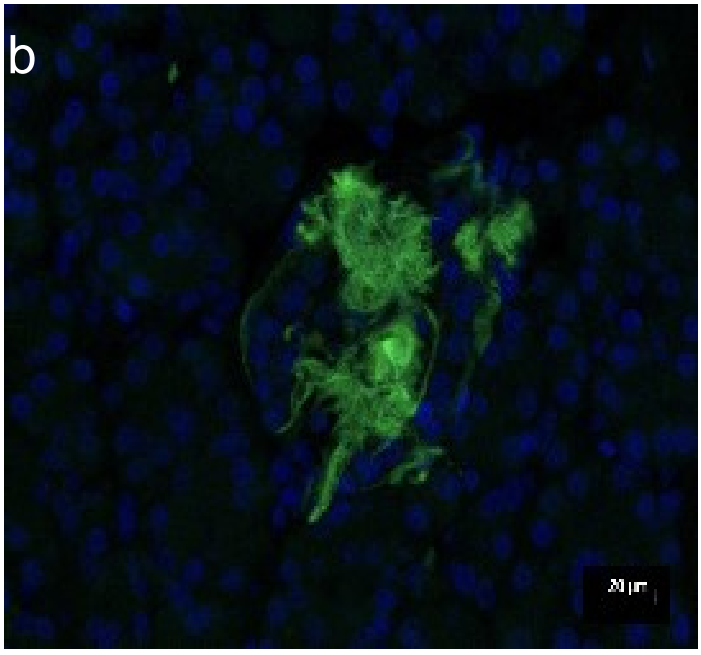


Figure 39: Pancreatic islet amyloid in a 70-year-old female with with type 2 diabetes (case 1). The pancreatic sections were stained for amyloid with Thioflavin S (green), insulin (cyan) and nuclei with DAPI (blue). Big aggregates of amyloid were found to take place in a wide extension in islets containing some insulin producing cells. The pictures were taken at 400-fold magnification.

8. DISCUSSION (II)

The present study was undertaken to examine the presence and quantity of amyloid aggregates and their role on beta cell apoptosis in patients with type 2 diabetes and patients with diabetes secondary to pancreatic diseases comparing them with control subjects.

The morphometric analysis of human pancreatic specimens from 10 patients with chronic pancreatitis or pancreatic carcinoma and secondary diabetes, 7 patients with type 2 diabetes and 11 non diabetic control subjects revealed a significant difference in the fractional beta-cell area between these 3 groups but no difference could be seen, instead, in the beta cell apoptosis and islet amyloid extent or area. The difference in the beta cell area observed between groups is in agreement to previous morphometric studies in patients with type 2 diabetes and patients with diabetes secondary to chronic pancreatitis. In patients with type 2 diabetes there is often a 40-60% reduction of beta cell mass (28, 44, 57), due to an increased beta cell apoptosis, that contributes to an impaired insulin secretion and a 30% reduction of beta cell mass has been shown, also, in subjects with chronic pancreatitis and diabetes compared to control subjects (144) without any difference in the beta cell turnover.

In our study, the difference in the beta cell area between the 3 groups does not depend on an increase of beta cell apoptosis as there is no difference in this parameter although a non significant trend to an increased cellular death is present in the patients with diabetes secondary to chronic pancreatitis or pancreatic carcinoma.

What decides the fate of a beta cell is the result of a mutual

influence between its genetic background and the external stimuli and environmental conditions. One of these stimuli, definitely important for the beta cell, is represented by the plasma glycemic levels that, normally, regulate the cell activity. It has been observed that an excessive quantity of glucose over a prolonged period has negative effects, called glucotoxicity, on beta cell function and insulin sensitivity. Chronic hyperglycemia causes an increased basal insulin release, a reduced response to stimulus to secrete insulin, a gradual depletion of insulin stores, a reduced transcription of insulin gene and an increase of beta cell apoptosis (41, 145). It seems that an high glucose metabolism take to an increase of apoptotic rate through an excessive mitochondrial production of reactive oxygen species (ROS) as oxidative stress markers, like nitrotyrosine, are in high levels in diabetic islets. Interventions on oxidative stress, like addition of anti-oxidant glutathione, lower the nitrotyrosine levels and improve the beta cell secretory function and increase insulin expression (41). Antioxidants administration increases also beta cell mass and decreases beta cell apoptosis rates in an animal model of type 2 diabetes (146). Then, pharmacological treatments to lower and control the plasma glycemic levels in type 2 diabetes can prevent the progressive beta cell dysfunction and death, through a reduction of oxidative stress. In our study patients with type 2 diabetes and those with diabetes secondary to chronic pancreatitis or pancreatic carcinoma are treated with hypoglycemic drugs that could help to prevent apoptosis in beta cells whose functionality is not fully compromised and that have not started a death internal programme yet.

Besides glucotoxicity, there are also other factors that, potentially, can influence the beta cell survival, like amyloid deposits, formed by the deposition of IAPP aggregates. The role of amyloid in the beta cell dysfunction is controversial as some studies have found an association between amyloid fibrils and a reduced beta cell mass and apoptosis (147) while others have not been able to show any association between amyloid and type 2 diabetes duration (148). In addition, some scientists believe that small oligomers of IAPP and not big fibrils aggregates are responsable for the beta cell death and experiments in vitro and in animal models seem to support that thesis (119-121, 123). Amyloid oligomers have been shown also in the Langerhans islets of diabetic patients where apoptotic beta cells are also present (114). Anyway, this hypothesis needs to be proved with quantitative studies on human islets and with more specific methods. In our study we have seen amyloid deposits, mainly of small size, in all patients of the 3 groups considered, without any significant difference. We expected to find a higher mean of amyloid deposition in the Langerhans islets of type 2 diabetes patients as reported in most of the literature. Anyway, Clark et al. found a mean value of 6.5% in islet amyloid area in diabetics (106) and lately Hai-Lu Zhao et al. have found patients with type 2 diabetes that do not show amyloid fibrils in their islets but rather a diffuse oligomer deposition that is present also in all non diabetic patients they considered for that study (114). Also, we detected in all our patients amyloid aggregates of a reduced size with an intracellular and extracellular localization. We used thioflavin S to stain the amyloid deposits, a fluorescent dye that, like its analogue thioflavin t, is more

sensible in detecting amyloid fibrils than the traditional Congo Red staining (149, 150). It is possible to speculate that thioflavin s binds not only the amyloid fibrils but also small aggregates of IAPP monomers, oligomers, as seen in experiments with the analogue compound, thioflavin t (149). The beta cells in Langerhans islets are highly active in the biogenesis and secretion of hormones and it is possible to think that, mainly, in the cellular compartments, ER, Golgi apparatus and secretory granules where these processes take place there is a temporary aggregation of IAPP due to its high concentration and its tendancy to assume in a part of its structure a beta sheet conformation fibril prone. This kind of aggregation and a further aggregation in a more complex structures, like fibrils, could be avoided by the cellular systems that assist the protein folding and by the interaction of IAPP with other molecules, like insulin (83). Then, this could explain why we observed small amyloid aggregates in non diabetic patients. Amyloid deposition was also present in all patients with diabetes secondary to chronic pancreatitis or pancreatic carcinoma and then caused by a different pathogenetic mechanism than that triggering type 2 diabetes. We did not report any difference in amyloid deposition and beta cell apoptosis between non diabetic subjects, type 2 diabetic patients and those with diabetes secondary to chronic pancreatitis and pancreas carcinoma, suggesting that amyloid deposition is not exclusively associated to type 2 diabetes and could not be a cause of beta cell apoptosis in this disease.

Anyway, our study shows some limitations: the number of patients in the 3 considered groups was not so high and, then, it is possible that differences in the beta cell apoptosis or islet amyloid extent or area could

have been, possibly, more pronounced with larger number of cases.

In addition, the control group was comprised of patients with benign pancreatic adenomas and extrapancreatic tumors that can produce alterations of pancreatic tissue. This was done by necessity, because human pancreatic biopsies from patients without any pancreatic diseases are ethically unacceptable. However, in the present cases, suspected pancreatic tissues were excluded.

We used thioflavin s staining to detect amyloid that can not be used to distinguish exactly between the species of amyloid deposits: different types of oligomers, protofibrils and fibrils.

9. CONCLUSIONS

The research activity focused on two research project.

The aim of the first project was to evaluate the effects of high local insulin levels on beta cell turnover in vivo, in a model of endogenous hyperinsulinism. We could not find any differences in beta-cell area and turnover between the tumor-free pancreas of insulinoma patients and control subjects, suggesting that high endogenous insulin levels have no effects on beta cells. Future in vivo studies, ideally employing larger animal models, are warranted to further evaluate the impact of exogenous insulin on beta-cell turnover.

The aim of the second project, that is still in progress, was to examine the presence and quantity of amyloid deposits and their role on beta cell death in a condition of reduced insulin secretion because of faulty beta cells or because of pancreatic diseases. We found a significant difference in beta cell area but no differences in beta cell apoptosis and amyloid deposition between type 2 diabetic patients, patients with diabetes secondary to chronic pancreatitis or pancreatic carcinoma and non diabetic control subjects, suggesting that amyloid deposition is not an exclusive phenomenon of type 2 diabetic islets and can not be a cause of beta cell death in this disease. Future larger studies, involving also appropriate animal models and sophisticated techniques, could be useful to better understand the aggregation mechanisms of IAPP and its role in beta cell death.

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