Insulin resistance alters islet morphology in non-diabetic humans

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No disclosures to declare.

Word count: 4851

Figures: 6
Tables: 2
Abstract

Type 2 diabetes is characterized by poor glucose uptake in metabolic tissues and manifests when insulin secretion fails to cope with worsening insulin resistance. In addition to its effects on skeletal muscle, liver, and adipose tissue metabolism, it is evident that insulin resistance also impacts pancreatic β cells. To directly examine the alterations that occur in islet morphology, as part of an adaptive mechanism to insulin resistance, we evaluated pancreas samples obtained during pancreateoduodenectomy from non-diabetic subjects who were either insulin resistant or insulin sensitive. We also compared insulin sensitivity, insulin secretion and incretin levels between the two groups. We report an increased islet size, and an elevated number of both β and α cells that resulted in an altered β:α cell area in the insulin resistant group. Our data suggest that neogenesis from duct cells and transdifferentiation of α cells are potential contributors to the β cell compensatory response to insulin resistance in the absence of overt diabetes.

INTRODUCTION

Insulin resistance, along with β cell inadequacy, represent the key features in the pathogenesis of type 2 diabetes, and it is generally accepted that both are essential for the full manifestation of the disease (1).

A feature that has been recognized in both rodents (2,3) and humans (4,5,6) is the ability of the pancreas to compensate for insulin resistance by an increase in β cell mass and insulin secretion. Indeed, β cell mass is dynamic and capable of adapting to physiological and pathological conditions to maintain normoglycemia (7,8,9). Studies in humans suggest that the number and mass of β cells increase in response to obesity; however, the time of onset of the increase and the precise origin of such new β cells are still unknown (7). It is also evident that a failure of this ability of the β cells to compensate for insulin resistance leads to progressive hyperglycemia and glucose toxicity (10) and
overt diabetes (11). A challenge to identifying the pathways and investigating the mechanisms that underlie compensatory changes in islets is the lack of longitudinal access to human tissue samples of appropriate quality for analyses, coupled with accurate metabolic and hormonal profiling.

We took advantage of the unique opportunity to collect pancreas samples from patients undergoing surgical removal of a tumor of the ampulla of Vater to explore the hypothesis that insulin resistance directly contributes to adaptive changes in β cell mass and function. To this end, we measured insulin sensitivity, insulin secretion and incretin levels, in non-diabetic, non-obese subjects both before and after pancreatoduodenectomy. We also evaluated markers of β cell proliferation, apoptosis, hypertrophy and islet neogenesis, and ductal cell markers. Our data indicate that alterations in insulin sensitivity are linked to markers of compensation in humans, and suggest ductal cells and α-cell transdifferentiation as sources for new β cells.

METHODS

Selection and description of participants

Eighteen patients (9 males and 9 females) scheduled to undergo pylorus-preserving pancreatoduodenectomy were recruited at from Hepato-Biliary Surgery Unit of the Department of Surgery (Agostino Gemelli University Hospital, Rome, Italy). The study protocol was approved by the local ethics committee and all participants provided written informed consent followed by a comprehensive medical evaluation. Indication for surgery was tumor of the ampulla of Vater. All patients enrolled did not have a family history of diabetes and were classified as non-diabetic as determined by both a 75-g oral glucose tolerance test and HbA1c according to the American Diabetes Association criteria (12). Only patients with normal cardiopulmonary and kidney functions, as determined by medical history, physical examination, electrocardiography, creatinine clearance and
urinalysis, were included. Altered serum lipase and amylase levels prior to surgery, as well as morphologic criteria for pancreatitis, were considered exclusion criteria. Potential patients who had severe obesity (BMI > 40), uncontrolled hypertension and/or hypercholesterolemia were excluded.

To assess differences in islet morphology in response to insulin resistance versus insulin sensitive states, patients were divided into 2 groups (insulin resistant and insulin sensitive) according to their insulin sensitivity, as measured with the euglycemic hyperinsulinemic clamp procedure before surgery. As previously described (13), the cut-off for insulin sensitivity was the median value of glucose uptake in the overall cohort (4.9 mg·Kg\(^{-1}\)·min\(^{-1}\)); therefore, subjects whose glucose uptake was above the median value were classified as “more insulin-sensitive” than subjects whose glucose uptake was below the median; for ease of comprehension, the two groups were defined “insulin sensitive” or “insulin resistant”. Clinical and metabolic characteristics of the two groups are shown in Table 2.

**Study design and experimental procedures**

Anthropometric parameters were determined according to standard procedures (14). BMI was calculated as weight divided by height squared (kg/m\(^2\)). All patients had blood samples drawn for serum lipid assays (total cholesterol, high and low-density cholesterol) in the morning after an overnight (8h) fast. All the procedures were performed with subjects in a supine position throughout the experiments. Each subject underwent a hyperinsulinemic euglycemic clamp, a hyperglycemic clamp and a mixed meal test one week before and after a variable period of recovery from the surgical procedure. A sufficient recovery period was judged on normalization of inflammatory parameters such as C-reactive protein, erythrocyte sedimentation rate, stability of weight, absence of symptoms of abnormal intestinal motility or exocrine pancreatic deficiency. During the clamp procedures an intravenous catheter was inserted into each arm, one for infusions and the other for blood sampling.
**Oral glucose tolerance test**

Normal glucose metabolism was confirmed by a standard 75-g oral glucose tolerance test, measuring glycaemia, insulin and C-peptide at 0, 30, 60, 90, 120 minutes after the glucose load.

**Hyperinsulinemic euglycemic clamp procedure**

The hyperinsulinemic euglycemic clamp test was performed after a 12h overnight fast using insulin 40 mIU/m²·min of body surface according to DeFronzo and colleagues (15). A primed constant infusion of insulin was administered (Actrapid HM, 40 mIU/m²·min; Novo Nordisk, Copenhagen, Denmark). The constant rate for the insulin infusion was reached within 10 min to achieve steady-state insulin levels; in the meantime, a variable infusion of 20% glucose was started via a separate infusion pump, and the rate was adjusted, on the basis of plasma glucose samples drawn every 5 min, to maintain the plasma glucose concentration at each participant’s fasting plasma glucose level. During the last 20 min of the Clamp procedure, plasma samples from blood drawn at 5–10 min intervals were used to determine glucose and insulin concentrations. Whole-body peripheral glucose utilization was calculated during the last 30-min period of the steady-state insulin infusion and was measured as the mean glucose infusion rate (as mg·Kg⁻¹·min⁻¹).

**Hyperglycemic clamp procedure**

The plasma glucose was clamped at a stable level of 125 mg/dl above the fasting blood glucose concentration. The hyperglycemic clamp was started with a bolus dose of dextrose 200 mg/mL (150 mg/kg) administered into the antecubital vein. Blood was drawn from a cannulated dorsal hand vein on the opposite arm. Every 5 min, venous plasma glucose was analyzed with a glucose analyzer and the infusion of 20% glucose was adjusted to achieve a stable glucose level of 125 mg/dl above the fasting
value. Serum samples for insulin, and C-peptide were drawn at 0, 2.5, 5, 7.5, 10, 15, 30, 60, 90, 120, 130, 140, and 150 min. First phase insulin release, reflecting the early insulin peak secreted from the pancreatic β cell in response to glucose stimulation, was calculated as the area under the curve (AUC) during the first 10 min of the clamp by using the trapezium rule. Second phase insulin release, reflecting β cell function under sustained elevated glucose levels, was calculated as AUC during the interval 10–120 min. Subsequently a 5-g arginine bolus was administered to measure maximum C-peptide secretory capacity at a steady-state blood glucose concentration of 250 mg/dl. Combined hyperglycemia- and arginine-stimulated β cell secretory capacity was calculated as the insulin AUC during the 30 min following the arginine bolus (AIRarg) (Figure 1A).

**Mixed meal test**

Patients were instructed to consume a meal of 830 kcal (107 kcal from protein, 353 kcal from fat, and 360 kcal from carbohydrates) within 15 min. Blood samples were drawn twice in the fasting state and at 30-min intervals over the following 240 min (sample time 0’, 30’, 60’, 90’, 120’, 150’, 180’, 210’ and 240’) for the measurement of plasma glucose, insulin, C-peptide, glucagon, GLP-1 or GIP concentrations. Blood samples for glucagon, total GLP-1, or intact GIP were sampled in tubes containing EDTA and a DPP-4 inhibitor (Millipore, MA, USA); after centrifugation (1000 rpm for 10 min at 4°C), they were stored at -80°C until analyses. Insulin levels were determined using a commercial RIA kit (Medical System, Immulite DPC, Los Angeles, CA). Plasma glucose concentrations were determined by the glucose oxidase technique, using a glucose analyzer (Beckman Instruments, Palo Alto, CA, USA). Plasma C-peptide was measured by autoDELPHIA automatic fluoroimmunoassay (Wallac, Turku, Finland), with a detection limit of 17 pmol/L. Immunoreactive glucagon was measured in ethanol-extracted plasma by RIA using antibody code no. 4305 which is
directed against the C-terminus of glucagon and reacts specifically with pancreatic glucagon (16). Total GLP-1 concentrations were measured using antiserum no. 89390, reacting equally with intact GLP-1 (7–36) amide and its primary N-terminally truncated metabolite GLP-1 (9–36) amide. Intact GIP was measured using antiserum no. 98171, reacting with the N-terminus of GIP, but not with the metabolite, GIP 3–42 (17).

**Surgical procedures**

Pancreatoduodenectomy was performed according to the pylorus preserving technique (18,19). Briefly, the pancreatic head, the entire duodenum, common bile duct, and gallbladder were removed en bloc, leaving a functioning pylorus intact at the gastric outlet. All adjacent lymph nodes were carefully removed. The continuity of the gastrointestinal tract was restored by an end-to-side invaginated pancreato-jejunostomy. Further downstream, an end-to-side hepatico-jejunostomy and side-to side gastro-entero-stomy or an end-to-side pylorus-jejunostomy were made. The removed volume of pancreas during the surgery is constant (~50%), as previously reported by Schrader et al. (20). A pancreatic sample was collected during the surgery, from the downstream edge of the surgical cut.

**Immunohistochemical analysis of pancreas samples**

**Pancreatic tissue processing**

Pancreas samples were fixed in formaldehyde and embedded in paraffin for subsequent analysis. Five µm sections were stained either with hematoxylin/eosin or by immunohistochemistry for islet hormones using a cocktail of antibodies to insulin, glucagon or somatostatin (21). In addition, sections were immunostained for insulin, Ki67 or DAPI (nucleus) to assess proliferation, TUNEL for apoptosis, and for duct marker using anti-CK19 antibodies, and GLP-1 to identify incretin immunoreactivity. The
hematoxylin/eosin slides were examined in all cases by two pathologists to exclude cases with pancreatitis, autolysis and tumor infiltration.

Primary antibodies included the following: Insulin (guinea pig antibody, Abcam 1:200), Glucagon (mouse mono, Sigma; 1:500), Somatostatin (Rabbit poly, Ab cam 1:500), or Ki67 (mouse mono antibody, 1:50, BD Biosciences), GLP-1 (Rabbit antibody, 1:1000 J.F. Habener MD, Massachusetts General Hospital, Boston), CK19 (rabbit poly, Abcam, 1:100). For TUNEL we used a Apoptag Fluorescein in Situ apoptosis detection kit (Roche), PDX1 (rabbit poly, Cell signaling, 1:200).

Secondary antibodies were: Donkey anti-GPb594, Donkey anti-mouse-AMCA, Donkey anti-rabbit-488, Donkey anti-mouse-AMCA and Biotinylated Donkey anti-rabbit (all from Jackson immunoResearch, West Grove, PA) and peroxidase labeled polymer, from Dako.

Morphometric analysis

β, α and δ cell area were analyzed as described previously (22). Each section was analyzed separately by measuring total insulin, glucagon or somatostatin positive areas, as well as the total pancreas section area, using Image Pro Plus software version 4. 5.1 (Media Cybernetics, Silver Springs, MD). The β, α or δ cell areas were expressed as percentage of total pancreas section area. The islet size was calculated as the sum of the individual areas (β, α and δ cell areas) divided by the number of islets counted in each pancreatic section. Islet density was quantified by measuring the total area of pancreas using Image Pro Plus and then counting the number of islets contained within that pancreatic area, the results being expressed as islets per mm². Islet size distribution was determined using the insulin stained sections of pancreas counterstained with DAPI. At least 100 islets per section were examined and classified according to the number of insulin-positive cells, i.e., one to eight cells, nine to 19 cells, 20 to 49 cells and 50 or more beta cells, and the data expressed as a percentage of islets. The ratio of the β to α cell
area (β:α) was evaluated for each section, dividing the individual percentage of β cell area by the α cell area. To evaluate β cell size and nuclear area, five randomly selected islets per case, were immunostained for insulin or DAPI, and imaged at 400x (40 x objective). Insulin positive area for each islet was measured and the number of nuclei present in the insulin-stained area (µm²) was manually counted to calculate the individual β cell cross-sectional area (µm²). The number of β cells was manually counted for each section and expressed as the ratio of β cells per total pancreas section. To measure the β cell nuclear area, insulin stained sections of pancreas counterstained with DAPI were used; five randomly selected islets per case were photographed at 400x magnification. Then 5 representative beta cell nuclei were identified in each islet. Selection criteria included: clear presence of the nucleus within a beta-cell, the ability to clearly visualize nuclear boundaries, circular shape (similar dimensions in all directions) and the appearance to the observer that the nucleus had been sectioned through its maximum diameter. Once the identified nucleus was encircled, nuclear area (µm²) was measured using Image Pro Plus software version 4. 5.1 (Media Cybernetics, Silver Springs, MD). The number of insulin and glucagon double-positive cells was manually counted in sections co-stained for insulin or glucagon. A mean (±SE) of 1172 ± 269 endocrine cells was evaluated per subject and resulting data were expressed as percentage of endocrine cells. The double positive cells were confirmed in randomly selected islets by confocal microscopy. All data were expressed as the mean ±SE for each group.

Quantification of scattered islets and exocrine ducts cells positive for insulin

As previously described (23), clusters of less than 8 endocrine cells were considered as new islets (neogenesis). On sections stained specifically for insulin, clusters with less than 8 insulin-positive cells were manually counted and considered as scattered islets, then expressed as the ratio of the number of
scattered islets per total pancreas area. Sections co-stained for pancreatic ductal marker CK19 or insulin were imaged by confocal microscopy and the number of CK19 and insulin-positive cells was manually counted. A mean (±SE) of 1107 ± 475 duct cells were evaluated for each section. The resulting data were expressed as a percentage of duct cells positive for insulin in each pancreas. All data were expressed as the mean ±SE for each group.

*Proliferation and Apoptosis*

To determine replication in β cells, the number of β cells co-staining with Ki67 was counted and expressed as percentage of total number of β cells (at least 2000 β cells were counted in each case). For the evaluation of apoptosis, the number of β cells co-staining with TUNEL was counted and expressed as percentage of total number of β cells (at least 2000 β cells for each case). The entire analysis was performed by a single observer in a blinded fashion.

*Statistics*

All data are expressed as mean ±SE, unless indicated otherwise. Since samples were normally distributed, differences in means were tested by 2-tailed Student’s t test. The relationship between variables was derived with linear regression analysis using SPSS version 9 (SPSS, Chicago, IL). A P value of less than 0.05 was considered statistically significant.

*Study approval*

This study was approved by the Ethical Committee of the Catholic University of the Sacred Heart, Rome, Italy. All study subjects provided written informed consent before screening and participation in the study.
RESULTS

Eighteen patients (9 female; 9 male; mean age 53±15 yrs.) undergoing pylorus-preserving pancreatoduodenectomy for a tumor of the ampulla of Vater were included in the present study. Clinical and metabolic characteristics of study subjects are provided in Table 1.

Hemipancreatectomy induced a marked reduction in insulin secretion, without affecting insulin sensitivity, and resulted in the onset of diabetes only in insulin resistant subjects

Subjects were evaluated one week before surgery and 40±7 days (range 34-48 days) after surgery. To evaluate the insulin secretory capacity, we performed hyperglycemic clamps over 2h followed by an acute stimulation with L-arginine (5 g). As expected, insulin secretion was significantly reduced after surgery (Figure 1B, p<0.001). The response to arginine (121 to 150 min after glucose infusion) revealed an even higher (76%) reduction of insulin secretion (Figure 1C). Conversely, insulin sensitivity, as assessed by the hyperinsulinemic euglycemic clamp (15), did not change significantly after surgery (Figure 1D).

Evaluation of glucose homeostasis by a standard oral glucose tolerance tests (75 g) in the overall cohort revealed worsening of glucose tolerance after surgery (Table 1).

To further characterize changes in glucose tolerance following removal of ~50% of pancreas, and to assess differences in islet morphology, patients were divided into insulin resistant and insulin sensitive groups (Table 2). Despite the removal of the head of the pancreas, which includes ~50% of β cell mass, patients identified as insulin sensitive before surgery preserved their glucose tolerance, whereas seven out of nine (77.7%) insulin resistant patients developed diabetes, as confirmed by a 75 g oral glucose tolerance test and a glycated hemoglobin higher than 7% (53 mmol/mol) (Table 2).
GLP-1 secretion in response to a mixed meal significantly increased (AUC GLP-1 before vs. after surgery: 10.4±3.2 nmol·l⁻¹·min vs. 15.6±4.3 nmol·l⁻¹·min, p=0.01; Figure 1E) while GIP response was significantly reduced (AUC GIP before vs. after surgery: 19.1± 8.1 nmol·l⁻¹·min vs. 7.7±2.8 nmol·l⁻¹·min, p<0.001; Figure 1E) after surgery.

Although 50% pancreatectomy led to a decrease in insulin secretion in both insulin sensitive and insulin resistant patients (Figure 2A), the latter exhibited a greater attenuation in all the phases of insulin secretion (insulin sensitive, 924±900 µIU·ml⁻¹·min vs. insulin resistant 6952±1951 µIU·ml⁻¹·min ∆AUC insulin secretion, p=0.04, Figure 2C). Furthermore, the increase in glucagon secretion during the mixed meal test after surgery was higher in insulin resistant patients (insulin sensitive 112±101 nmol·l⁻¹·min versus insulin resistant 1812±326.3 nmol·l⁻¹·min ∆AUC glucagon secretion, p=0.02, Figure 2B and D).

**Insulin resistant individuals exhibit increased islet size**

To evaluate changes in islet morphology, we performed immunohistochemical analyses of sections of pancreas removed during surgery. As compared to insulin sensitive individuals, insulin resistant subjects exhibited an increased % insulin area (insulin sensitive 0.58±0.17 % vs. insulin resistant 1.10±0.23 %, p=0.05), % glucagon area (insulin sensitive 0.04±0.01 % vs. insulin resistant 0.23±0.06 %, p<0.01) as well as % somatostatin area (insulin sensitive 0.01±0.00 %, vs. insulin resistant 0.03±0.01 %, p=0.01) (Figure 3A). Overall, these differences resulted in an increased mean islet size in insulin resistant subjects compared to the insulin sensitive ones (mean islet size: insulin sensitive, 2456±332 µm² versus insulin resistant 5156±944 µm², p<0.001) (Figure 3B). Further, we observed a strong inverse correlation between islet size and glucose uptake in the entire cohort (r= -0.74, p<0.01; Figure 3C), which suggests that changes in islet morphology are influenced by insulin sensitivity.
Increased islet size is likely due to β cell hyperplasia

To identify potential mechanism(s) underlying the increased islet size in insulin resistance, we examined β cell replication, apoptosis and cell size. Among a total of 37,845 β cells counted in the entire cohort, replication was undetectable, as determined by Ki67 immunostaining. β-cell apoptosis was also infrequent among the 39,600 cells examined in the entire cohort. While positive cells were undetectable in the insulin sensitive group, we detected 24 positive apoptotic cells in 3 insulin resistant patients (mean percentage of β cell apoptosis among insulin resistant group 0.1%). However, there were no significant differences between groups (p=0.20). By measuring mean individual β cell cross-sectional area in the two groups, we also ruled out a possible contribution of cell hypertrophy to the increase in islet size (insulin sensitive, 127±9 µm² vs. insulin resistant, 129±17 µm², p=0.79; Figure 3D). Insulin resistant patients showed an increased number of β cells per mm² of pancreas area (insulin sensitive, 22.7±2.7 β cells/mm² vs. insulin resistant, 80.9±15.8 β cells/mm², p<0.01). These data suggest that the increase in islet size is due to increased number of cells, i.e. hyperplasia rather than altered β cell volume. Further, β cell nuclear size was increased in the insulin resistant group (insulin sensitive, 34.5±1.1 µm² vs. insulin resistant, 42.4±6.2 µm², p=0.03; figure 3E) suggesting that secretory β-cells are relatively young (24,25).

Insulin resistance is associated with increased islet neogenesis

Based on the results discussed above and the finding of increased islet density (insulin sensitive 3.5±0.5 vs. insulin resistant 7.5±1.4 islets per mm², p<0.01; figure 3F), we hypothesized that neogenesis, rather than proliferation, contributes to β-cell hyperplasia in insulin resistant patients. To explore whether the pancreas shows evidence of neogenesis, we quantified the number of scattered islets with less than 8 nuclei (23), and observed an increase of such islets in insulin resistant subjects.
(insulin sensitive 1.80±0.18 vs. insulin resistant 4.65±1.16 nuclei/mm², p=0.04, Figure 4A). Although these scattered islets were distributed within the exocrine tissue, we cannot ascertain whether these cells arise directly from acinar cells without further detailed investigation.

Further, since previous studies have reported potential formation of new islets from duct cells, we evaluated the number of cells that were double positive for the duct marker CK19 and insulin. The percentage of CK19/insulin double positive cells was increased in insulin resistant subjects (mean percentage of CK19/insulin double positive cells: insulin sensitive 0.28±0.12 % vs. insulin resistant 1.47±0.26 %, p<0.001 Figure 4 B and D). As shown in figure 4C, insulin resistant subjects displayed a greater number of both small clusters and islets with more than 50 cells.

**Insulin resistance and alterations in α cells**

The fractional α-cell area was greater in the insulin resistant compared to the insulin sensitive group, and was inversely correlated with glucose uptake (r=-0.65, p=0.03, Figure 5A). The ratio of β/α cell areas was lower in the insulin resistant subjects (mean β:α ratio: insulin sensitive 0.13±0.01 vs. insulin resistant 0.08±0.01, p=0.05, Figure 5B), suggesting a relative increase in the α cell area.

Since previous reports have suggested transdifferentiation of α cells as a mechanism that contributes to alterations in β cell mass, we immunostained pancreas sections to identify insulin and glucagon co-expressing cells. Interestingly, we detected an increased number of double positive cells in insulin resistant subjects as compared to insulin sensitive subjects (mean percentage of double positive cells: insulin sensitive, 4.51±1.07 % vs. insulin resistant, 10.86±2.17 %, p=0.02, Figure 5 C and D, and supplementary Figures 1 and 2). We also examined PDX1 immunoreactivity in the insulin/glucagon double positive cells and detected PDX1 positive cells in both insulin sensitive and insulin resistant subjects (supplementary Figure 3).
Further immunohistochemical analyses using a specific anti-GLP-1 antibody, which is highly selective for processed amidated GLP-1 directed to the C-terminal, revealed that glucagon co-localizes with GLP-1 in both groups (Figure 6A). Interestingly, the α cell area correlated with GLP-1 (r=0.63, p=0.04) (Figure 6B) but not GIP secretion (r=0.08, p=0.79). The AUC of GLP-1 secretion during the mixed meal test also correlated with glucose uptake in the entire cohort (r=-0.57, p<0.01). Whether these correlations indicate a link between circulating GLP-1 and α-cell biology requires further study.

The relative increase in α cell area could lead to an increase in β cells by transdifferentiation, and also to an increase in intra islet GLP-1 production. Furthermore, it is possible that this change in the relative proportion of α cells could be a first step towards hyperglucagonemia, a hallmark of type 2 diabetes.

**DISCUSSION**

In the present study, we evaluated pancreas samples obtained from non-diabetic subjects to investigate the effects of altered insulin sensitivity on islet morphology. The major finding of our study is that insulin resistant subjects exhibited increased islet size, which was strongly inversely correlated with insulin sensitivity (r=-0.74; p<0.001). This suggests that insulin resistance directly impacts islet biology in non-diabetic humans by inducing an increase in β cell area to compensate for the increased insulin demand. These findings are consistent with reports in humans and mouse models (26,27) wherein defects in insulin signaling pathways in β cells have been suggested to be responsible for a decrease in mass and reduced secretory function. Indeed, it has been shown that insulin resistant patients have impaired β cell responsiveness to insulin (28). Consistent with previous reports in insulin resistant obese patients (7), who exhibit an increase in islet size due to increased cell number, β cells from insulin resistant individuals in our study also exhibited an increased number. In addition, the β cells
showed an increased nuclear area, suggesting that cells were relatively young and with increased secretory capacity (29).

Several studies (7,30-31) have investigated proliferation of β cells in humans mostly in pancreata from autopsy samples. Although some variability is evident, most reports agree that the rate of β cell proliferation is extremely low in adult human pancreas. Consistently, we noted virtually undetectable β cell proliferation by Ki67 immunostaining in pancreas sections from the entire group. We also failed to detect β cell apoptosis, in contrast to the increase seen in patients with type 2 diabetes (5). Based on the lack of alterations between groups in Ki67 and TUNEL staining, our data suggest that neither proliferation nor apoptosis contributes significantly to the β cell adaptive response to insulin resistance in this cohort of patients.

Recent reports have suggested that plasticity of adult β cell mass is linked to neogenesis during different periods of life (early postnatal life, pregnancy, and aging) as well as in obesity, impaired glucose tolerance and in individuals with newly diagnosed diabetes (32,33). Other reports suggest that cells lining the ducts or acinar cells may serve as a source of new β cells (34). Indeed our findings of higher number of islet clusters and presence of insulin-positive duct cells suggest that these pathways underlie the alteration in islet size associated with insulin resistance.

Since inappropriate glucagon secretion is a feature of patients with diabetes (4,35), and previous reports indicate a role for insulin signaling in the regulation of α cell function (36), we explored the link between α cells and insulin sensitivity. Analyses of islet morphology revealed that the ratio between β and α cell area was lower in insulin resistant subjects due to a relatively greater increase in α cell area. The relative low α cell number in the insulin sensitive group is a feature of the significantly higher insulin sensitivity while the increased number of α cells in insulin resistant subjects occurs as
compensation for insulin resistance. Similar changes are also evident in diabetic patients (37) and insulin resistant primates (38). Furthermore, we found a strong inverse correlation between α cell area and insulin sensitivity (r:-0.65, p=0.003). These observations raise several questions: for example, do alterations in α cell biology precede changes in β cell mass?; could transdifferentiation of α cells contribute to an increase in β cells?; does the imbalance between β and α cells result from dedifferentiation (39)? Despite the cross-sectional nature of our study, it is worth noting that our data are timely and highly relevant to islet biology because an ideal longitudinal study on human pancreata is extremely difficult to undertake due to ethical limitations.

A second link between insulin sensitivity and α cell mass was evident from the correlation with GLP-1 secretion. As previously reported by our group (19), patients who underwent pancreatoduodenectomy and were subjected to a mixed meal exhibited a significant increase in GLP-1 secretion in contrast to a significant decrease in GIP. While the latter is likely due to removal of the duodenum, a major site of production of GIP (40), the mechanism contributing to greater GLP-1 secretion is not fully understood and suggests either hypersecretion by existing intestinal L-cells and/or other potential sources of the incretin hormone.

Our observations on GLP-1 gain significance in light of previous reports (41) suggesting α cells are a potential source of the incretin hormone, which, in turn, can exert a local paracrine effect on islet function as previously suggested by Marchetti et al. (41). Indeed, the presence of GLP-1 in the islet has been suggested to have multiple effects, including differentiation of progenitor cells into β cells in the pancreatic duct epithelium (42,43), and direct stimulation of β cell proliferation and inhibition of apoptosis (44,45). In addition, pancreas extracts from glucagon receptor knockout mice (46) exhibit an increase in GLP-1 that is associated with an up to 10-fold increase in circulating GLP-1 amide,
active form of the incretin hormone. The lack of change in GLP-1 in intestinal extracts suggests that the pancreas is one of the sources contributing to circulating GLP-1.

In our study, none of the insulin sensitive patients developed diabetes after surgery, whereas 77.7% of insulin resistant patients became diabetic. The latter displayed a greater reduction in all the phases of insulin secretion and a higher increase in glucagon secretion in response to a mixed meal test after surgery. It is tempting to speculate that these alterations are secondary to insulin resistance in α cells and, as a consequence, to an inability of ambient insulin to adequately suppress glucagon secretion (34,47).

In conclusion, our findings suggest that neogenesis from duct cells and/or transdifferentiation from α cells are likely explanations for the alterations in β cell mass observed in insulin resistant subjects. Our study provides an example of a unique approach in the investigation of islet morphology in non-diabetic patients. A strength of this approach is the comprehensive evaluation of metabolic parameters in conjunction with analyses of pancreatic tissue from living donors which allows comparison of in vivo and ex vivo studies that would otherwise not be possible unless sequential biopsies throughout life are performed. These findings provide a platform to plan studies to directly identify the source of new β cells and determine the molecular mechanisms responsible for the dynamic changes that impact β cell mass over the time course of progression of type 2 diabetes with the long-term goal of enhancing islet compensation to insulin resistance.

**Author contribution**
T.M. generated the data and wrote the manuscript. R.N.K. and A.G. reviewed/edited manuscript. G.M. and A.P. contributed to discussion, reviewed/edited manuscript. G.C., G.P.S. and J.H. researched data, J.J.H. generated data. R.N.K supervised the work at the Joslin Diabetes Center. T.M., A.G. and R.N.K. are guarantors of this work and, as such, had full access to all the data in the study and take the responsibility for the integrity of the data and the accuracy of the data analyses.

ACKNOWLEDGEMENTS

This study was supported by grants to AG from Università Cattolica del Sacro Cuore (Fondi Ateneo Linea D.3.2 Sindrome Metabolica), from the Italian Ministry of Education, University and Research (PRIN 2010JS3PMZ_011) and from Fondazione Don Gnocchi. TM is the recipient of the Albert Reynolds Travel Fellowship by European Association for the Study of Diabetes and Fellowship Prize by the Società Italiana di Diabetologia. GPS is the recipient of fellowship from Laboratori Guidotti. Some of the reagents used in the study were supported by NIH RO1DK 67536 to RNK. Authors have no conflict to declare.

We thank S. Bonner-Weir PhD (Joslin Diabetes Center) for advice and assistance with histological examination of the samples. J.F. Habener MD (Mass General Hospital) for the gift of the anti- GLP-1 antibody. C. Cahill and S. Sioletic MD, PhD for expert technical assistance; A. Molven PhD, A. El Ouaamari and C. Conte MD for constructive comments.

TABLE AND FIGURE LEGENDS

Figure 1 Decreased insulin secretion but unaltered insulin sensitivity after pancreateoduodenectomy.
(A) Schematic of the hyperglycemic clamp experiment. L-arginine bolus (5 g) was injected at 120 min.
(B) Insulin secretion during hyperglycemic clamp. (C) Changes in the Area Under the Curve (AUC) of first phase, second phase and phase after L-arginine stimulus (Arginine) detected during hyperglycemic clamp. A significant reduction was found in all phases of insulin secretion. (D) Hyperinsulinemic euglycemic clamp performed before and after surgery. (E) Mixed meal test performed before and after surgery. Changes in the Area Under the Curve (AUC) of GLP-1, GIP and Glucagon. *p<0.05, ** p ≤ 0.001.

**Figure 2 After pancreatoduodenectomy insulin resistant subjects exhibited reduction in insulin secretion and increase in glucagon secretion**

(A) Area Under the Curve (AUC) of insulin secretion evaluated during the hyperglycemic clamp before and after surgery in insulin sensitive and insulin resistant subjects. Insulin secretion was reduced in both groups and for all the insulin secretion phases.(B) Area under the curve (AUC) of glucagon secretion evaluated during the mixed meal test before and after surgery in both insulin sensitive and insulin resistant subjects. (C) Absolute reductions from baseline (before surgery) in insulin secretion AUCs during the hyperglycemic clamp. (D) Absolute increases from baseline (before surgery) in glucagon secretion AUCs during the mixed meal test. *p<0.05

**Figure 3 Increased islet size, β-cell nuclear size and glucagon area in insulin resistant subjects.**
(A) Insulin, glucagon and somatostatin areas, evaluated as fraction of total pancreatic section area. (B) Mean islet size in insulin sensitive and insulin resistant subjects. (C) Glucose uptake versus islet size. Correlation between insulin sensitivity index and islet size in all the subjects. (D) Mean β cell area in insulin sensitive and insulin resistant subjects. (E) Mean β cell nuclear area in insulin sensitive and
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**Figure 4 Increased neogenesis in insulin resistant patients.** (A) Clusters of islets (with less than 8 nuclei) were counted and expressed per mm² of pancreas section. The number of scattered islets was increased in insulin resistant subjects. (B) Percentage of duct cells marked by CK19 positive for insulin. (C) Frequency distribution of beta cells per islet on section in insulin resistant and insulin sensitive subjects. There was a marked shift towards small islets and very large islets in insulin resistant compared with the insulin sensitive subjects. (D) Insulin immunoreactivity in duct cells marked by CK19. Confocal microscopy analysis of CK19 (Green) and Insulin (Red) showed duct cells positive for insulin. Insulin resistant (Upper panel) and insulin sensitive (Lower panel). Scale bars 100 µm. *p<0.05, **p<0.01.

**Figure 5 Insulin resistance is associated with decreased β:α ratio.** (A) Correlation between percentage of glucagon area and insulin sensitivity. (B) The ratio of β/α cell areas was significantly lower in the insulin resistant subjects, suggesting an unbalanced proportion between β and α cell areas. (C) Percentage of insulin and glucagon double positive cells in the sections of insulin sensitive and insulin resistant subjects. (D) Confocal microscopy analysis of insulin (Red) and glucagon (Green) immunostaining. 25X objective, scale bar 100 µm (1,2,3,4,5,6); 3x Amplification. Scale Bar 20 µm(7,8). * p ≤ 0.05.

**Figure 6 GLP-1 costains with glucagon, and its plasma values correlate with islet glucagon area and (inversely) with whole body insulin sensitivity.**

Immunostaining for Glucagon (Red), GLP-1 (Green) and DAPI (Blue). (A) Representative picture of an islet showing an overlap of glucagon and GLP-1 immunoreactivity. Scale Bar 100 µm. (B)
Correlation between percentage of glucagon area and GLP-1 secretion evaluated during the mixed meal test and expressed as AUC. (C) Correlation between glucose uptake and GLP-1 secretion evaluated during the mixed meal test and expressed as AUC and insulin sensitivity.

Table 1: Clinical and metabolic characteristics of patients before and after surgery. Glucose, Insulin and C-peptide AUC were measured during OGTT. Data are means ± SD or n. (sex distribution and clinical diagnoses), p- value significant <0.05.

Table 2: Clinical and metabolic characteristics of insulin sensitive and insulin resistant patients before and after surgery. Data are means ± SD, * p- value significant <0.05 vs. before surgery.

REFERENCES


<table>
<thead>
<tr>
<th>Subject characteristics</th>
<th>Before Surgery</th>
<th>After Surgery</th>
<th>P value</th>
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<tbody>
<tr>
<td>Mean Age (y)</td>
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<td>Sex (female/male)</td>
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<td>Clinical Diagnoses (n)</td>
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<td>Ampullary tumor</td>
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<td>BMI (kg/m²)</td>
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<td>26.5±4.7</td>
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<td>Waist-to-hip ratio</td>
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<td>Fasting glucose (mg/dl)</td>
<td>90.7±11.5</td>
<td>113±32.1</td>
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<td>Fasting Insulin (µUI/ml)</td>
<td>8.85±3.32</td>
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<td>Fasting C-peptide (ng/ml)</td>
<td>2.63±0.58</td>
<td>2.28±1.15</td>
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<td>Glucose AUC (mg/dl x 120’ x 10³)</td>
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<td>Insulin AUC (µUI/ml x 120’ x 10³)</td>
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<td>C-peptide AUC (ng/ml x 120’ x 10³)</td>
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<td>HDL cholesterol (mg/dl)</td>
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<td>LDL cholesterol (mg/dl)</td>
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<td>Total cholesterol (mg/dl)</td>
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<td>HbA1c % (mmol/mol)</td>
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<td>6.76±1.20 (50±13.1)</td>
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</table>

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<table>
<thead>
<tr>
<th>Subject characteristics</th>
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<td>After Surgery</td>
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<tr>
<td>BMI (kg/m^2)</td>
<td>27.7±3.22</td>
<td>26.4±2.54</td>
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<tr>
<td>Waist to hip ratio</td>
<td>0.93±0.05</td>
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<td>Fasting glucose (mg/dl)</td>
<td>89.8±11.8</td>
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<td>Fasting Insulin (µUI/ml)</td>
<td>7.83±2.07</td>
<td>7.41±6.52</td>
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<td>Fasting C-peptide (ng/ml)</td>
<td>2.7±0.44</td>
<td>2.6±1.13</td>
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<td>Glucose AUC (mg/dl x 120' x 10^3)</td>
<td>194±33.7</td>
<td>160±13.2</td>
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<tr>
<td>Insulin AUC (µUI/ml x 120' x 10^3)</td>
<td>33.9±6.8</td>
<td>11.27±3.27</td>
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<tr>
<td>C-peptide AUC (ng/ml x 120' x 10^3)</td>
<td>0.9±0.2</td>
<td>0.4±0.1</td>
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<td>HbA1c (%)</td>
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<td>HbA1c (mmol/mol)</td>
<td>37±6.3</td>
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Table 2: Clinical and metabolic characteristics of insulin sensitive and insulin resistant patients before and after surgery. Data are means ± SD, * P-value significant <0.05 vs. before surgery.
Figure 1 Decreased insulin secretion but unaltered insulin sensitivity after pancreateoduodenectomy.

(A) Schematic of the hyperglycemic clamp experiment. L-arginine bolus (5 g) was injected at 120 min. (B) Insulin secretion during the hyperglycemic clamp. (C) Changes in the area under curve (AUC) of first phase, second phase and after L-arginine stimulus (Arginine) detected during the hyperglycemic clamp. A significant reduction was found in all the phases of insulin secretion. (D) Hyperinsulinemic euglycemic clamp performed before and after surgery. (E) Mixed meal test performed before and after surgery. Changes in the area under curve (AUC) of GLP-1, GIP and Glucagon. * p ≤ 0.05, ** p ≤ 0.001.
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**Supplemental figure 1:**

Insulin + Glucagon double + cell. Insulin (Red), Glucagon (Green) and DAPI (Blue). Confocal microscopy. Objective 63X, magnification 2x. On the right profile curves showing the intensity distribution of the image along the straight red line traced on the cell.

**Supplemental figure 2:**

Confocal microscopy analysis Z-stack image, on the edges the transversal sections corresponding to the vertical and horizontal arrows on the picture.
Supplemental figure 3:

PDX1 (brown) Immunoreactivity in double positive cells. Microscopy analysis of glucagon (green) and insulin (red) staining (left pictures) overlapped to PDX1 immunoreactivity with chromagen (DAB) for PDX1 (right pictures) showed duct cells positive for PDX1. Insulin resistant (lower panel) and insulin sensitive (upper panel). Objective 400x