

EXHIBIT 5

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One year of sitagliptin treatment protects against islet amyloid-associated β -cell loss and does not induce pancreatitis or pancreatic neoplasia in mice

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¹Division of Metabolism, Endocrinology and Nutrition, Department of Medicine, Veterans Affairs Puget Sound Health Care System and University of Washington, Seattle, Washington; ²Metabolic Research Unit, School of Medicine, Deakin University, Victoria, Australia; and ³PhenoPath Laboratories, Seattle, Washington

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Aston-Mourney K, Subramanian SL, Zraika S, Samarasekera T, Meier DT, Goldstein LC, Hull RL. One year of sitagliptin treatment protects against islet amyloid-associated β -cell loss and does not induce pancreatitis or pancreatic neoplasia in mice. *Am J Physiol Endocrinol Metab* 305: E475–E484, 2013. First published June 4, 2013; doi:10.1152/ajpendo.00025.2013.—The dipeptidyl peptidase-4 (DPP-4) inhibitor sitagliptin is an attractive therapy for diabetes, as it increases insulin release and may preserve β -cell mass. However, sitagliptin also increases β -cell release of human islet amyloid polypeptide (hIAPP), the peptide component of islet amyloid, which is cosecreted with insulin. Thus, sitagliptin treatment may promote islet amyloid formation and its associated β -cell toxicity. Conversely, metformin treatment decreases islet amyloid formation by decreasing β -cell secretory demand and could therefore offset sitagliptin's potential proamyloidogenic effects. Sitagliptin treatment has also been reported to be detrimental to the exocrine pancreas. We investigated whether long-term sitagliptin treatment, alone or with metformin, increased islet amyloid deposition and β -cell toxicity and induced pancreatic ductal proliferation, pancreatitis, and/or pancreatic metaplasia/neoplasia. *hIAPP* transgenic and nontransgenic littermates were followed for 1 yr on no treatment, sitagliptin, metformin, or the combination. Islet amyloid deposition, β -cell mass, insulin release, and measures of exocrine pancreas pathology were determined. Relative to untreated mice, sitagliptin treatment did not increase amyloid deposition, despite increasing hIAPP release, and prevented amyloid-induced β -cell loss. Metformin treatment alone or with sitagliptin decreased islet amyloid deposition to a similar extent vs untreated mice. Ductal proliferation was not altered among treatment groups, and no evidence of pancreatitis, ductal metaplasia, or neoplasia were observed. Therefore, long-term sitagliptin treatment stimulates β -cell secretion without increasing amyloid formation and protects against amyloid-induced β -cell loss. This suggests a novel effect of sitagliptin to protect the β -cell in type 2 diabetes that appears to occur without adverse effects on the exocrine pancreas.

DPP-4 inhibitor; IAPP; β -cell mass; amyloid; exocrine pancreas pathology

DEFICITS IN β -CELL FUNCTION AND MASS underlie the pathogenesis of type 2 diabetes (6, 28). One contributing factor is islet amyloid, which occurs in the majority of subjects with type 2 diabetes (7, 25). Thus, development of interventions that can improve β -cell function and mass under conditions of amyloid formation, are desirable.

Dipeptidyl peptidase-4 (DPP-4) inhibitors improve glycemic control in type 2 diabetes, acting by blocking degradation of

incretins, including glucagon-like peptide-1 (GLP-1). Thus, these drugs increase active GLP-1 levels and thereby enhance insulin release and lower glucose levels. In rodent models of diabetes that do not develop islet amyloid, DPP-4 inhibition has been shown to preserve or even increase β -cell mass (11, 32, 35, 36, 42). Whether these beneficial effects also occur in the presence of amyloid deposition, however, remains unknown.

Investigation of islet amyloid in rodents requires transgenic models that express the amyloidogenic human form of islet amyloid polypeptide (IAPP); mouse and rat IAPP are not amyloidogenic. IAPP is a normal product of the β -cell that is cosecreted with insulin (27). Using transgenic mice that express human IAPP (hIAPP) at physiological levels, we have shown that reducing insulin/IAPP release with metformin or rosiglitazone treatment reduces islet amyloid deposition and the associated β -cell loss (22). Conversely, most (2, 31) but not all (39) studies show that interventions that increase hIAPP secretion increase amyloid deposition in cultured islets. Whether DPP-4 inhibition increases amyloid formation in vivo and/or offsets amyloid's toxic effects remains unknown. Additionally, whether metformin's ability to reduce amyloid formation still occurs when given with a DPP-4 inhibitor is an important unanswered question, as this combination is used clinically (16).

One study has investigated the effect of DPP-4 inhibition in hIAPP transgenic rats. Twelve weeks of treatment with the DPP-4 inhibitor sitagliptin alone or with metformin did prevent the loss of β -cells (34). However, since islet amyloid deposition occurs over many months in rodent models (5, 20, 23, 44), the extent of islet amyloid deposition and its contribution to the observed effects were not evaluated. A longer-term study is therefore necessary to answer this question.

Although beneficial effects of DPP-4 inhibition on glucose metabolism in humans have been demonstrated, recent reports have raised some concerns regarding the safety of this class of therapeutics, particularly in situations where amyloidogenic hIAPP is expressed. In transgenic rats with significant overexpression of hIAPP, 12 wk of sitagliptin treatment resulted in increased pancreatic ductal proliferation, pancreatitis, and ductal metaplasia (34). In mice with an activating mutation of the *KRAS* protooncogene (but in the absence of hIAPP expression), treatment with a GLP-1 analog resulted in exacerbation of chronic pancreatitis and proliferative effects on the exocrine pancreas (14). Conversely, long-term GLP-1 analog treatment of nonhuman primates (which express amyloidogenic IAPP) have not reported such changes (38).

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Case studies in humans have reported that use of DPP-4 inhibitors is associated with acute pancreatitis in subjects with type 2 diabetes (12, 13, 15, 43). Conversely, other studies in human type 2 diabetes have shown that treatment with the DPP-4 inhibitors sitagliptin or vildagliptin was not associated with an increased risk of pancreatitis above that of diabetes alone (10, 13, 30). A recent study on samples from humans with type 2 diabetes reported that those treated with DPP-4 inhibitors or GLP-1 mimetics had increased exocrine and endocrine mass and increased numbers of pancreatic intraepithelial neoplasia (PanIN) 1 and 2 lesions (4).

Thus, the data regarding adverse effects of DPP-4 inhibitors on the exocrine pancreas in humans and animal models remain mixed. An animal study utilizing clinically relevant, long-term administration of a DPP-4 inhibitor in the presence of physiological hIAPP expression, and with appropriate controls that do not express amyloidogenic hIAPP, would shed light on this issue. In the present study, we used our transgenic mice, which express hIAPP at physiological levels, and nontransgenic littermate controls to determine whether 1 yr of sitagliptin treatment, alone or with metformin, increases islet amyloid deposition, offsets the toxic effects of amyloid, and/or results in increased ductal proliferation and exocrine pancreas pathology.

MATERIALS AND METHODS

Study mice and treatments. Studies were approved by the VA Puget Sound Health Care System Institutional Animal Care and Use Committee. Male hemizygous C57/DBA.hIAPP transgenic mice (9) and nontransgenic littermates received a high-fat diet (45% kcal from fat, D12451; Research Diets, New Brunswick, NJ) starting at 10 wk of age throughout the year-long study. Mice were randomly assigned to one of four treatment groups: untreated ($n = 21$ hIAPP transgenic and 25 nontransgenic), sitagliptin (Merck Research Laboratories, Rahway, NJ; 3.1 g/kg in food; $n = 25$ hIAPP transgenic and 25 nontransgenic), metformin (US Biological, M3009-75A, Swampscott, MA; 10 g/l in drinking water; $n = 24$ hIAPP transgenic and 26 nontransgenic), or sitagliptin plus metformin ($n = 24$ hIAPP transgenic and 23 nontransgenic). Doses of sitagliptin and metformin were chosen based on published studies (22, 35). Due to the large number of endpoints in this study, not all measurements were made on all mice. The sample size for each measurement is given below in each respective section.

Body weight, food, water, and drug intake, plasma drug levels, energy expenditure, and physical activity. Body weight was determined monthly. Food and water intakes were monitored for 2-wk periods at 0, 3, 6, 9, and 12 mo of treatment. Plasma levels of metformin and sitagliptin were measured throughout the light-dark cycle at 6 AM, 12 PM, 6 PM, and 10 PM following 1 mo of treatment ($n = 6-7$), using an LCMS-based assay at Merck Research Labs. Briefly, plasma protein was extracted, purified, and analyzed by Sciex API 5000 triple quadrupole mass spectrometer at the positive ionization mode, coupled with Waters Acquity UPLC HSSS T3 50 \times 2.1-mm column (1.8 mm). Concentrations of metformin and sitagliptin were determined by the product ions and quantified against a calibration curve. Drug levels were expressed as average daily exposure (mean of data from each time point \times 24 h).

DPP-4 activity was measured in plasma after 25 wk of treatment ($n = 9-14$) by fluorometric assay at Merck Research Labs. Assay samples were blinded with respect to treatment and genotype. DPP-4 inhibition was calculated as the ratio of DPP-4 activity in treated samples vs. control samples, as previously described (29).

After 17 wk of treatment, a time where body weight was already significantly different among groups and weight gain was still occurring, energy expenditure and physical activity were assessed using the Comprehensive Lab Animal Monitoring System (Columbus Instru-

ments, Columbus, OH) ($n = 4-7$). Ambulatory activity was calculated as the number of beam breaks per day. Oxygen consumption (VO_2) was calculated using standard approaches. No differences were observed in weight gain between hIAPP transgenic and nontransgenic mice for a given treatment; thus, genotypes were combined for these analyses.

Intravenous glucose tolerance test. After 12 mo of treatment, following an overnight fast, glucose (1 g/kg iv) was administered under pentobarbital anesthesia (100 mg/kg ip) (22). Retroorbital blood samples were drawn before and 2, 5, 10, 20, 30, and 45 min following glucose injection ($n = 10-19$). The acute insulin response to glucose was calculated as the average of insulin values 2 and 5 min after glucose stimulation, with the basal insulin (before glucose administration) value subtracted. Glucose tolerance was assessed by the rate of glucose disappearance (K_{it}), the slope of the regression line for the relationship between natural logarithm of glucose levels and time from 5 to 20 min.

Intraperitoneal insulin tolerance test. After 12 mo of treatment, after a 3- to 4-h fast, insulin (1.5 U/kg ip) was administered to conscious mice, and blood samples were drawn before and 15, 30, 45, and 60 min following insulin injection via tail tipping ($n = 4-7$).

Pancreatic peptide measurements. At euthanasia, a small portion of the pancreas was snap-frozen and homogenized in 50% (vol/vol) isopropanol/1% (vol/vol) trifluoroacetic acid (22). Insulin and hIAPP contents were measured and expressed as a proportion of total protein content.

Glucose, hIAPP, insulin, and protein assays. Plasma glucose was determined using the glucose oxidase method (22) for all procedures except intraperitoneal insulin tolerance test (IPITT), where blood glucose was measured using an AlphaTRAK glucometer (Abbott Laboratories, Abbott Park, IL). hIAPP and insulin concentrations were determined by ELISA using the Total Human Amylin ELISA (Millipore, Billerica, MA) and Insulin Ultrasensitive (Mouse) ELISA (Alpco, Salem, NH), respectively (2, 22). Total protein was determined using the BCA assay (Thermo Scientific, Rockford, IL).

Quantification of amyloid deposition and β -cell mass. Pancreata were weighed and then fixed in 4% (wt/vol) phosphate-buffered paraformaldehyde and embedded in paraffin. Five-micrometer sections were stained with thioflavin S to visualize amyloid and insulin antibody (I-2018, diluted 1:2,000; Sigma-Aldrich, St. Louis, MO) to visualize β -cells, as we have done previously (3, 20, 22, 23, 46). Assessments were made in a blinded manner on an average of 26 islets per mouse from three different sections of the pancreas. We have previously shown this sampling technique to be representative of a whole mouse pancreas (46). Section, β -cell, and amyloid areas were determined. Amyloid severity was calculated as percent amyloid-positive area/section area. β -Cell mass was calculated as (insulin area/section area) \times pancreas weight.

Quantification of pancreatic ductal proliferation. Ductal proliferation was assessed in three pancreas sections per mouse following antigen retrieval (EDTA buffer, pH 9.0, 100°C for 20 min) and immunolabeling for Ki67 (clone TEC-3, 1:50; Dako, Carpinteria, CA) and cytokeratin (Z0622, 1:2,000; Dako). Whole slides were scanned (NanoZoomer Virtual Microscopy, Olympus, Center Valley, PA), and the images were examined in a blinded manner. The number of Ki67-cytokeratin double-positive cells was counted manually from an average of 1,640 \pm 73 total duct cells. The total number of ductal cells was computed based on a correlation-relating duct perimeter (acquired using the NanoZoomer software) to the number of cells per duct (counted manually) using data from 70 ductal structures containing $>3,500$ ductal cells (no. of cells = [perimeter-31.7]/5.3; $r^2 = 0.98$). Ductal proliferation was expressed as percent Ki67-positive duct cells per total duct cells.

Histological determination of exocrine pancreas abnormalities. Three different pancreas sections per mouse were stained with hematoxylin and eosin and examined by an experienced pathologist (L. C. Goldstein) in a blinded manner. Sections were examined for ductal

abnormalities (including abnormal duct morphology and evidence of neoplasia or metaplasia), hemorrhage, fibrosis, inflammatory cell infiltrates, and necrosis in the exocrine pancreas. Abnormalities were graded as absent, mild, moderate, or severe (definitions in Table 1). In addition, specimens were independently examined, in a blinded manner, by two veterinary pathologists to provide validation of this grading system and specifically to identify the presence of any lesions suggesting possible neoplastic transformation.

Gross lesions were observed in the vicinity of the pancreas in five mice (two untreated nontransgenic, two sitagliptin-treated *hIAPP* transgenic, and one sitagliptin-treated nontransgenic). No such lesions were observed in any other treatment groups. For two of these (untreated nontransgenic and sitagliptin-treated nontransgenic), no specimen was available for analysis. For the remaining three, hematoxylin and eosin-stained sections of the lesions and of the adjacent pancreas were independently examined by L. C. Goldstein and the two veterinary pathologists.

Statistical analyses. Data are expressed as means \pm SE unless otherwise indicated and were tested for normality using the Kolmogorov-Smirnov and Shapiro-Wilk tests. Data that were not normally distributed were log or square root transformed prior to analysis. Data were compared by Pearson's χ^2 test or analysis of variance (ANOVA) with least significant difference post hoc analysis. Exocrine tissue pathology data were compared using χ^2 test. A *P* value \leq 0.05 was considered significant.

RESULTS

Drug delivery and efficacy. All groups of mice consumed drug(s) at or above the target doses (0.3 g·kg⁻¹·day⁻¹ sitagliptin, 0.6 g·kg⁻¹·day⁻¹ metformin; data not shown), consistent with previous findings from our group and others (22, 35). Plasma sitagliptin exposure did not differ between groups treated with sitagliptin alone or together with metformin (124 \pm 8 μ M/day sitagliptin alone, 122 \pm 8 μ M/day sitagliptin plus metformin, *P* = 0.9) or across the light-dark cycle (*P* = 0.2 sitagliptin, *P* = 0.9 sitagliptin plus metformin). Similarly, plasma metformin levels did not differ between mice treated with metformin alone or in combination with sitagliptin (979 \pm 127 μ M/day metformin alone, 1,222 \pm 162 μ M/day sitagliptin plus metformin, *P* = 0.3) or over the light-dark cycle (*P* = 0.1 metformin, *P* = 0.6 sitagliptin plus metformin). Virtually complete DPP-4 inhibition was observed in both sitagliptin (99 \pm 0.1%) and sitagliptin plus metformin-treated mice (99 \pm 0.1%) relative to untreated or metformin treated mice (21 \pm 9 and 22 \pm 8%, respectively).

Body weight, food intake, energy expenditure, and physical activity. Untreated and sitagliptin-treated *hIAPP* transgenic and nontransgenic mice gained weight to a similar degree through-

out the study (Fig. 1A). Mice treated with metformin with or without sitagliptin gained significantly less weight than untreated mice (Fig. 1A). No differences in body weight were observed between genotypes for any treatment group. Despite decreased weight gain, mice treated with metformin alone or in combination with sitagliptin exhibited increased food intake throughout the treatment period (Fig. 1B). Mice treated with metformin alone or in combination with sitagliptin exhibited increases in ambulatory activity (Fig. 1C) and Vo₂ (Fig. 1D).

Plasma glucose, insulin, and *hIAPP* levels, insulin sensitivity, and pancreatic hormone content. Fasting plasma glucose and insulin levels were comparable in untreated *hIAPP* transgenic and nontransgenic mice (Table 2). In nontransgenic mice, all treatments resulted in decreased fasting plasma glucose levels. Findings were similar in *hIAPP* transgenic mice, but not all reached statistical significance (Table 2). Fasting plasma insulin levels did not differ with sitagliptin treatment but were decreased with metformin alone and metformin plus sitagliptin (Table 2). Fasting plasma *hIAPP* levels were significantly increased with sitagliptin treatment, tended to be decreased with metformin treatment alone (*P* = 0.06), and were significantly decreased with metformin plus sitagliptin treatment (Table 2). Insulin sensitivity was similar in untreated, sitagliptin-treated, and sitagliptin plus metformin-treated mice, whereas metformin treatment significantly increased insulin sensitivity (Fig. 2). Insulin sensitivity did not differ between *hIAPP* transgenic and nontransgenic mice in each treatment group (Fig. 2).

Pancreatic insulin content was decreased in untreated *hIAPP* transgenic compared with nontransgenic mice (Table 2). Insulin content did not differ with sitagliptin treatment but was decreased with metformin treatment with or without sitagliptin (Table 2). *hIAPP* content and the ratio of *hIAPP* to insulin contents were comparable in all *hIAPP* transgenic mice, the content of *hIAPP* being 1–2% that of insulin, which is consistent with physiological *hIAPP* production (20, 27, 37).

Glucose tolerance and insulin secretion. During the IVGTT, sitagliptin-treated mice had increased glucose disappearance rates, whereas glucose disappearance did not differ between untreated mice and those on metformin (Fig. 3A).

Consistent with our previous studies (20, 23), untreated *hIAPP* transgenic mice had decreased insulin release in response to glucose (Fig. 3B) compared with untreated nontransgenic mice (*P* < 0.05). Sitagliptin treatment significantly increased insulin release in *hIAPP* transgenic and nontrans-

Table 1. Scoring definitions of exocrine pancreas abnormalities

	Mild	Moderate	Severe
Ductal Abnormalities	1 instance of abnormal duct morphology/neoplasia/metaplasia	2 instances of abnormal duct morphology/neoplasia/metaplasia	>2 instances of abnormal duct morphology/neoplasia/metaplasia
Hemorrhage	1 instance of hemorrhage	2 instances of hemorrhage	>2 instances of hemorrhage
Fibrosis	Fibrosis without inflammatory infiltrates in <10% of ducts	Fibrosis with or without inflammatory infiltrates in 10–25% of ducts	Fibrosis with or without inflammatory infiltrates in >25% of ducts
Inflammatory cell infiltration	Single focal occurrence of <100 mononuclear cells	Diffuse occurrence including <100 mononuclear cells or focal occurrence of 100–200 mononuclear and/or polymorphonuclear cells	Diffuse occurrence including >100 and/or >2 focal occurrences of >200 mononuclear and/or polymorphonuclear cells
Necrosis	1 small (<0.1 mm ²) region of necrosis	2 small (<0.1 mm ²) or 1 large (>0.1 mm ²) region of necrosis	>2 small (<0.1 mm ²) or >1 large (>0.1 mm ²) region of necrosis

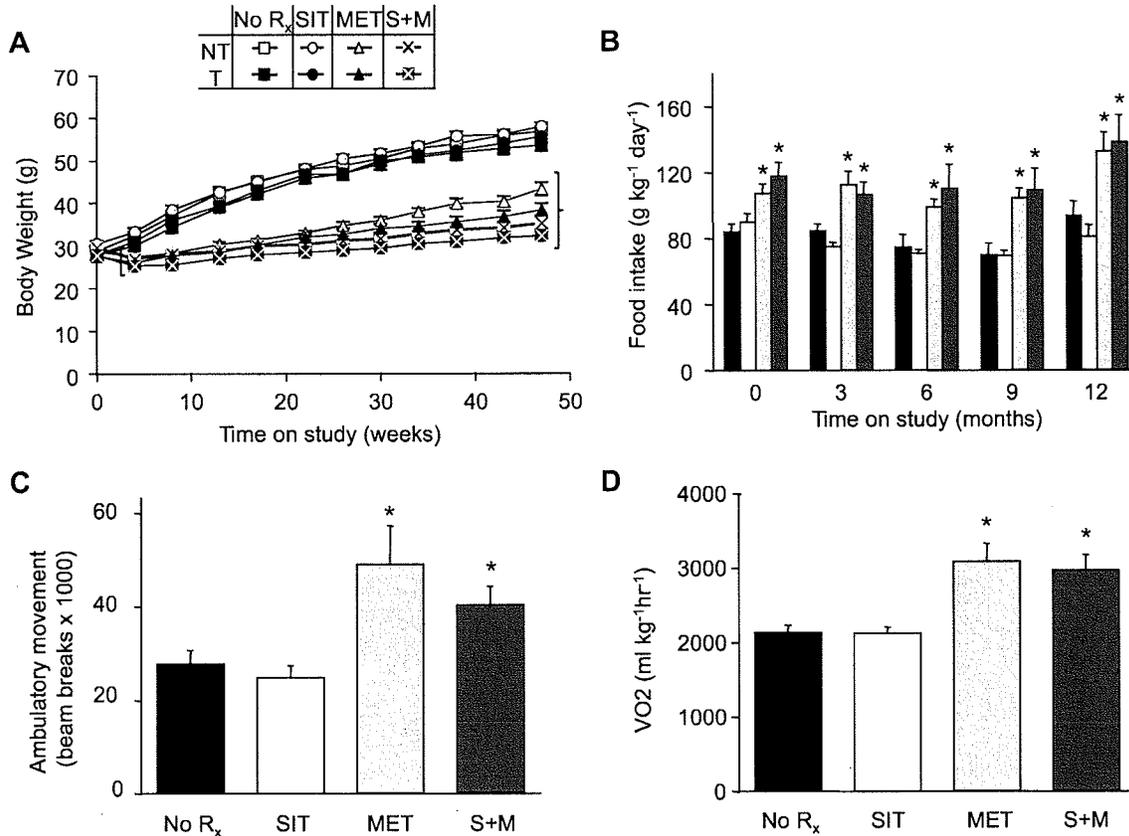


Fig. 1. Body weight (A; $n = 18-24$) throughout the study in human islet amyloid polypeptide (*hIAPP*) transgenic (T; filled symbols) and nontransgenic (NT; open symbols) mice following 1 yr of no treatment (No Rx; squares) or treatment with sitagliptin (SIT; circles), metformin (MET; triangles), or sitagliptin + metformin (S+M; crosses), ANOVA $P < 0.001$. Food intake at 0, 3, 6, 9, and 12 mo of treatment (B; $n = 4-13$ cages, 8-35 mice, ANOVA $P < 0.001$), ambulatory activity (C; $n = 4-7$, ANOVA $P < 0.01$), and oxygen consumption (D; $n = 4-7$, ANOVA $P < 0.001$) after 17 wk of treatment. B-D: No Rx, filled bars; SIT, open bars; MET, light gray bars; S+M, dark gray bars. * $P < 0.05$ vs. No Rx. In A, for all treatment groups, all points between parentheses are significantly different from the corresponding time point for No Rx.

genic mice ($P < 0.005$; Fig. 3B). Insulin responses were comparable between sitagliptin-treated nontransgenic and *hIAPP* transgenic mice (Fig. 3B). Insulin release was decreased with metformin treatment alone or in combination with sitagliptin for both genotypes (Fig. 3B).

Amyloid deposition. Untreated *hIAPP* transgenic mice developed islet amyloid deposits (Fig. 4A) while, as expected, nontransgenic mice did not, regardless of treatment. Sitagliptin treated *hIAPP* transgenic mice had similar amyloid formation to untreated *hIAPP* transgenic mice (Fig. 4, A and B). As seen

previously (22), metformin-treated *hIAPP* transgenic mice had greatly reduced amyloid deposition (Fig. 4, A and B). The combination of sitagliptin plus metformin treatment also resulted in a significant reduction in amyloid deposition in *hIAPP* transgenic mice (Fig. 4, A and B).

β -Cell mass. Untreated *hIAPP* transgenic mice had decreased β -cell mass compared with untreated nontransgenic mice (Fig. 4C), consistent with the toxic effect of islet amyloid deposition. Unexpectedly, nontransgenic mice treated with sitagliptin had decreased β -cell mass compared with untreated

Table 2. Fasting plasma glucose, insulin, and *hIAPP* levels and pancreatic insulin and *hIAPP* content

Genotype	Treatment	Fasting glucose (mmol/l)	Fasting insulin (pmol/l)	Fasting <i>hIAPP</i> (pmol/l)	Insulin content (nmol/mg protein)	<i>hIAPP</i> content (pmol/mg protein)	Pancreatic <i>hIAPP</i> : insulin ratio (%)
Nontransgenic	Untreated	9.44 ± 0.44	1510 ± 517		2.85 ± 0.55		
	Sitagliptin	8.22 ± 0.28*	774 ± 100		1.79 ± 0.24		
	Metformin	7.83 ± 0.39*	204 ± 39*		0.61 ± 0.07*		
	Sitagliptin + Metformin	7.50 ± 0.33*	163 ± 35*		0.42 ± 0.07*		
<i>hIAPP</i> transgenic	Untreated	8.94 ± 0.50	646 ± 103	46 ± 10	1.57 ± 0.32†	13.8 ± 4.0	1.18 ± 0.17
	Sitagliptin	8.00 ± 0.44	1105 ± 196	71 ± 5*	1.78 ± 0.32	19.7 ± 4.5	1.29 ± 0.19
	Metformin	7.89 ± 0.50	171 ± 47*	27 ± 7	0.57 ± 0.10*	12.0 ± 5.6	1.79 ± 0.41
	Sitagliptin + Metformin	7.44 ± 0.33*	137 ± 27*	26 ± 3*	0.57 ± 0.05*	7.0 ± 0.7	1.63 ± 0.34

Values are means ± SE. *hIAPP*, human islet amyloid polypeptide. * $P \leq 0.05$ vs. Untreated; † $P < 0.05$ vs. Nontransgenic.

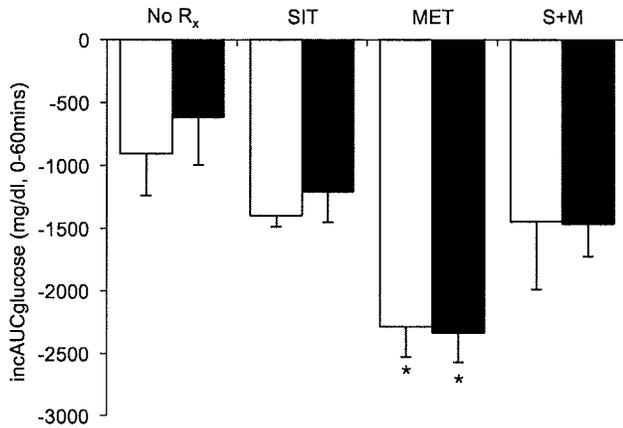


Fig. 2. Inverse incremental AUC glucose during an ip insulin tolerance test (ANOVA $P < 0.01$) in *hIAPP* transgenic (filled bars) and nontransgenic mice (open bars). Mice were No R_x or treated with SIT, MET, or S+M for 1 yr; $n = 4-7$. * $P < 0.05$ vs. No R_x .

nontransgenic mice (Fig. 4C). In contrast, *hIAPP* transgenic mice treated with sitagliptin had increased β -cell mass compared with untreated *hIAPP* transgenic mice; there was no difference in β -cell mass between sitagliptin-treated *hIAPP* transgenic and nontransgenic mice ($P = 0.3$; Fig. 4C). As seen previously (22), metformin-treated *hIAPP* transgenic and nontransgenic mice had significantly lower β -cell mass than untreated mice (Fig. 4C). Sitagliptin plus metformin treatment also resulted in significantly reduced β -cell mass in both genotypes (Fig. 4C).

Pancreatic ductal proliferation, pancreas mass, and exocrine pancreas abnormalities. Proliferating ductal epithelial cells were detected in all mice at a low level (<3% K167-positive duct cells). No statistically significant differences were observed in ductal proliferation among treatment groups or between *hIAPP* transgenic and nontransgenic mice (Fig. 5; $P = 0.18$).

In untreated animals, *hIAPP* transgenic mice had lower pancreas mass than nontransgenic controls (Table 3). Sitagliptin treatment did not alter pancreas mass in either genotype. Conversely, metformin treatment alone or in combination with sitagliptin resulted in decreased pancreas mass. However, when data were normalized to body weight, pancreas mass was increased with metformin or metformin plus sitagliptin treatment.

Histological pancreas specimens were examined for the presence and severity of ductal abnormalities (including abnormal duct morphology, metaplasia, or neoplasia), hemorrhage, fibrosis, inflammatory cell infiltration, and necrosis in the exocrine pancreas. None of the mice in this study exhibited any ductal abnormalities, including evidence of metaplasia, neoplasia, or presence of potentially premalignant lesions such as PanINs (Fig. 6A). Similarly, no evidence of hemorrhage was observed in the exocrine tissue (Fig. 6B). However, hemorrhage was observed in one to two islets each from two nontransgenic mice treated with sitagliptin, but not from any other group (data not shown).

All groups demonstrated mild to moderate periductal fibrosis (Fig. 6C); this did not differ significantly by treatment or genotype ($P = 0.4$). Similarly, mild to moderate inflammatory

cell infiltrates were also observed in all treatment groups (Fig. 6D), occurring both around ducts and around blood vessels. The frequency of these inflammatory cell infiltrates did not differ by treatment or genotype ($P = 0.3$). Focal necrosis was observed in only six mice: two untreated *hIAPP* transgenic, one sitagliptin-treated *hIAPP* transgenic, and three sitagliptin-treated nontransgenic mice (Fig. 6E). When the frequency of focal necrosis was compared across treatment/genotype groups, it was not statistically significant ($P = 0.4$). Gross lesions in the vicinity of the pancreas in three mice (one untreated nontransgenic and two sitagliptin-treated transgenic) were examined and found to be pseudocysts with fat necrosis, saponification, and calcification. Pancreas tissue in close proximity to these lesions was examined and showed no signs of pancreatitis or other exocrine abnormalities. One of these three mice (untreated nontransgenic) showed mild periductal fibrosis and inflammatory infiltration, similar to many animals in the study; the others showed no such abnormalities.

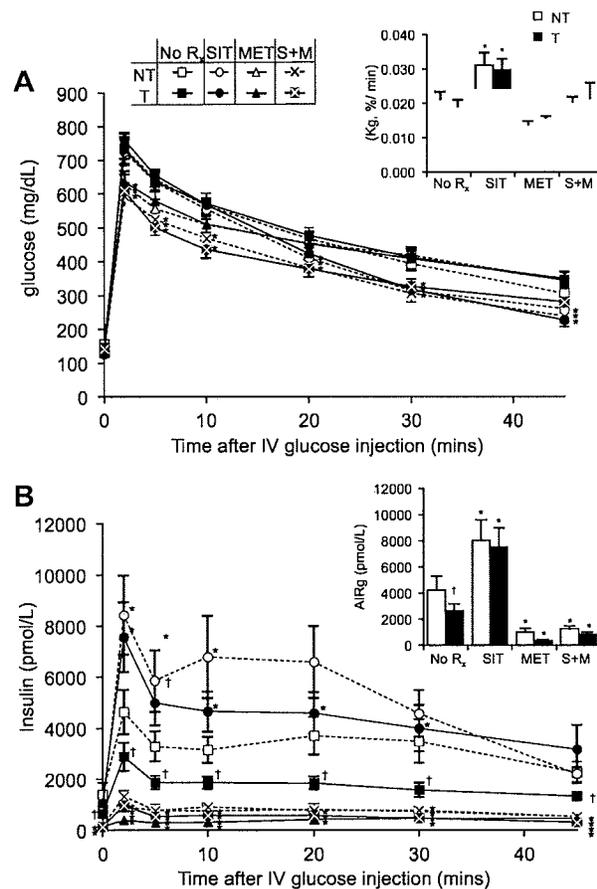


Fig. 3. Plasma glucose levels (A), rate of glucose disappearance (K_g ; inset in A, ANOVA $P < 0.001$), plasma insulin levels (B), and the acute insulin response to glucose (AIRg; inset in B, ANOVA $P < 0.001$) during an iv glucose tolerance test in *hIAPP* transgenic (T; filled symbols) and nontransgenic mice (NT; open symbols). Mice were No R_x (squares) or treated with SIT (circles), MET (triangles), or S+M (crosses) for 1 yr; $n = 10-18$. * $P < 0.05$ vs. No R_x ; † $P < 0.05$ vs. NT.

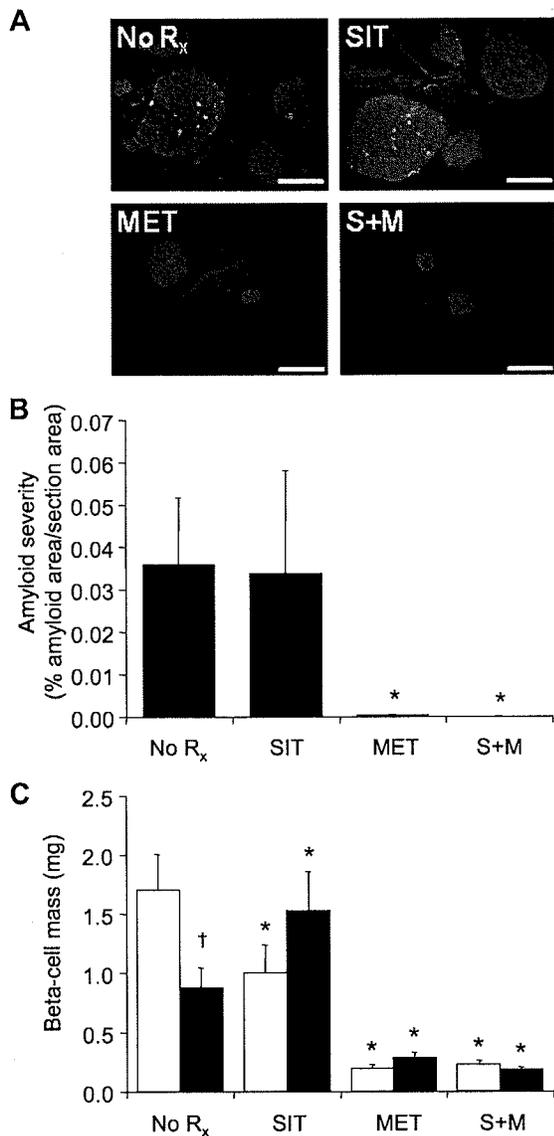


Fig. 4. A: representative photomicrographs of pancreas sections showing amyloid deposits (green) and insulin immunostaining (red). B: amyloid deposition in *hIAPP* transgenic mice (ANOVA $P = 0.01$). Nontransgenic mice did not develop any islet amyloid, as expected; thus, amyloid data are not shown for those groups. C: β -cell mass (ANOVA $P < 0.001$) in *hIAPP* transgenic (filled bars) and nontransgenic mice (open bars). Pancreata were analyzed following 1 yr of No R_x or treatment with SIT, MET, or S+M; $n = 10$ –21. Scale bar, 100 μ m. * $P < 0.05$ vs. No R_x; † $P < 0.05$ vs. NT.

DISCUSSION

In this study, we found that 1 yr of sitagliptin treatment in *hIAPP* transgenic mice did not increase islet amyloid deposition despite increasing *hIAPP* release. However, although amyloid still formed with sitagliptin treatment, the expected decrease in β -cell mass did not occur. Our data therefore strongly suggest that sitagliptin treatment can offset the toxicity of islet amyloid formation in vivo, in keeping with our and others' in vitro observations with exendin-4 (2, 39). Furthermore, we observed that the combination of sitagliptin plus

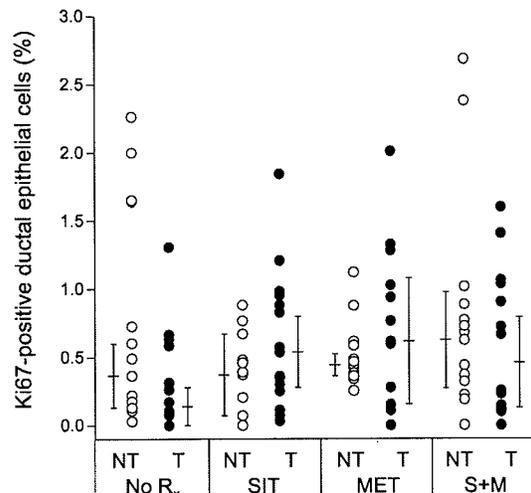


Fig. 5. Ductal proliferation in *hIAPP* transgenic (filled symbols) and nontransgenic mice (open symbols). Mice were No R_x or treated with SIT, MET, or S+M for 1 yr; $n = 10$ –15, ANOVA $P = 0.18$. Individual points (circles) represent data for each mouse, with mean \pm interquartile range for each group also depicted (lines).

metformin resulted in decreased islet amyloid deposition, similar to that of metformin treatment alone, the latter consistent with our previous study (22).

We also examined the effects of long-term exposure to these treatments on the exocrine pancreas. Ductal proliferation in our model was low and did not significantly differ among treatment groups. Pancreas mass did not change with sitagliptin treatment but was increased with metformin treatment. Noteworthy, in contrast to a previous report in rats overexpressing *hIAPP* (34), we found that long-term treatment with sitagliptin alone or in combination with metformin was not associated with exocrine pancreas pathology, including lack of evidence of pancreatitis, abnormal duct morphology, metaplasia, or neoplasia.

As we have previously observed (20, 22, 23, 45), *hIAPP* transgenic mice exhibited islet amyloid formation following a year of high-fat feeding, which in the present study was associated with reduced insulin release and decreased β -cell mass. Sitagliptin treatment did not increase amyloid formation compared with that in untreated animals, despite a significant increase in insulin and *hIAPP* release. This was an unexpected finding, as the magnitude of *hIAPP* secretory output has been

Table 3. Pancreatic mass

Genotype	Treatment	Pancreatic Mass, g	Pancreatic Mass/Body Weight, %
Nontransgenic	Untreated	0.46 \pm 0.02	0.82 \pm 0.03
	Sitagliptin	0.42 \pm 0.02	0.73 \pm 0.03
	Metformin	0.38 \pm 0.01*	0.90 \pm 0.04
	Sitagliptin + Metformin	0.32 \pm 0.01*	0.96 \pm 0.05*
<i>hIAPP</i> transgenic	Untreated	0.40 \pm 0.02†	0.75 \pm 0.03
	Sitagliptin	0.43 \pm 0.02	0.79 \pm 0.03
	Metformin	0.36 \pm 0.02	0.93 \pm 0.04*
	Sitagliptin + Metformin	0.33 \pm 0.01*	1.01 \pm 0.04*

Values are means \pm SE. * $P \leq 0.05$ vs. Untreated; † $P < 0.05$ vs. Nontransgenic.

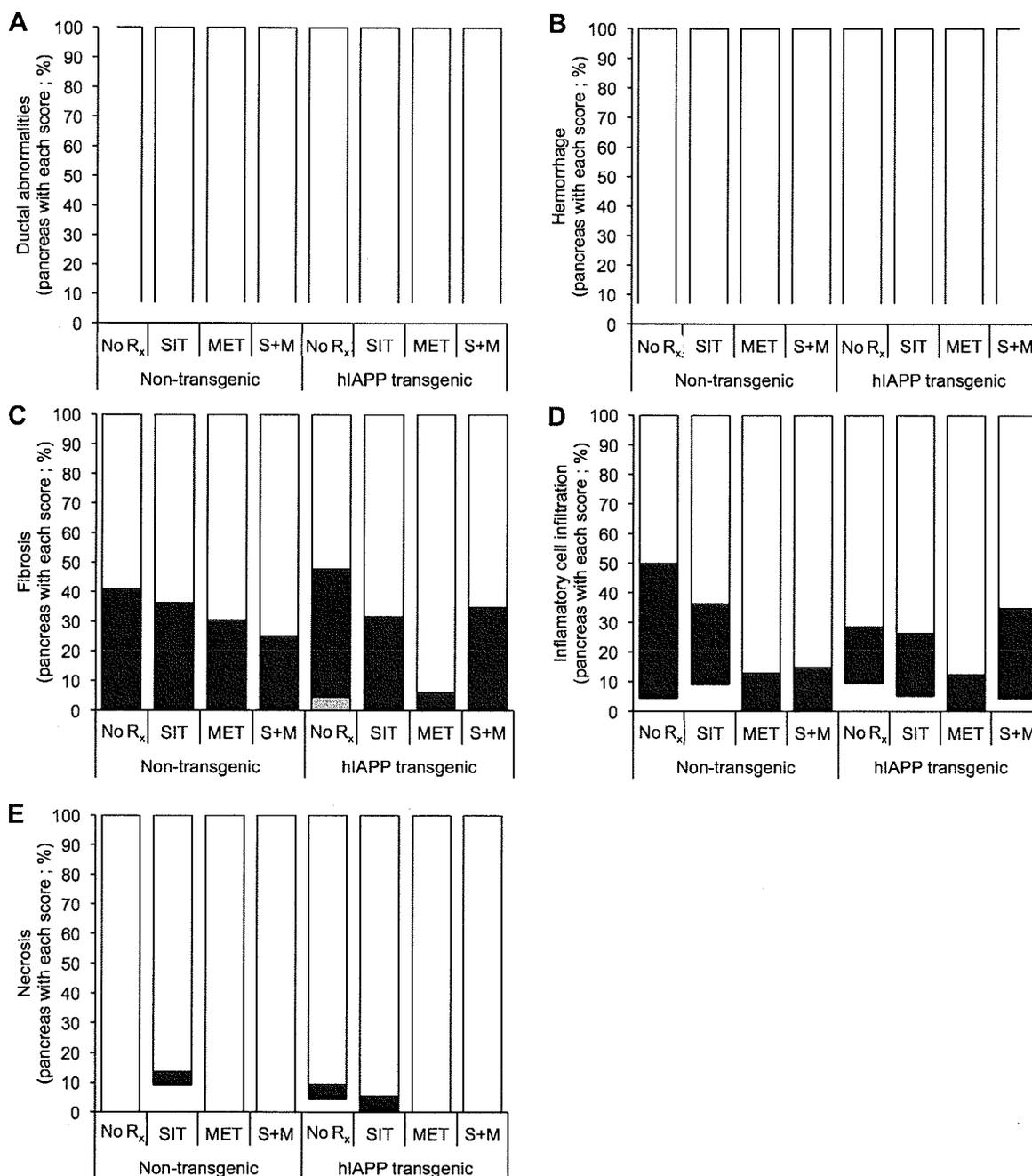


Fig. 6. Percentage of mice with exocrine pancreas pathology, assessed as ductal abnormalities (A), hemorrhage (B), fibrosis (C, $P = 0.4$), inflammatory cell infiltration (D, $P = 0.3$), and necrosis (E, $P = 0.4$) in *hIAPP* transgenic and nontransgenic mice. Mice were No Rx or treated with SIT, MET, or S+M for 1 yr; $n = 10$ –21. Absence of abnormalities is denoted by open bars, with mild abnormalities shown in gray hatched bars and moderate abnormalities shown in filled bars. Note: no mice exhibited any severe abnormalities.

shown to be a critical determinant of amyloid deposition both in vitro (2, 31) and in vivo (17, 18, 23, 44). Moreover, treatment of *hIAPP* transgenic islets with the GLP-1 analog exendin-4 resulted in increased amyloid formation (2). This difference in findings between exendin-4 and sitagliptin most likely occurs because GLP-1 analogs increase β -cell function to a far greater degree than DPP-4 inhibitors (40); therefore,

the former would be expected to stimulate insulin and *hIAPP* release to a much greater degree. We believe it is therefore possible that this effect of GLP-1 analogs to exacerbate amyloid formation will also occur in vivo, although this remains to be tested. In contrast, sitagliptin appears to dissociate increased β -cell output from amyloid formation, suggesting that it may have a beneficial effect in diabetes since it may stimulate

insulin release without increasing amyloid deposition and its resulting toxicity.

Interventions that decrease insulin and hIAPP release are associated with decreased amyloid formation both in vitro (2) and in vivo (1, 22). In the present study, metformin treatment markedly decreased amyloid formation and β -cell mass, as we demonstrated previously (22). Here, we now also show that the combination of sitagliptin plus metformin resulted in similar decreases in amyloid deposition and β -cell mass. These effects almost certainly occur due to metformin's action to limit weight gain, which while consistent with our previous study (22), occurred to a greater extent in the present study. Importantly, reduced weight gain with metformin treatment alone or with sitagliptin occurred in the face of increased food intake and energy expenditure, suggesting that the body weight phenotype was not secondary to an aversive effect of the drug. We do not believe that the observed decrease in β -cell mass following metformin treatment reflects islet pathology. Rather, we believe this is an appropriate response to the prevailing β -cell secretory demand. Previously, we (21) reported that long-term high-fat feeding in rodents stimulated β -cell expansion in an attempt to respond to the increased demand for insulin release due to obesity and insulin resistance. In the present study, as in our previous study (22), the decreased body weight gain and improved insulin sensitivity seen with metformin treatment would therefore be expected to reduce insulin demand, thus reducing islet hyperplasia and leading to decreased β -cell mass. The β -cell mass following metformin treatment is remarkably similar to what we previously observed following 12 mo of a low-fat diet (21), an intervention also associated with lower weight gain and β -cell secretory demand relative to high-fat-fed mice.

In *hIAPP* transgenic mice, sitagliptin treatment resulted in increased β -cell mass, consistent with a beneficial effect to offset the toxic effects of amyloid deposition. The reported anti-inflammatory effects of sitagliptin may have contributed to this effect. In fact, we and others (33, 47) have shown that hIAPP aggregation can be proinflammatory, inducing cytokine and chemokine production in macrophages and dendritic cells in vitro. Thus, sitagliptin treatment may antagonize this effect, contributing to its effects to block amyloid toxicity. Our observation that increased β -cell mass can occur in the face of islet amyloid deposition in vivo provides new information and further supports a potential beneficial effect of sitagliptin on the endocrine pancreas in diabetes.

We also determined, in nontransgenic mice, the effect of long-term sitagliptin treatment on β -cell mass in the absence of amyloid deposition. On the basis of the literature from relatively short-term studies (3–25 wk) in younger animals, one would expect an increase in β -cell mass (32, 34–36, 42). Rather, in the present study sitagliptin treatment resulted in decreased β -cell mass relative to that in untreated high-fat-fed nontransgenic mice. We believe that, similarly to our findings with metformin treatment, nontransgenic mice treated with sitagliptin exhibit differences in the manner of β -cell adaptation to increased secretory demand with high-fat feeding. As mentioned, long-term high-fat feeding stimulates β -cell expansion in mice (21). In the present study, sitagliptin treatment stimulated insulin release pharmacologically, which we believe in turn decreased the need for β -cell expansion in order to meet secretory demand. Consistent with our findings, a recent study

showed that sitagliptin treatment, while increasing β -cell mass in mice fed a low-fat diet, actually decreased β -cell mass in mice fed a high-fat diet relative to mice receiving high-fat diet alone (41).

Some studies have suggested that sitagliptin has detrimental effects on the exocrine pancreas (4, 13, 15, 34). In the present study, exocrine periductal fibrosis and inflammatory infiltrates were detected, but this was observed in all groups, suggesting that this occurred in response to age and/or high-fat feeding rather than to any specific genotype or treatment. Focal necrosis was detected in a few mice, but this did not differ among groups. Interestingly, however, no necrosis was detected in metformin-treated mice, alone or in combination with sitagliptin, suggesting that this compound may have a protective effect. Interestingly, metformin-treated mice were the only group in which an increase in pancreas mass was observed (when normalized to body weight). The lack of exocrine pancreas abnormalities in this group suggests that increased pancreatic mass in this study did not constitute evidence of pathology.

Ductal proliferation was low in all groups but was somewhat variable and did not differ by genotype or treatment. Consistent with these neutral effects on ductal proliferation, drug treatment was not associated with evidence of pancreatitis, abnormal duct morphology, metaplasia, or neoplasia. Gross lesions were observed in the vicinity of the pancreas of a few mice (again, none of these were treated with metformin); these were pseudocysts with fat necrosis. Pseudocysts are, in human disease, sequelae of pancreatitis. In the present study, detailed characterization of the pancreas adjacent to/in the vicinity of the pseudocysts was performed, and no evidence of pancreatitis was found. Thus, the etiology of the pseudocysts in these animals is unclear.

Our findings in relation to exocrine pancreas pathology are in contrast to those of Matveyenko and colleagues (34), which suggested a link between sitagliptin but not metformin treatment and ductal metaplasia in *hIAPP* transgenic rats. However, there are several differences between that study and the present one that may explain the disparate findings. The model used by Matveyenko and colleagues had significant overexpression of hIAPP (8), whereas our mouse model expressed hIAPP at a 1:1 ratio with endogenous mouse IAPP and at levels comparable to those in humans (20, 37). Therefore, the overexpression of *hIAPP* in the model used by Matveyenko and colleagues may, per se, be associated with islet/pancreas pathology. For example, *hIAPP* overexpression in the Matveyenko model is associated with endoplasmic reticulum (ER) stress in islets (19), whereas a physiological level of *hIAPP* expression in our model is not (24). Furthermore, we have shown that although ER stress is present in islets from humans with type 2 diabetes, this is not associated with amyloid formation (24).

Another difference between models is that the Matveyenko model exhibits overt hyperglycemia (5), whereas our model displays several features of β -cell loss and dysfunction without overt hyperglycemia. Thus, sitagliptin may induce adverse pancreas pathology only under conditions of significant overexpression of hIAPP and/or hyperglycemia. A recent study by Butler et al. (4) using samples from brain-dead organ donors with type 2 diabetes showed increased exocrine cell proliferation and mass in those treated with incretin therapy (seven with sitagliptin and one with exenatide). Our study in mice

showed no evidence of an increase in pancreas weight with long-term sitagliptin treatment. Given that the vast majority of the pancreas is comprised of exocrine tissue, this implies that exocrine mass was not altered under our experimental conditions. Furthermore, we found no evidence of an increase in neoplasia, which Butler et al. reported in their human specimens. Whether hyperglycemia and/or other aspects of the diabetes milieu may be playing a role in the effects of incretin therapy on the exocrine pancreas or whether other confounding aspects of the Butler study (26) are responsible for the differences in findings between that study and ours remains to be determined. Thus, the present study clearly demonstrates that long-term sitagliptin treatment in the face of physiological hIAPP production and in the absence of overt hyperglycemia does not result in exocrine pancreas pathology. In agreement with these findings, a recent study by Nyborg et al. demonstrated that two years of GLP-1 analog treatment in *Macaca fascicularis* did not induce any pancreatitis or preneoplastic proliferative lesions (38). As nonhuman primates naturally express amyloidogenic IAPP, this study also indicates that a long-term increase in GLP-1 signaling in the presence of physiological IAPP does not result in exocrine pancreas pathology.

In conclusion, we show that 12 mo of sitagliptin treatment does not increase islet amyloid deposition despite increasing hIAPP release and can additionally protect against amyloid-induced β -cell loss. This suggests a novel mechanism by which sitagliptin may protect the β -cell in type 2 diabetes. Furthermore, we have not observed any evidence of increases in pancreatic mass, pancreatitis, or ductal proliferation, metaplasia, or neoplasia with long-term sitagliptin administration. However, whether sitagliptin treatment may result in adverse exocrine pancreas pathology under hyperglycemic conditions requires further analysis.

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AUTHOR CONTRIBUTIONS

Author contributions: K.A.-M., S.L.S., S.Z., and R.L.H. conception and design of research; K.A.-M., S.L.S., T.S., D.T.M., L.C.G., and R.L.H. performed experiments; K.A.-M., S.L.S., T.S., D.T.M., L.C.G., and R.L.H. analyzed data; K.A.-M., S.L.S., S.Z., and R.L.H. interpreted results of exper-

iments; K.A.-M. and R.L.H. prepared figures; K.A.-M. and R.L.H. drafted manuscript; K.A.-M., S.L.S., S.Z., T.S., D.T.M., L.C.G., and R.L.H. edited and revised manuscript; K.A.-M., S.L.S., S.Z., T.S., D.T.M., L.C.G., and R.L.H. approved final version of manuscript.

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EXHIBIT 6

Re-analysis of study of pancreatic effects of incretin therapy: Methodological deficiencies

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Abstract

A recently published study by Butler et al concluded that incretin treatment had adverse effects on the human type 2 diabetic pancreas including “a marked expansion of the exocrine and endocrine pancreatic compartments, the former being accompanied by increased proliferation and dysplasia and the latter by α -cell hyperplasia with the potential for evolution into neuroendocrine tumors”. Incretin therapy has become widely used for type 2 diabetes, so these conclusions have instigated major concerns with regard to patient safety. We reassessed both the clinical case information and virtual microscopy images of the same 34 cases that were used in the Butler study as well as nPOD cases that were not included. While we would like to stress that it is important to investigate in depth any indication that incretin treatment may lead to inflammation or dysplasia in the pancreas, we find that the data presented in the Butler paper have serious methodological deficiencies that preclude any meaningful conclusions.

In the July issue of *Diabetes*, a study was published by Butler et al (1) that examined potential adverse effects of incretin treatment on the human type 2 diabetic pancreas. The study, using samples obtained from the Network for Pancreatic Organ Donation (nPOD)(2), concluded that “incretin therapy in humans resulted in a marked expansion of the exocrine and endocrine pancreatic compartments, the former being accompanied by increased proliferation and dysplasia and the latter by α -cell hyperplasia with the potential for evolution into neuroendocrine tumors”. Since incretin therapy has become widely used for type 2 diabetes, these conclusions have instigated major concerns in the medical community with regard to patient safety (3). These concerns have led us to examine the data in more detail.

While we would like to stress that it is important to investigate in depth any indication that incretin treatment may lead to inflammation or dysplasia in the pancreas, we find that the data presented in this paper (1) have serious methodological deficiencies that preclude any meaningful conclusions. Others (4-6) have already raised some of the issues: some inherent in a human postmortem study (small number of cases; lack of detailed premortem history), others specific to this study, including the heterogeneity in subject age, and differences in the incretin drugs used, dosage employed and duration of diabetes. Harja et al (7) in reexamining the clinical information of the cases in the nPOD database detailed even more differences between the study groups. As members of the nPOD consortium (SBW and PAIV), we had access to the same 34 cases that were used in the Butler study as well as nPOD cases that were not included, allowing us to reanalyze the cases using both clinical case information and virtual microscopy images available on the nPOD website (<http://path-aperio.ahc.ufl.edu>); the images and data we present are directly from this unique database. We have reevaluated the pathology of the cases (Supplemental Table) and have identified several methodological concerns that are summarized in Table 1 and detailed below.

Inappropriate cohorts for comparisons

The Butler study(1) is based on a comparison of three groups stated to be “matched for age, sex and BMI”: the first group consists of type 2 diabetes subjects with incretin therapy (T2D + I, n=8), the second group of type 2 diabetes without incretin therapy (n=12), and the third group of non-diabetic (ND) controls (n=14). As seen in **Figure 1**, the age distribution is significantly mismatched: all of the incretin-treated type 2 diabetes subjects were at least 45 years old, whereas 6/12 “type 2 diabetes” controls and 4/14 ND were younger than age 40. This age mismatch is particularly problematic because many pancreatic pathologies, in particular focal areas of pancreatitis-associated changes, pancreatic intraductal neoplasms (PanINs) (8) and microadenomas and large hyperplastic islets (9), are seen with increasing age.

Additionally, the diagnoses of several of the control “type 2 diabetes” cases are open to question. Some seem more likely to have had type 1 diabetes; in particular, 4 cases were diagnosed with diabetes at a young age and one of these had documented diabetic ketoacidosis (DKA) (nPOD 6059) and two others had positive auto-antibodies (nPOD 6149, nPOD 6142); a fifth case nPOD 6109 (age 48.8 y), who was listed as “preclinical” in the database, had insulin auto-antibodies and hemoglobin A_{1c} of 8. Even several of the non-diabetic controls are problematic since nPOD 6015 had had a gastric bypass, nPOD 6158 was positive for both GAD and IAA auto-antibodies and was classified as “prediabetic type 1 diabetes,” and nPOD 6097 was listed as “preclinical type 2 diabetes” with hemoglobin A_{1c} of 7.1.

Certainly there can be difficulty making a distinction between type 1 diabetes and type 2

diabetes, but cases with an ambiguous diagnosis should not have been included in the study. If such ambiguous patients and those under 40 years of age are omitted from the study, only 5 out of 12 type 2 diabetes controls and 8 of 14 non diabetic controls remain. Yet, within the nPOD database, there are 2 subjects with type 2 diabetes and 9 without diabetes (ND) above the age of 40 that were not included in the study with no explanation provided as to why they were excluded.

Methodological problems with quantification

Two important methodological issues erode the accuracy of the quantitative data presented in the study. The first is the variability in intensity of staining with chromogen Fast Red, which when attached to antibodies can be used to immunostain alpha or beta cells; the intensity of staining ranged from very faint to severe overstaining, often with little distinction between positive and negative cells (**Figure 2, 3**). Moreover, the variability was not random. Very strong staining was found in cases from Feb 2012 to December 2012 that included most (7/8) of the incretin-treated cases and none of other groups, while a number of cases processed in the first half of 2011 had very faint staining, including only one (nPOD 6157) incretin therapy case. The staining variability is particularly problematic since their automatic quantification system relies on color differences to determine the area of positive staining for specific hormones.

The second issue is a potential overestimation of the relative areas of the specific cell types. The total tissue area is used as the denominator for determining the relative area of glucagon- and insulin-expressing cells. Since the relative area for each cell type is multiplied by the pancreatic weight to estimate the mass of that cell type, all tissues included in the section must be accounted for in the relative area calculations. Quantification of the “total tissue area defined by the hematoxylin counterstain” may give an underestimation since hematoxylin only weakly stains the extracellular matrix of the connective tissue and does not stain the lipid-extracted adipocytes; yet these tissues contribute significantly to the weight to the pancreas. Because the proportion of fat within the pancreas increases with age (10), it cannot be excluded that the older subjects, including all of the incretin treated, may selectively have had such a problem, resulting in erroneously increased relative areas of their beta and alpha cells and subsequent overestimation of the mass of those cells.

Questionable conclusions due to the above problems

Most of the provocative claims of the Butler paper appear to be insufficiently supported by the data due to the above methodological issues and the use of the inappropriately age-matched cohorts. Several specific issues include:

“Pancreatic weight was 40% increased in DM-I compared with DM ($P < 0.05$).” As illustrated in **Figure 4** when the individual pancreatic weights are plotted, incretin-therapy cases do not show substantially increased pancreatic weight, except for one (nPOD 6185) that weighed a remarkable 204 g, an outlier well beyond 2 standard deviations from the mean of its group or that of the whole study group. In fact, in the Brussels database only one of 1238 pancreases donated for islet isolation weighed over 200 g (In’t Veld, unpublished). The average adult human pancreas weighs between 70-95 g, based on the autopsied measurements of Rahier (11), a study employing CT scans (10), the nPOD study (12) and this Butler study (1). Without the outlier case nPOD 6185, the incretin-therapy cases had a mean (\pm SEM) of 100 ± 13 g, which did not significantly differ from that of the non diabetic controls (91 ± 4 g) nor of that of the DM group (79 ± 6 g, $p < 0.1$).

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“ β -cell mass was decreased [55%] in [control] DM compared with ND but was approximately sixfold increased in DM-I compared with DM.” The reported decreased mass in type 2 diabetes controls versus ND is comparable to that widely reported (11; 13-17), however, only two ND cases with known pancreatic weights remain after those cases younger than 40 year old and those of questionable diagnosis are excluded. The 6-fold increase after incretin therapy is very unexpected, and if true, important. However, the use of inappropriate cohorts (including cases with probable type 1 diabetes), the flawed morphometric measurements and the variable staining as detailed above, leave this finding very much in doubt.

Pancreas from type 2 diabetes cases with incretin therapy have “alpha cell hyperplasia and glucagon microadenomas (3/8)...” The reported increased percentage of alpha cell relative area and mass after incretin therapy are both questionable due to the above mentioned issues of mismatched age comparisons and methodological issues. However, in many of the included older cases with diabetes (with or without incretin therapy) we observed significant numbers of alpha cells adjacent to or within the ductal epithelium associated with PanINs and focal chronic pancreatitis-like changes (**Figure 5**); there was no obvious difference between type 2 diabetes +I and control type 2 diabetes pancreas. Additionally the criteria used to distinguish between microadenoma and large hyperplastic islets were not given although this remains an unsettled issue in the field (9). We observed microadenoma/ hyperplastic glucagon-rich islets (300 μ m or larger) in about half of the over 40 year-old cases, including the non diabetic cases.

One type 2 diabetes case with incretin therapy had “a neuroendocrine tumor”. The presumably asymptomatic endocrine tumor expressing mainly glucagon is likely to be an incidental finding. In a study of 800 consecutive autopsies of persons at least 60 years with no history of pancreatic problems, 10% of pancreases were found to have asymptomatic endocrine tumors when the pancreas was thoroughly examined (9).

Type 2 diabetes with incretin therapy reported to have increased PanINs. PanINs are microscopic noninvasive epithelial neoplasms within the pancreatic ducts; they are graded (PanIN-1 to -3) according to degree of cytological and architectural atypia (www.path.jhu.edu/pc/professionals/DuctLesions.php). In autopsied pancreas from non-neoplastic pancreas, PanINs are rarely seen before age 35 but are found in 60% of pancreases by age 45 and in 75% by age 55 (8). The increased occurrence of PanINs reported in the Butler study(1) in the type 2 diabetes +I cases may thus be solely due to their older age. The PanINs that we observed were mainly PanIN-1, without clear signs of dysplasia, and would be more fittingly described as metaplasia. The prognostic value of PanIN-1 with regard to progression to invasive pancreatic adenocarcinoma is considered to be very low (19). A more detailed analysis in larger and better matched patient groups using PanIN subclassification will be necessary before any link between incretin treatment and increased dysplasia can be established.

Type 2 diabetes with incretin therapy reported to have “increased exocrine proliferation”: Immunostaining for the antigen Ki67 indicates cells that are in cell cycle, which is equated with cell division (proliferation). We observed Ki67 positive cells to be preferentially located within PanINs in all study groups, with little to no Ki67 positivity in other pancreatic cell types. Since the occurrence of PanINs is age-dependent (8), age-matched comparisons for proliferating cells are critical. Without well-matched controls, no link between incretin therapy and increased Ki67 positivity can be established. Additionally Ki67 positivity has

been reported increased in all pancreatic cell types under conditions of prolonged life support (18), but such clinical data are not available for these nPOD cases.

The only finding we were not able to reanalyze was the increased co-localization of insulin and glucagon (16% in incretin treated vs 3% in type 2 diabetes)(1); although the slides are from the same nPOD cases, images of the immunofluorescent staining are not in the nPOD database. Increased incidence of hormone co-localizing cells in type 2 diabetes was recently reported using surgical resections (20) but at a much lower frequency, only 0.82% in newly diagnosed type 2 diabetes and 0.33% in long standing type 2 diabetes. The finding in itself is thus interesting and should be examined in more detail, provided suitably matched groups are used.

In conclusion, our reanalysis of the patient data and histopathological observations in cases of the Butler study(1) finds that most of the major conclusions of this study are insufficiently supported by the data and that no meaningful conclusions can be drawn. Nonetheless, the potential dangers of (prolonged) incretin therapy for pancreatic changes remain a legitimate concern. These concerns call for extended and rigorously controlled studies in large and well-matched patient groups. Such studies could be facilitated by pooling all patient-level data from ongoing incretin-therapy trials and adding a separate process for identifying and adjudicating pancreatic cancer events. Such population-based studies should be complemented by a system of biobanks that will collect high quality tissue samples and clinical data necessary for an extended histopathological analysis.

Acknowledgements

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Figure legends

Figure 1. Graphic display of ages of nPOD cases used in Butler et al (1) showing the lack of age matching of studied cohorts. Solid black circles= type 2 diabetes +Incretin (I) therapy; white circles = type 2 diabetes; grey circles = non-diabetic cases. The dotted line indicates 45 years of age, which is that of the youngest case in the type 2 diabetes + incretin group.

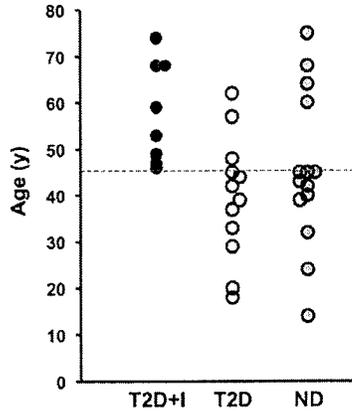


Figure 2. Variability of immunostaining is problematic for accurate quantification of relative areas of the α - and β -cells. **A, B.** Staining with Fast Red chromogen was excessive and “bleeds over” adjacent non-endocrine tissue and luminal space (asterix). Here are shown images from two type 2 diabetes +I cases with overstaining for glucagon. **C, D** In other sections staining was very faint for insulin (**C**) or glucagon (**D**). In the case shown staining for both insulin and glucagon are very faint as seen in the same field of adjacent sections from the glucagon-rich tail of the pancreas stained for each (arrows indicate the islets).

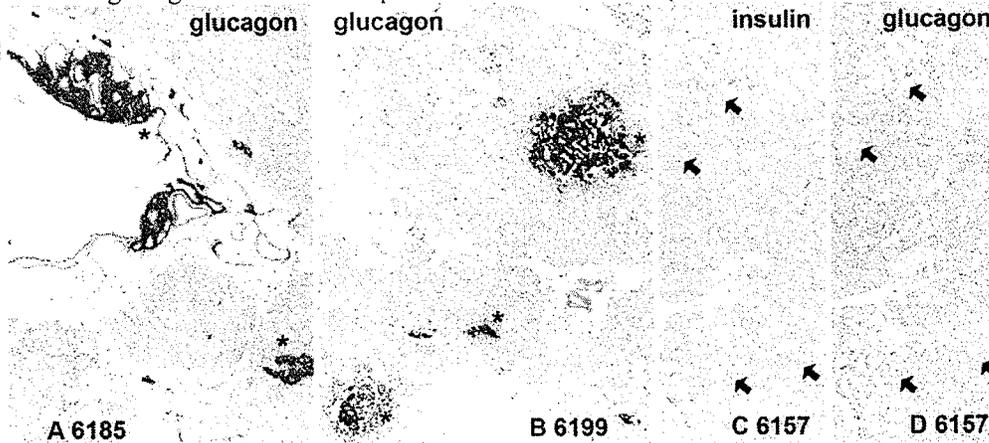


Figure 3. Overstaining for both insulin and glucagon distorts the quantification of relative areas of beta and alpha cells respectively. The same islets on adjacent sections stained for insulin (A) and glucagon (B) show imprecise discrimination of cell types due to overstaining.

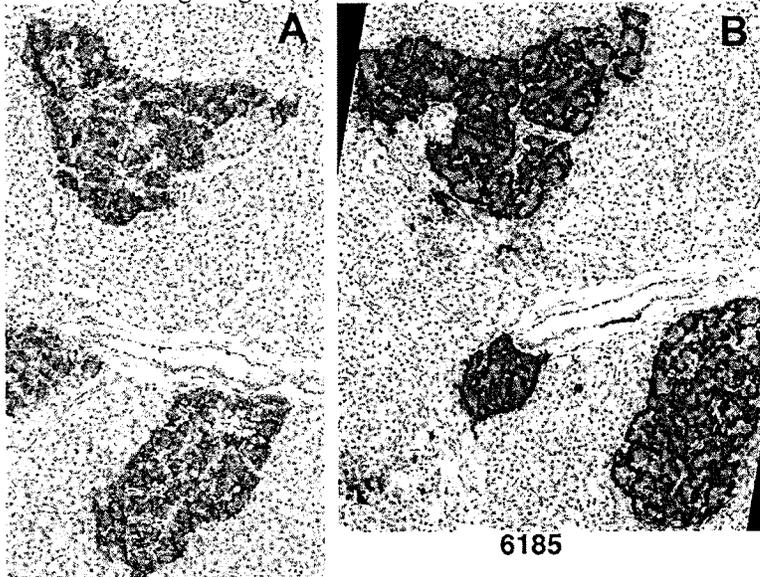


Figure 4. Graphic display of the pancreatic weights of the nPOD cases used Butler et al (1) suggesting the 204 g pancreas (nPOD 6185) as an outlier. Solid black circles= type 2 diabetes +I; white circles = type 2 diabetes; grey circles = non-diabetic. The dashed line indicates of the mean (92.4 g) of all the cases used in Butler et al (1); the dotted lines indicate \pm two standard deviations.

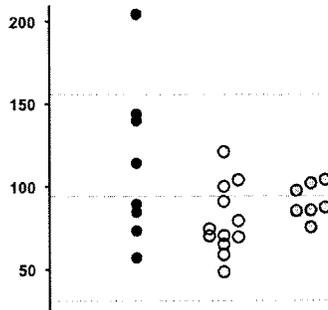


Figure 5. Substantial hormone-positive cells (here shown glucagon: red) associated with PanINs and focal areas of pancreatitis-associated changes in type 2 diabetes were present in subjects with (**A**, case nPOD 6206) and without (**B**, case nPOD 6139) incretin therapy. In both, characteristic focal areas of pancreatitis-associated changes are seen with multiple ductal profiles, many of which have tall columnar ductal cells typical of early PanINs. In **B**, the large duct has the papillary lesion characteristic of PanIN1B.

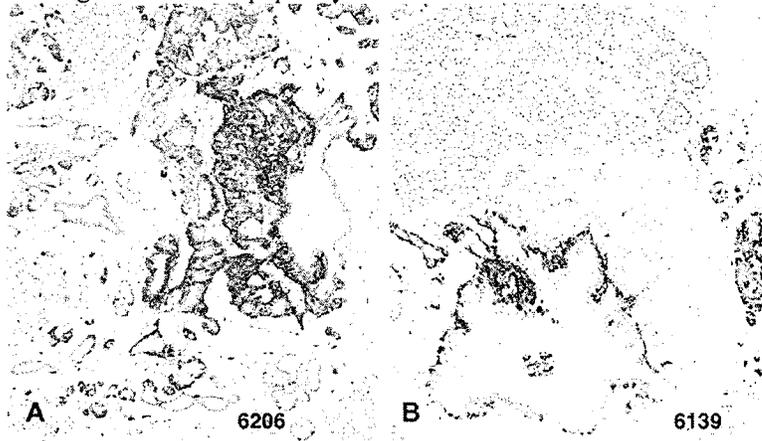


Table 1. Summary of our reanalysis of major points of Butler et al.

Conclusions of Butler paper	Our reanalysis
~40% increased pancreatic mass in T2D with incretin therapy compared to T2D controls	One outlier pancreas skewed data; others are within normal range. T2D control group may have included T1D subjects with lower pancreas weight.
Increased exocrine proliferation	Proliferation marker Ki67 seen almost exclusively in PanINS, which occur more frequently with increasing age. The treated group was older than controls.
Increased pancreatic intraepithelial neoplasia (PanINs)	PanIN occurrence increases with age with few seen under age of 40, and the treated group was older than controls.
Alpha cell hyperplasia	<ul style="list-style-type: none"> • Problematic morphometric measurements due in part to variable intensity of staining • Similar findings in age matched non-incretin treated subjects
Occurrence of glucagon-expressing neuroendocrine tumor	Probably an incidental finding since 10% of pancreases from subjects over 60 yr have silent endocrine tumors.
B cell mass increased 6 fold in incretin-treated subjects compared to T2D conventional treatment	<ul style="list-style-type: none"> • Inappropriate cohort of T2D controls (only 6 age matched T2D) with some subjects likely to be T1D. • Problematic morphometric measurements due in part to variable intensity of staining.
Increased number of cells coexpressing insulin and glucagon	No reanalysis by us; images of immunofluorescent sections were not in nPOD database.

EXHIBIT 7

Marked Expansion of Exocrine and Endocrine Pancreas With Incretin Therapy in Humans With Increased Exocrine Pancreas Dysplasia and the Potential for Glucagon-Producing Neuroendocrine Tumors

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Controversy exists regarding the potential regenerative influences of incretin therapy on pancreatic β -cells versus possible adverse pancreatic proliferative effects. Examination of pancreata from age-matched organ donors with type 2 diabetes mellitus (DM) treated by incretin therapy ($n = 8$) or other therapy ($n = 12$) and nondiabetic control subjects ($n = 14$) reveals an $\sim 40\%$ increased pancreatic mass in DM treated with incretin therapy, with both increased exocrine cell proliferation ($P < 0.0001$) and dysplasia (increased pancreatic intraepithelial neoplasia, $P < 0.01$). Pancreata in DM treated with incretin therapy were notable for α -cell hyperplasia and glucagon-expressing microadenomas (3 of 8) and a neuroendocrine tumor. β -Cell mass was reduced by $\sim 60\%$ in those with DM, yet a sixfold increase was observed in incretin-treated subjects, although DM persisted. Endocrine cells costaining for insulin and glucagon were increased in DM compared with non-DM control subjects ($P < 0.05$) and markedly further increased by incretin therapy ($P < 0.05$). In conclusion, incretin therapy in humans resulted in a marked expansion of the exocrine and endocrine pancreatic compartments, the former being accompanied by increased proliferation and dysplasia and the latter by α -cell hyperplasia with the potential for evolution into neuroendocrine tumors. *Diabetes* 62:2595–2604, 2013

Type 2 diabetes mellitus (DM) is characterized by defective insulin secretion in the setting of insulin resistance, leading to hyperglycemia. This defect in insulin secretion is accompanied by a deficit in β -cell mass. However, the extent and relevance of this β -cell deficit has been questioned, in part due to the paucity of human pancreatic studies as well as to methodological differences among such efforts (1–3). Therapeutic hope for DM has recently been raised by the introduction of a glucagon-like peptide-1 (GLP-1) mimetic class of drugs widely referred to as incretins. Interestingly, beyond their effects on improved metabolic regulation, GLP-1 mimetic therapy was also noted to induce β -cell

regeneration in rodents, thus portending the remarkable notion that the deficit in β -cell mass in DM might be overcome with such agents (4–7). However, given this ability was most evident in the period coincident with the postnatal expansion of β -cell mass in rodents, questions arose as to the relevance of this property of GLP-1 in adult humans (8,9). Moreover, β -cell replication was not detected in human islets exposed to high concentrations of GLP-1 in vitro (10). In contrast, there are concerns that the proliferative actions of GLP-1 might induce deleterious effects on the exocrine pancreas, in which the capacity for the proliferative actions of GLP-1 appears to be better retained into adult life (5,11,12).

To address this, we analyzed a series of high-quality (i.e., transplant grade) human pancreata obtained from brain-dead organ donors with and without DM, including a subgroup of the latter who underwent ≥ 1 year of incretin therapy (13). Our goals were to confirm that β -cell mass was indeed decreased with DM overall and to establish whether incretin therapy induced an expansion of the endocrine and/or exocrine pancreas.

RESEARCH DESIGN AND METHODS

Study subjects. All pancreata were procured from brain-dead organ donors by the JDRF Network for Pancreatic Organ Donors with Diabetes (nPOD) coordinated through the University of Florida in Gainesville, Florida (Table 1) (13). All procedures were in accordance with federal guidelines for organ donation and the University of Florida Institutional Review Board. Pancreata were procured from 20 individuals with DM. These were subdivided into 12 who did not receive GLP-1 drugs (DM) and 8 who received incretin therapy (DM-I) for 1 year or more, 7 being treated with the dipeptidyl peptidase-4 (DPP-4) inhibitor sitagliptin (Januvia) and 1 with the GLP-1 mimetic exenatide (Byetta). Pancreata were also obtained from 14 nondiabetic (ND) control subjects matched by age, sex, and BMI with the two DM treatment groups.

Pancreas fixation, embedding, and sectioning. nPOD uses a standardized preparation procedure for pancreata recovered from cadaveric organ donors (13). The pancreas is divided into three main regions (head, body, and tail), followed by serial transverse sections throughout the medial to lateral axis, allowing for sampling of the entire pancreas organ while maintaining anatomical orientation. Because preparation is completed within 2 h, tissue integrity is maintained. Tissues intended for paraffin blocks are trimmed to pieces no larger than 1.5×1.5 cm and fixed in 10% neutral buffered formalin for 24 ± 8 h. Fixation is terminated by transfer to 70% ethanol, and samples are subsequently processed and embedded in paraffin. Mounted transverse sections were obtained from the head, body, and tail of the paraffin-embedded tissue blocks.

Immunostaining

In Florida. Formalin-fixed paraffin-embedded sections were stained according to established procedures, as previously described (14). In brief, serial sections ($4 \mu\text{m}$) were deparaffinized and incubated with primary antibodies to Ki67 and insulin, or CD3 and glucagon, with antibody localization visualized with peroxidase-DAB and alkaline phosphatase-Fast Red polymer systems. Assays included positive and negative controls for each antibody, and new lots of reagents were validated to reproduce previous staining intensities using

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See accompanying commentary, p. 2178.

TABLE 1
Clinical characteristics of brain-dead organ donors

Case	Age (years)	Duration of DM (years)	Sex	BMI (kg/m ²)	Treatments	Cause of death
DM-I						
6157	74	1	F	39	Januvia	ICH/stroke
6185	46	15	M	41	Januvia, metformin	Anoxia
6186	68	5	M	21	Januvia, metformin	ICH/stroke
6189	49	26	F	36	Byetta, metformin, glipizide	Stroke
6199	53	20	M	30	Januvia, insulin pen	ICH/stroke
6194	47	13	M	24	Humulin, NovoLog, Januvia	ICH/stroke
6203	68	5	M	33	Januvia, metformin	Stroke
6206	59	10	M	42	Januvia, metformin	Stroke
Mean (SEM)	58 (4)	12 (3)		33 (3)		
DM						
6028	33	17	M	30	Insulin	Gunshot wound to head
6059	18	0.3	F	39	None	Cardiovascular
6108	57	2	M	30	Metformin	ICH/stroke
6110	20	0.2	F	40	None	ICH/stroke, DKA
6109	48	—	F	33	None	ICH/stroke, DKA
6114	42	2	M	31	Metformin, noncompliant	Asphyxiation
6124	62	3	M	34	Metformin	ICH/stroke
6127	44	10	F	30	Insulin	ICH/stroke
6133	45	20	F	40	Insulin	Cardiovascular
6139	37	1.5	F	45	None	Seizure
6142	29	14	F	34	None	Bacterial meningitis
6149	39	20	F	29	Insulin	ICH/stroke
Mean (SEM)	40 (4)	8 (3)		35 (2)		
ND						
6009	45		M	31		Anoxia
6015	39		F	32		Anoxia
6012	64		F	31		Cerebrovascular/stroke
6016	42		M	31		Cerebrovascular/stroke
6019	68		F	24		Head trauma
6020	60		M	30		Cerebrovascular/stroke
6022	75		M	31		Cerebrovascular/stroke
6034	32		F	25		Head trauma
6060	24		M	33		Head trauma
6097	43		F	36		Cerebrovascular/stroke
6099	14		M	30		Head trauma
6102	45		F	35		Cerebrovascular/stroke
6158	40		M	30		Head trauma
6165	45		F	25		Cerebrovascular/stroke
Mean (SEM)	45 (5)			30 (1)		

DKA, diabetic ketoacidosis; F, female; ICH, intracerebral hemorrhage; M, male.

normal human spleen for Ki67 and CD3 and pancreata from the ND donors for insulin and glucagon.

In Los Angeles. Sequential paraffin tissue sections from each region of pancreas from each subject were stained for 1) Ki67, insulin, and Alcian blue by immunohistochemistry and 2) Ki67 and glucagon by immunohistochemistry. In addition, a section of pancreas from each of the DM-I subjects and a section from a subset of the DM subjects not treated with incretin therapy ($n = 5$) and ND subjects ($n = 6$) were stained for insulin and glucagon by immunofluorescence and additional sections for glucagon, insulin, cytokeratin, and DAPI.

Briefly, the cytokeratin, insulin, and glucagon slides were stained as follows: CK-19 (mouse 1:50, 4°C overnight; Dako, Carpinteria, CA), glucagon (rabbit 1:1000, 4°C overnight; ImmunoStar Inc, Hudson, WI), and insulin (guinea pig 1:100, 4°C overnight; Invitrogen, Grand Isle, NY). FITC, Cy3, and Cy5 followed incubation of each primary antibody, respectively (1:100, The Jackson Laboratory—West, Sacramento, CA). Slides were coverslipped with Vectashield with DAPI (Vector Laboratories, Burlingame, CA).

Morphometric analysis

In Florida. Stained slides were scanned to create whole digital slide images with an Aperio ScanScope CS (Aperio Technologies, Vista, CA) and Spectrum Plus version 11 at ×20 magnification. Each image was reviewed and annotated using the Aperio ImageScope image viewing program. Regions with edge artifact or nonspecific staining were excluded from analysis. The Spectrum

colocalization algorithm was used to estimate the proportion of insulin- and glucagon-stained area compared with total tissue area defined by the hematoxylin counterstain. Software parameter settings were optimized for detection of both hematoxylin and Fast Red chromogen. Data were averaged within regions and then averaged among all regions for a given donor pancreas. Cytonuclear IHC quantification software (Indica Laboratories, Albuquerque, NM) was used to quantify total cell numbers per pancreas section and total cell replication rates using Ki67 nuclear immunopositivity. Input parameters were set using an ImageScope plug-in tool that calculates optical densities of the underlying structures in RGB OD values. Total cell counts were estimated from nuclei stained with hematoxylin. Nuclei costained with Ki67 were counted and expressed as a percentage of the total.

In Los Angeles. Whole sections of pancreas stained for insulin, Ki-67, and Alcian Blue and hematoxylin counterstain were digitally scanned using Aperio ScanScope. Analysis was performed using Aperio ImageScope version 11.0.2.725. With this software, the total area of the tissue was measured.

Full cross-sections of the pancreas head, body, and tail were evaluated for pancreatic intraepithelial neoplasia (PanIN) by a gastrointestinal pathologist blinded to clinical information and using the established consensus classification system for these precursor lesions (15). The total number of PanIN lesions and their grade were determined per lobular unit and scored as the highest-grade lesion within that lobule. The number of PanINs per pancreas section was then computed per unit area of pancreas ($\text{mm}^2 \times 10^3$).

Using the insulin-, Ki-67-, and Alcian blue-stained sections, 100 islets were analyzed per section, with an Olympus CX41 microscope (Olympus America, Center Valley, PA), to determine the frequency of Ki67 in the β -cells of islets and in the non- β -cell compartment of those islets. Similarly, using the glucagon- and Ki-67-stained sections, 100 islets per section were analyzed to determine the frequency of Ki67 in the α -cells of islets and in the non- α -cell compartment of those islets. The mean β - and α -cell diameter was determined in each individual using the insulin and glucagon immunostained sections from the pancreas body, as previously described (16). A total of 475 α -cells and 475 β -cells were evaluated.

To determine the percentage of β - and α -cells within pancreatic ducts, the insulin-Ki67-Alcian blue-stained slides and the glucagon-Ki67-hematoxylin-stained slides were used. The total number of duct cells per pancreatic section was counted manually, as was the total number of cells within the ducts that stained for insulin or glucagon, respectively. The results are expressed as the percentage of duct cells positive for insulin or glucagon.

Using the sections stained by immunofluorescence for insulin, glucagon, and DAPI, a minimum of 30 islets per section (range 31–45, mean 38.0 ± 1.0) were randomly selected, imaged at $\times 20$ magnification, and stored using a Leica DM6000 fluorescent microscope (Leica Microsystems, Deerfield, IL) connected to a Macintosh computer loaded with Openlab software (Improvision, Lexington, MA). Each islet was analyzed to determine the number of β -cells, α -cells, and cells costaining for insulin and glucagon.

Confocal microscopy was performed where cells costaining for insulin and glucagon were detected for confirmation that insulin and glucagon granules were indeed both present in the same cells. Imaging was performed using a scanning confocal microscope equipped with argon, green and red helium-neon lasers (Leica). Images were acquired by sequential scanning using a $\times 20$ objective and the appropriate filter combination. Z sections were captured with a $0.25\text{-}\mu\text{m}$ step size.

Statistical analysis. Statistical analysis was performed using the Student *t* test with GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA). Data in graphs and tables are presented as means \pm SEM. Findings were assumed statistically significant at $P < 0.05$.

RESULTS

Pancreatic mass was increased ($P < 0.05$) $\sim 40\%$ in DM-I patients compared with that in individuals with DM not treated with such agents (Fig. 1A). The pancreatic fractional insulin area was $\sim 60\%$ reduced in DM patients not treated with incretin therapy compared with ND control subjects (0.34 ± 0.10 vs. $0.79 \pm 0.10\%$; $P < 0.001$) (Fig. 1B). In contrast, the pancreatic fractional insulin area was approximately fivefold increased in DM-I patients compared with individuals not treated with incretin therapy (1.60 ± 0.41 vs. $0.34 \pm 0.10\%$; $P < 0.0001$).

The β -cell mass, computed from the product of the pancreatic fractional insulin-positive area and pancreatic mass, was 55% decreased in DM patients not on incretin therapy in comparison with ND control subjects (0.29 ± 0.08 vs. 0.60 ± 0.10 g; $P < 0.05$) (Fig. 1D). In contrast, β -cell mass was increased sixfold in DM-I patients (1.81 ± 0.56 vs. 0.29 ± 0.08 g; $P < 0.01$). Moreover, β -cell mass was threefold greater in DM-I individuals compared with ND control subjects (1.81 ± 0.56 vs. 0.60 ± 0.10 g; $P < 0.05$). The sixfold increase in β -cell mass in DM-I subjects was almost all due to increased β -cell numbers rather than β -cell size, although there was a 3% increase in β -cell size with incretin therapy (8.9 ± 0.1 vs. 8.6 ± 0.1 μm , DM vs. DM-I; $P < 0.05$) (Fig. 1C). The pancreatic fractional area immunostained for glucagon was comparable between DM individuals not subject to incretin therapy and ND control subjects (0.57 ± 0.12 vs. $0.52 \pm 0.08\%$; $P = \text{NS}$) (Fig. 1E). However, the pancreatic fractional area immunostained for glucagon was markedly increased in DM-I individuals compared with DM patients on other therapy (1.65 ± 0.39 vs. $0.57 \pm 0.12\%$; $P < 0.0001$) as well as compared with ND control subjects (1.65 ± 0.39 vs. $0.52 \pm 0.08\%$; $P < 0.0001$). The pattern of the calculated glucagon mass followed that of the pancreatic fractional area, being comparable in individuals with

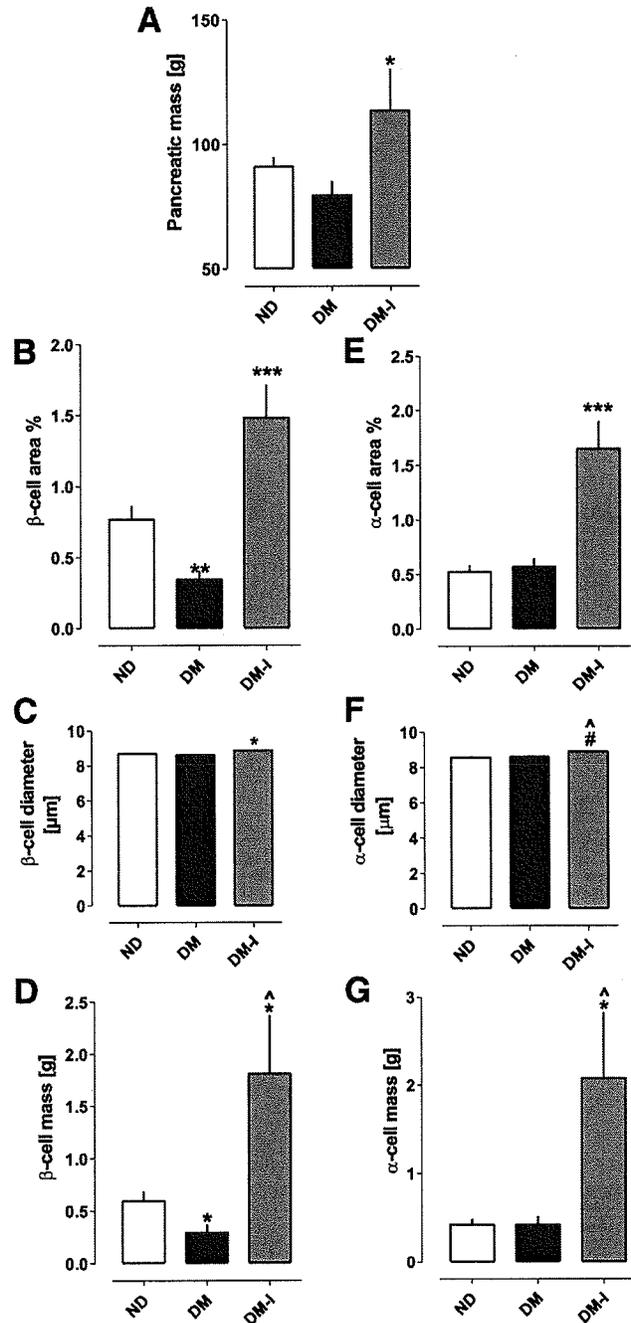


FIG. 1. Pancreas, β -cell, and α -cell mass. **A:** Mean pancreatic weight. * $P < 0.05$ DM-I vs. DM. **B:** Pancreatic fractional insulin area. *** $P < 0.0001$ DM-I vs. DM, ** $P < 0.001$ DM vs. ND. **C:** β -Cell diameter. * $P < 0.05$ DM-I vs. DM. **D:** β -Cell mass. * $P < 0.01$ DM-I vs. DM, * $P < 0.05$ DM-I vs. ND, * $P < 0.05$ DM vs. ND. **E:** Pancreatic fractional glucagon area. *** $P < 0.0001$ DM-I vs. DM and ND. **F:** α -Cell diameter. * $P < 0.01$ DM-I vs. DM, # $P < 0.005$ DM-I vs. ND. **G:** α -Cell mass. * $P < 0.01$ DM-I vs. DM, * $P < 0.05$ DM-I vs. ND. Pancreatic weight was 40% increased in DM-I compared with DM ($P < 0.05$). β -Cell mass was decreased in DM compared with ND but was approximately sixfold increased in DM-I compared with DM. α -Cell mass was comparable in DM and ND but was approximately fivefold increased in DM-I compared with DM. The increase in β -cell and α -cell mass with incretin treatment was predominantly due to endocrine hyperplasia rather than hypertrophy.

DM not treated with incretin therapy and control subjects, but fivefold increased in DM-I individuals compared with DM patients not treated with incretin therapy (2.08 ± 0.75 vs. 0.45 ± 0.10 g, DM-I vs. DM; $P < 0.01$) (Fig. 1G). The marked increase in α -cell mass, like that of β -cell mass, was almost completely due to an increase in the number of α -cells, although again, there was a 4% increase in α -cell size (8.9 ± 0.1 vs. 8.6 ± 0.1 μm , DM-I vs. DM; $P < 0.01$) (Fig. 1F).

There were two immediately striking findings on inspection of the pancreatic sections of the DM-I subjects: a subset of enlarged and often eccentrically shaped islets as well as increased numbers of endocrine cells in association with duct structures (Figs. 2–4). The impression of a subset of enlarged islets was confirmed quantitatively, the 12 largest islets per section in the DM-I subjects being almost twice the cross-sectional area of those in the DM subjects not treated by incretins ($82,270 \pm 10,330$ vs. $44,770 \pm 6,986$ $\mu\text{mol/L}^2$, $P < 0.01$). While examples of insulin

immunoreactive cells related to ductal structures could be found in individuals from all three groups, the percentage of such cells within ducts was not increased in DM-I versus DM subjects without incretin therapy (0.30 ± 0.09 vs. $0.27 \pm 0.07\%$; $P = \text{NS}$) (Figs. 3 and 4). However, glucagon-immunoreactive cells were frequently found in long linear groups or solid nests of cells either within the duct itself or in the immediate periductal location. In addition, these glucagon-positive cells also formed intraductal luminal projections, as previously described in chronic pancreatitis (17) (Fig. 2A). There were also regions with multiple small ducts that had glucagon-expressing tubular- and islet-like structures directly contiguous to the ducts. Of note, there were cells expressing both cytokeratin and glucagon at the interface of these α -cell tubular projections from ducts (Fig. 2F–I). The percentage of cells immunoreactive for glucagon in ducts was increased in DM-I versus DM with no incretin therapy (2.8 ± 0.9 vs. $0.5 \pm 0.2\%$; $P < 0.05$)

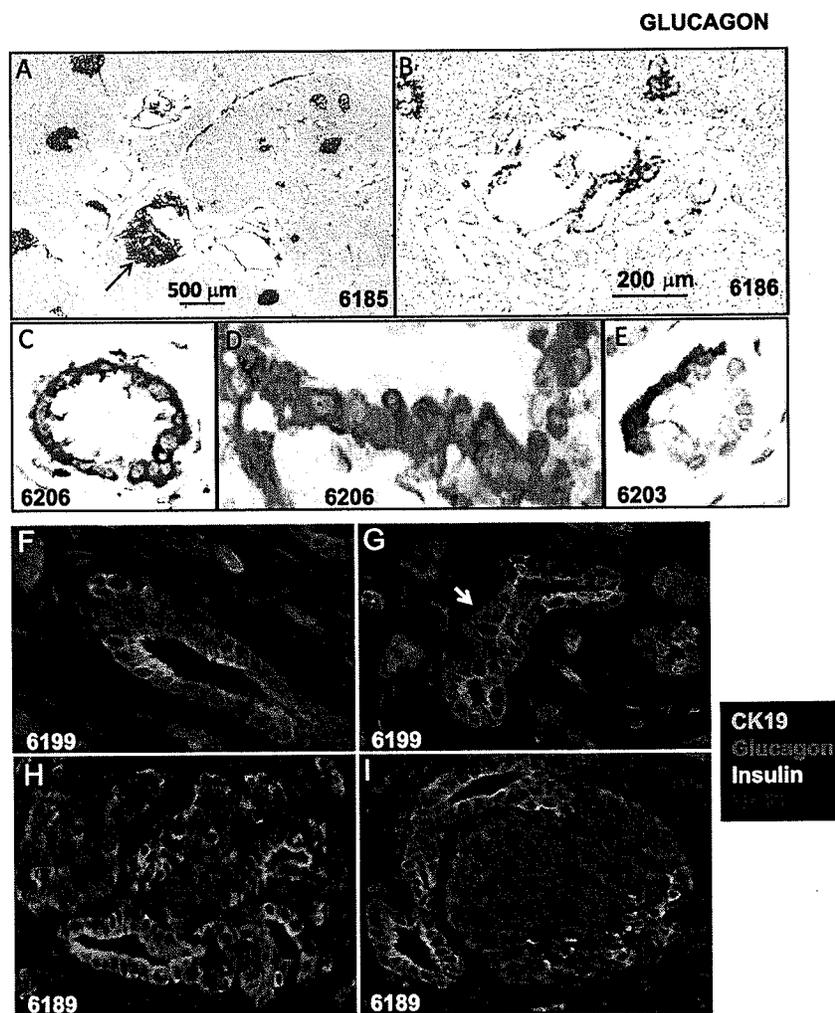


FIG. 2. Glucagon immunohistochemistry in pancreas in DM after incretin therapy. *A–E*: Sections of pancreas from DM-I donors (cases 6185, 6186, 6206, and 6203 with sitagliptin) immunostained for glucagon (pink) with hematoxylin counterstain. Exuberant expansion of glucagon immunoreactive cells is seen as enlarged eccentrically shaped islets as well as nodular and linear aggregates of cells intimately associated with ducts and demonstrating variable extension into duct lumens (arrow). *C–E*: Higher-power images show glucagon immunoreactivity in cells lining ducts. *F–I*: Pancreas sections from DM-I donors (case 6199, sitagliptin; 6189, exenatide) show immunofluorescent costaining for cytokeratin (green), glucagon (red), and DAPI nuclear counterstain (blue). Glucagon-expressing cells are present within and adjacent to keratin-positive duct structures. *G*: One cell costaining for cytokeratin and glucagon is indicated by the arrow.

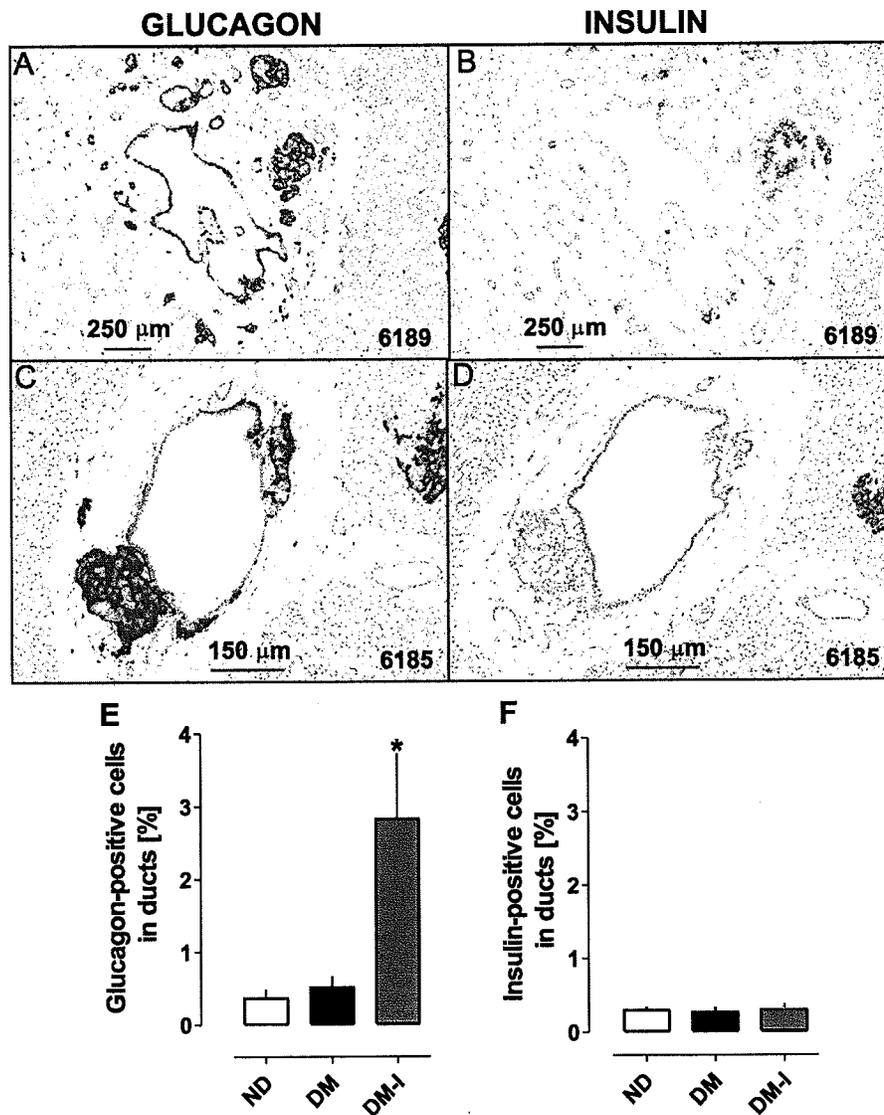


FIG. 3. Distribution of α -cells and β -cells in relation to ducts in DM-I donors. Serial adjacent sections of pancreas from DM-I donors (case 6189, exenatide; 6185, sitagliptin) were immunostained for glucagon (A and C) or insulin (B and D) with hematoxylin counterstain. These serial sections indicate that the exuberant endocrine growth associated with ducts is predominantly comprised of glucagon immunoreactive cells. The percentage of glucagon-positive cells in ducts (E) and insulin-positive cells in ducts (F) is shown. The percentage of glucagon-positive cells in ducts was increased in the DM-I group compared with the DM and ND groups (* $P < 0.05$). In contrast, the percentage of insulin-positive cells in ducts was unchanged in the DM-I group.

(Fig. 3E). Thus, while the exuberant increase in glucagon immunoreactive cells with incretin treatment was frequently observed in the periductal areas, the increased numbers of insulin immunoreactive cells with incretin therapy tended to be most abundant more remote from these periductal endocrine complexes (Fig. 3B and D). The pancreas from the single individual treated with exenatide also revealed exuberant α -cell hyperplasia (Fig. 4).

In one individual with DM treated with sitagliptin (nPOD case 6185), a 1.5-cm α -cell/glucagon-producing neuroendocrine tumor (grade 1, World Health Organization, 2010) not appreciated in life was identified in the body of the pancreas after resection at brain death (Fig. 5). Glucagon-producing microadenomas were also detected in the same individual (case 6185) and in two other DM-I individuals (cases 6157 and 6206) (Supplementary Fig. 3), whereas

hyperplastic islets with predominant glucagon staining were noted in seven of eight of the DM-I individuals. No neuroendocrine tumors or glucagon-producing microadenomas were detected in ND control subjects or in DM subjects not treated with incretin therapy.

Inspection of pancreatic sections immunostained with either insulin or glucagon from the DM-I individuals gave the impression that numerous cells within these islets were immunoreactive for both hormones. When quantified in sections double-immunostained for glucagon and insulin by confocal microscopy for this purpose (Fig. 6, Supplementary Figs. 1 and 2), the percentage of insulin-positive cells in DM-I individuals that were also glucagon immunoreactive were indeed markedly increased when compared with those with DM not treated with incretin therapy (16.8 ± 5.0 vs. $3.2 \pm 1.4\%$; $P < 0.05$). Interestingly, there was also an

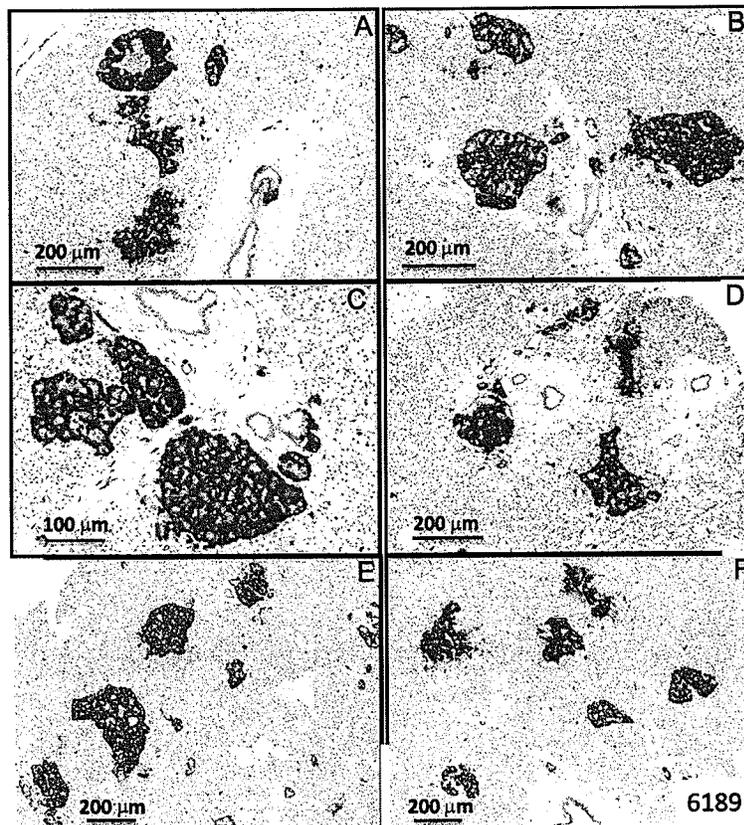


FIG. 4. Evidence for a direct role of GLP-1 mimetic action in α -cell hyperplasia. Images of pancreatic sections (A–F) are provided to illustrate a similar pattern of α -cell hyperplasia in the donor treated with exenatide (6189) as in the sitagliptin-treated donors, implying a role for GLP-1 action independent of DPP-4 inhibition in α -cell hyperplasia. Enlarged and often eccentrically shaped islets are apparent, as well as increased numbers of glucagon immunoreactive cells associated with and surrounding ductal structures.

increase in double immunoreactive-positive cells in individuals with DM not treated with incretin therapy when compared with the ND control subjects (3.2 ± 1.4 vs. $0.4 \pm 0.1\%$; $P < 0.05$). As in prior human studies (2), the frequency of Ki67-positive nuclei in islet endocrine cells was extremely rare (all less than 0.01 cells per islet section), with no significant differences in this very low frequency of β - or α -cell replication among any of the three groups studied.

The increased pancreatic mass in DM induced by incretin therapy was accompanied by increased whole pancreas cell proliferation (0.25 ± 0.03 vs. $0.12 \pm 0.01\%$, DM-I vs. DM; $P < 0.0001$) and an increase in the presence of PanINs (11.9 ± 2.6 vs. 4.9 ± 1.7 , DM-I vs. DM, PanINs/mm² $\times 10^3$; $P < 0.01$) (Fig. 7). PanIN lesions were also not infrequently detected in or close to these areas with ductular α -cell complexes (Fig. 7). Inspection of pancreas sections in DM-I individuals revealed small foci of increased Ki67 immunostaining in and around ducts and sometimes in areas of exocrine dysplasia (Supplementary Fig. 4). Given the focal and often dysplastic nature of these areas of increased cell replication, it was not possible to attribute the increased replication to a particular tissue compartment of the pancreas (e.g., ducts, acinar cells).

DISCUSSION

In this study, we took advantage of the unique resource of pancreata procured by the JDRF nPOD program (13) to

re-evaluate the question of β -cell mass in DM and, in addition, to address the potential actions of the incretin drugs on the endocrine and exocrine compartments. In the individuals with DM not using incretin drugs, we report a 60% deficit in β -cell mass but unchanged α -cell mass. Perhaps not surprisingly, given methodological differences, previous reports suggest a wide range of change in β - and α -cell mass (or pancreatic fractional area) in DM (1–3,18–20).

A striking finding in the present studies is the marked expansion of the exocrine and endocrine compartments of the pancreas with incretin therapy. The DPP-4 inhibitor sitagliptin acts to increase endogenous GLP-1 levels (21). It is not known whether the actions of sitagliptin reported here were mediated by increased circulating GLP-1 levels, increased GLP-1 levels produced by the newly formed α -cells acting in a paracrine manner, or due to the actions of signaling peptides other than GLP-1 that are degraded by DPP-4. The one individual treated by exenatide available to us here showed a pattern of changes comparable to those observed in the sitagliptin-treated individuals (Fig. 4), implying that a direct action of GLP-1 is likely involved. However, to fully address this question, it will be important to obtain more pancreata from individuals who have been treated with GLP-1 mimetic therapy.

The pancreatic mass of the DM-I individuals was increased by 40% compared with patients with DM not treated with incretin therapy, consistent with prior rodent

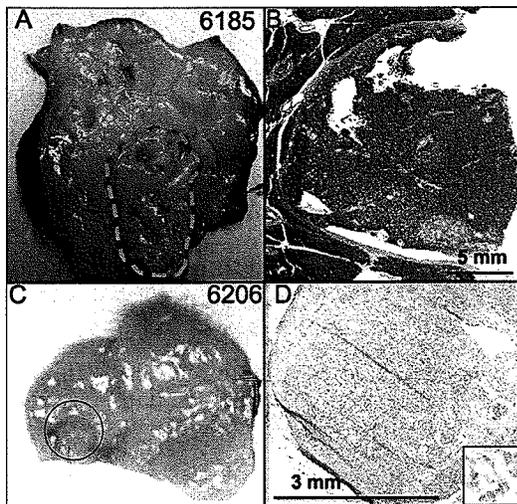


FIG. 5. Pancreatic glucagon-expressing neuroendocrine tumor and microadenoma. Grossly visible lesion (A) and corresponding section stained with hematoxylin and eosin (B) of the clinically undetected glucagon-expressing neuroendocrine tumor in the pancreas of a DM-I donor (nPOD case 6185) after sitagliptin therapy. Gross specimen (C) and corresponding hematoxylin and eosin-stained section (D) of a glucagon-expressing microadenoma in nPOD case 6206, DM after sitagliptin therapy. The inset shows high-power view of representative cells stained for glucagon by immunohistochemistry. (See Supplementary Fig. 3 for an additional image of microadenoma in case 6206.)

studies that revealed proliferative actions of GLP-1 on the exocrine pancreas now extended here to humans (5,12). Also of particular concern, incretin therapy was associated with an increase in pancreatic dysplastic PanIN lesions, consistent with the prior finding that GLP-1 receptors are expressed not only in the human exocrine pancreas but also in PanINs and that GLP-1 induces proliferative signaling in human pancreatic duct epithelial cells (12). Moreover, GLP-1 accelerated mPanIN formation in the *Kras^{G12D}* mouse model (12). Of interest, PanINs and ductal endocrine complexes in DM-I donors were often seen in close proximity (Fig. 7), which could suggest a common underlying dysplastic process that perhaps adds insight into the admixture of endocrine cells often noted within pancreatic adenocarcinoma (22). The increased cellular proliferation observed in the whole pancreas sections of DM-I patients (Fig. 7E) could theoretically be due to a contribution of intrapancreatic GLP-1 released by newly formed α -cells. It is unlikely that brain-dead individuals in an intensive care unit setting for the week before organ procurement would be treated by incretin therapy. To more precisely elucidate the direct action of those therapies on proliferation in the human pancreas and to investigate the cellular subcompartments in which they induce this action, it will also be important to seek opportunities to obtain pancreata from individuals who had incretin therapy up until the pancreas was obtained, perhaps via surgery.

The marked α -cell hyperplasia, glucagon-expressing microadenomas, and glucagon-expressing neuroendocrine tumor noted in DM-I individuals are also of concern. These findings reproduce the α -cell hyperplasia, abnormal α -cell distribution, and predisposition to glucagon-expressing neuroendocrine tumors previously reported with suppressed glucagon secretion or signaling (23–25). It is of note that

a consistent action of incretin therapy is to suppress glucagon secretion or action. As in prior reports of decreased glucagon secretion or action, the expansion in α -cell mass in DM-I subjects was prominently distributed as tubular outgrowths of glucagon-expressing cells from small duct-like structures within the lining of (or surrounding) larger ducts occasionally forming intraductal projections within the lumen. The latter possibly contribute to the reported increased incidence of pancreatitis in incretin-treated patients (11). Pancreatitis has previously been reported in humans with unexplained α -cell hyperplasia (26), and intraductal endocrine cell projections have been reported previously in chronic pancreatitis (17). Therefore, incretin therapy-induced intra- and periductal α -cell proliferations are now added to incretin therapy-induced expansion of PanIN lesions as plausible mechanistic links to the induction of pancreatitis through obstruction of the outflow of pancreatic enzymes.

The source of new α -cells in prior studies of impaired glucagon secretion or signaling was deemed most likely as arising from progenitor cells because no increase in α -cell replication was detected (23–25). We were also unable to detect any increase in α -cell replication in DM-I specimens to account for α -cell hyperplasia. Although again it should be noted that the study subjects were likely not exposed to incretin drugs immediately before pancreas procurement, a prior study suggests that sitagliptin therapy suppresses rather than enhances α -cell replication (27). Given that α -cells are epigenetically relatively unstable (28) and that most glucagonomas display malignant behavior, the present finding calls into question the safety of long-term suppression of glucagon secretion or action. Moreover, because the standard of care of a pancreatic neuroendocrine tumor is surgical resection, due to the risk of conversion to malignancy, even if benign, patients exposed to incretin therapy would seem to be at increased risk of requiring pancreatic surgery.

The present finding of an increased pancreatic mass, increased PanIN lesions, and endocrine proliferations encircling and sometimes encroaching on pancreatic ducts in response to GLP-1 mimetic therapy does add to concerns already raised regarding the potential adverse actions of GLP-1 mimetic therapy to induce pancreatitis and accelerate pancreatic dysplasia (11). Prior reports of potentially concerning pancreas changes with incretin therapy were confined to studies of rodent pancreas but are now here extended to humans with the added concern of the risk of neuroendocrine tumors. Because newly formed α -cells in response to impaired glucagon secretion have been shown to secrete high levels of GLP-1 (23–25), the local GLP-1 concentrations in the pancreas in patients treated with DPP-4 inhibitors may be very high, particularly where these cells are abundant in and around dysplastic ducts. This may account for the observed increase in pancreatitis and pancreatic tumors registered with the U.S. Food and Drug Administration's adverse event reporting system for GLP-1 mimetics and DPP-4 inhibitors, in contrast to the signal for thyroid tumors being restricted to GLP-1 mimetics (11). It also may explain the expansion of the exocrine pancreas in glucagon receptor-null mice (and mutant human), exocrine proliferation being noted in both, as well as high GLP-1 release by newly formed α -cells (23–25).

At first sight, the increase in β -cell mass with incretin therapy in DM in ND subjects would appear to be an exciting finding in relation to the potential for disease reversal.

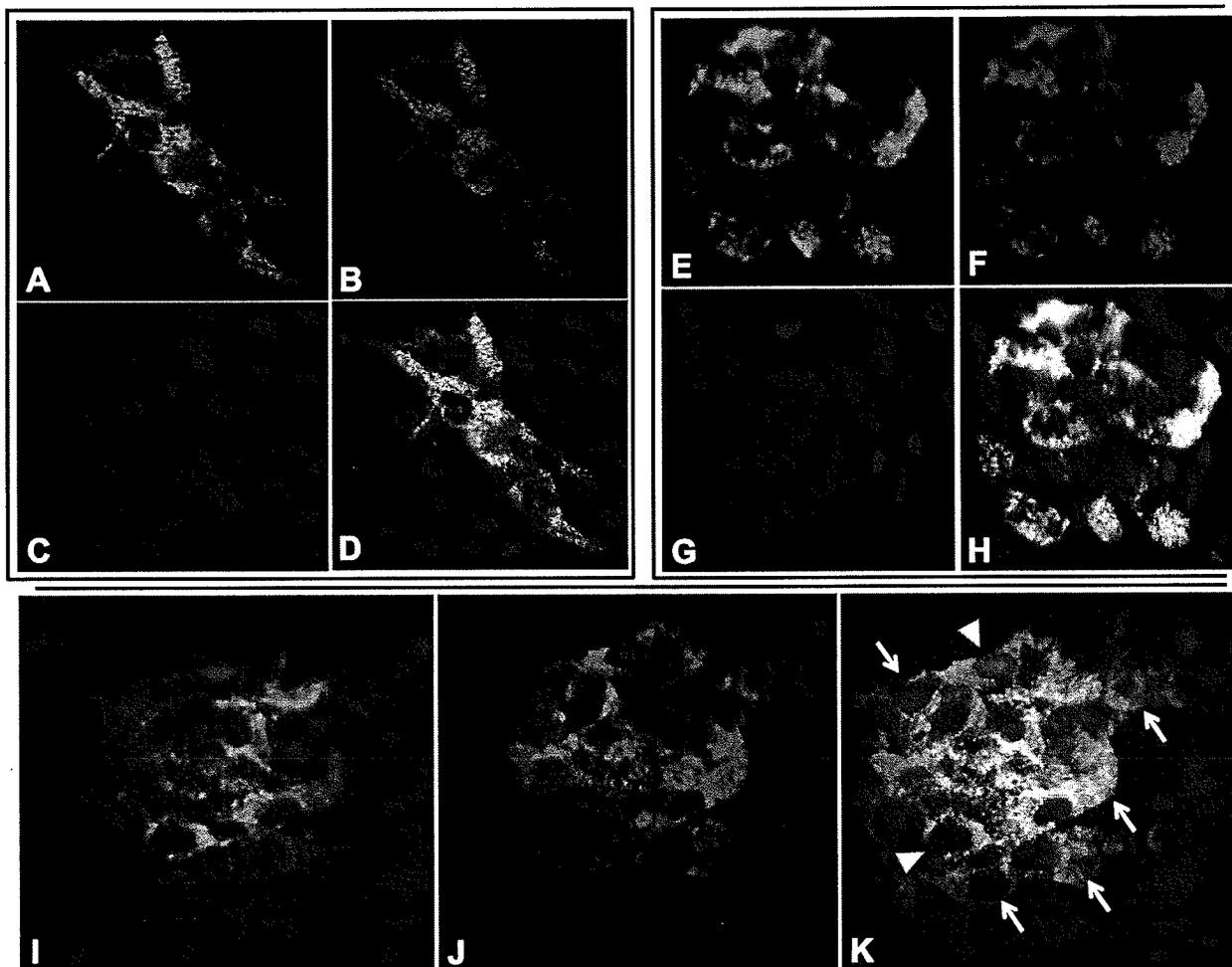


FIG. 6. Insulin and glucagon coexpression. Co-immunofluorescent images of islets from case 6185, sitagliptin (A–H) show endocrine cells coexpressing insulin (green) (A and E), glucagon (red) (B and F), and merged (D and H). The proportion of endocrine cells per islet that were thus detected as coexpressing insulin and glucagon was markedly increased in DM-I. Confocal images are shown of an islet from case 6185 stained for insulin (green) (I) and glucagon (red) (J), and a merged image (K) shows a mixture of cells, some expressing insulin or glucagon only (arrowheads) and some showing coexpression (arrows). DAPI nuclear counterstain (blue).

Unfortunately, these insulin-expressing cells are presumably not functionally mature because the DM-I individuals still had DM. It will be important to establish the molecular signature of the insulin-expressing cells in future studies. The marked expansion of the β -cell mass in individuals with DM treated with sitagliptin or exenatide for a year or more is in contrast to the findings of the effects of these drugs on β -cell function in DM (29,30). Although β -cell function benefits to some extent in patients with DM with any therapy that lowers blood glucose values (31,32), no data to date suggest that there is a disease-modifying action of incretin therapy in DM over that of any other glucose-lowering agent with regard to recovery of β -cell function (29,30). It is plausible that GLP-1 directly induced proliferation of β -cells, as has been reported in animal studies (4,5). However, these studies were in juvenile rodents, and GLP-1 did not induce proliferation in human islets *in vitro* (10). Also, β -cell replication did not differ among the groups in this study. However, as stated previously, because incretin therapy was likely not administered during the last week of life in

the present donors, it is not possible to exclude incretin-induced β -cell replication from this study. Likewise, it is conceivable that the increase in β -cell number in DM-I may be due in part to GLP-1-mediated inhibition of β -cell apoptosis.

Another striking finding in the islets of the DM-I individuals is a high proportion of cells that expressed both insulin and glucagon. This has been described in the newly forming endocrine pancreas at week 20 of gestation in humans (33) and thus might be an indication of newly formed immature endocrine cells from presumptive progenitors. Alternatively, lineage-tracing studies in diabetic mice have established that α -cells have the capacity to transdifferentiate toward a β -cell phenotype (34). A third possible source of these double hormone-expressing cells could be β -cells that have partially transdifferentiated toward an α -cell fate. The epigenetic basis of this interconversion has been established (35).

In summary, we confirm that in humans with DM, β -cell mass is deficient while α -cell mass is no different from that in ND individuals. We note that both the exocrine and

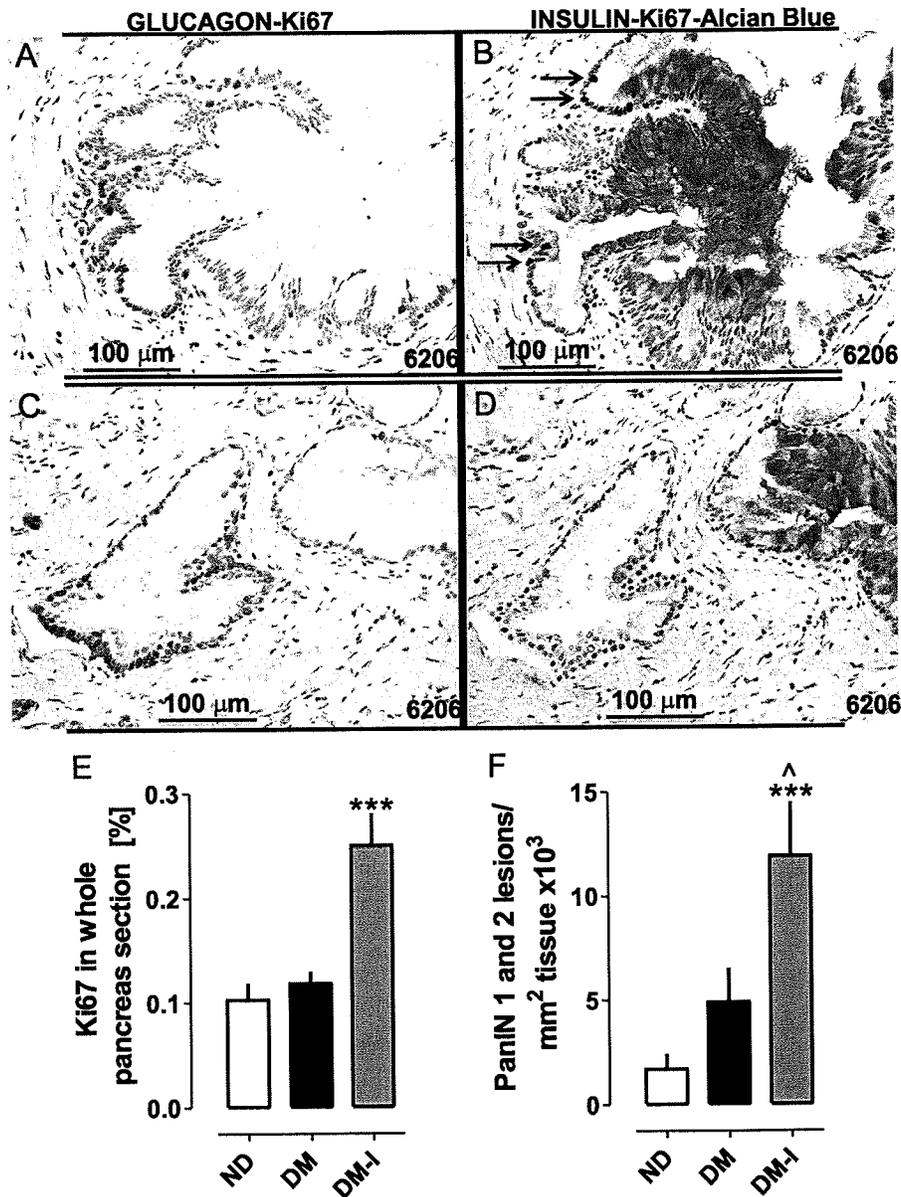


FIG. 7. Pancreatic intraepithelial neoplasia, endocrine complexes, and cellular replication. Photomicrographs show sections with immunohistochemical staining for Ki67 (brown) and glucagon (pink) in PanIN lesions (*A* and *C*) with hematoxylin counterstain or Ki67 (brown) and insulin (pink) (*B* and *D*) with Alcian blue counterstain to highlight mucin. Glucagon-expressing endocrine cells are shown intimately associated with PanIN lesions to varying degrees. Foci of replication (arrows, Ki67 nuclei) are also apparent. (See Supplementary Fig. 4 for additional examples of foci of increased replication in incretin treated pancreas.) *E*: Pancreas cell replication is increased in DM-I (Ki67). *** $P < 0.0001$ DM-I vs. DM and ND. *F*: Frequency of PanIN1 and 2 (lesions/mm² $\times 10^3$ of pancreas) is increased in DM-I. [^] $P < 0.01$ DM-I vs. DM, *** $P < 0.0001$ DM-I vs. ND.

endocrine pancreas are markedly enlarged in DM-I individuals, with increased exocrine cell proliferation. The α -cell hyperplasia and neuroendocrine tumor and microadenoma formation is consistent with chronic inhibition of glucagon secretion by GLP-1. These findings lend additional weight to concerns regarding the effects of long-term GLP-1-related therapy with respect to unintended proliferative actions on the exocrine pancreas and now also a possible increased risk of neuroendocrine tumors. In addition to the surveillance previously recommended for the potential association of GLP-1-based therapy and pancreatic cancer risk, the current data imply that surveillance

for a possible increased risk of pancreatic neuroendocrine tumors is warranted. On the other hand, the finding that there is a capacity to form large numbers of insulin-expressing cells in adult humans offers an impetus to the hopes of the goal of inducing β -cell regeneration in either type 1 or 2 diabetes. It is clear that a priority will be to establish the pathway by which these cells are formed and how to favor an endocrine versus exocrine cell lineage and, within the latter, a β -cell lineage. Moreover, further investigation on how to foster the maturation of the newly formed β -cells to function is of vital therapeutic importance.

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A.E.B. and M.C.-T. independently evaluated the morphometric analysis of the pancreas. M.C.-T. and M.A. supervised procurement of the pancreata by the nPOD program and the immunostaining procedures in Florida and participated in interpretation of the data. P.C.B. and T.G. supervised the immunostaining procedures in Los Angeles and participated in interpretation of the data. D.W.D. performed pathologic evaluation of pancreas sections. A.E.B., M.A., and P.C.B. contributed to writing the manuscript and preparation of the figures. P.C.B. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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EXHIBIT 8

A Critical Analysis of the Clinical Use of Incretin-Based Therapies: Are the GLP-1 Therapies Safe?

How safe are the GLP-1-based therapies?

There is no question that incretin-based glucose-lowering medications have proven to be effective glucose-lowering agents. Glucagon-like peptide 1 (GLP-1) receptor agonists demonstrate an efficacy comparable to insulin treatment and appear to do so with significant effects to promote weight loss with minimal hypoglycemia. In addition, there is significant data with dipeptidyl peptidase 4 (DPP-4) inhibitors showing efficacy comparable to sulfonylureas but with weight neutral effects and reduced risk for hypoglycemia. However, over the recent past there have been concerns reported regarding the long-term consequences of using such therapies, and the issues raised are in regard to the potential of both classes to promote acute pancreatitis, to initiate histological changes suggesting chronic pancreatitis including associated preneoplastic lesions, and potentially, in the long run, pancreatic cancer. Other issues relate to a potential risk for the increase in thyroid cancer. There is clearly conflicting data that has been presented in preclinical studies and in epidemiologic studies. To provide an understanding of both sides of the argument, we provide a discussion of this topic as part of this two-part point-counterpoint narrative. In the point narrative below, Dr. Butler and colleagues provide their opinion and review of the data to date and that we need to reconsider the use of incretin-based therapies because of the growing concern of potential risk and based on a clearer understanding of the mechanism of action. In the counterpoint narrative following the contribution by Dr. Butler and colleagues, Dr. Nauck provides a defense of incretin-based therapies and that the benefits clearly outweigh any concern of risk.

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The clinical value of new therapies for diabetes tends to be overestimated at launch, whereas the disadvantages emerge more slowly. Possible reasons include inflated expectations, marketing pressures, and the limited number of people exposed to the drug prior to launch. Full recognition of unwanted effects has also been delayed by inadequate postmarketing surveillance, especially when the unwanted effect is difficult to pinpoint or slow to emerge.

Off-target or unwanted effects pose a particular problem when a new class has wide-ranging effects. This was exemplified by the thiazolidinediones, nuclear receptor agonists with useful glucose-lowering properties but pleiotropic and unpredictable pathophysiological actions. Some undesirable outcomes such as osteopenia, redistribution of body fat, and fluid retention emerged as class effects, whereas others such as acute liver failure, increased cardiovascular morbidity, and bladder cancer appear specific to individual agents. Although the potential for unwanted effects was recognized at an early stage of development, it took years for them to be identified, analyzed, and acted upon. This meant that millions of people were exposed

to agents whose potential long-term consequences were incompletely understood.

The glucagon-like peptide 1 (GLP-1)-based therapies have comparably pleiotropic actions. GLP-1 is a peptide hormone that enhances insulin secretion, inhibits glucagon release, delays gastric emptying, and suppresses appetite. Other potentially useful properties include enhanced growth and proliferation of pancreatic β -cells in immature (but not adult) rodents. GLP-1 receptors are however present in many other tissues, including thyroid, exocrine pancreas, meninges, renal tubules, and bone, and their activation results in changes entirely unrelated to glucose homeostasis. High levels of vigilance are therefore justified.

GLP-1 has a very short half-life and is therefore "seen" by its receptors in a transient and tightly regulated fashion in healthy individuals. The incretin effect is deficient in type 2 diabetes, and GLP-1-based therapy addresses this deficit. Its full glucose-lowering effect is however achieved at supraphysiological (DPP-4 inhibition) or pharmacological (GLP-1 mimetic) dosing levels. GLP-1 analogs thus achieve pharmacologic override of normal physiologic function and have the

potential to produce unexpected off-target effects, whereas DPP-4 inhibition enhances the release of gastric inhibitory polypeptide (GIP) as well as GLP-1, and the long-term impact of DPP-4 inhibition upon other regulatory systems is unknown.

Regulatory authorities have expressed concerns about the potential risk of acute pancreatitis, thyroid cancer, and renal failure with some or all of the GLP-1-based therapies, warnings that are (as appropriate) conveyed in every pack that is handed to a patient. These concerns are however largely discounted by the manufacturers and those representing their views to physicians, who typically maintain that the risk of pancreatic inflammation is illusory.

Pancreatitis: Now you see it, now you don't

—Exenatide, the first GLP-1-based therapy, was launched in the U.S. on April 29, 2005. A single case report of acute pancreatitis appeared in 2006 and was spotted by investment advisors who conducted their own search of the U.S. Food and Drug Administration (FDA) database and reported a potential risk of acute pancreatitis on October 2, 2006. The company made a change to its label on October 8, but the FDA did not issue its first alert until October 2007 (1). This was followed by a series of publications, mostly sponsored by the manufacturers, which reported that pancreatitis is more common in established diabetes than previously appreciated, together with pharmacoepidemiological studies using administrative databases that indicated that pancreatitis is no more common with exenatide than with other therapies for diabetes (2–4).

It is not easy to estimate the prevalence of acute pancreatitis, let alone assign a probable cause, and there are genuine difficulties in ascertaining the prevalence of acute pancreatitis in a population with diabetes. Reverse causation is an important confounder since both acute and chronic pancreatitis may give rise to

diabetes. Chronic pancreatitis may present with acute episodes of pancreatic pain. The formal criteria for diagnosis—typical pain, enzyme rises, and changes on computed tomography (CT) examination—may not be satisfied or adequately recorded in administrative databases, and unequivocal CT abnormalities may not be present. The source documentation is often inadequate and pharmacoepidemiologic analyses may reach differing rate estimates because of differing criteria. Last but not least, a plausible mechanism to explain the occurrence of pancreatitis was initially lacking. This is no longer the case.

Emergence of a mechanism

—GLP-1 receptors are abundantly expressed in the pancreatic ducts as well as in the pancreatic islets, and the intense interest in GLP-1–based therapies as a potential stimulus to β -cell regeneration has overshadowed the possibility that exocrine pancreatic cells might be similarly affected. Acinar and duct cells proliferate in response to GLP-1 therapy (5) and cause an increase in pancreatic weight (6,7) (Fig. 1). Such observations attracted little attention prior to 2009 when one of eight HIP rats, a model of type 2 diabetes, developed hemorrhagic pancreatitis following exposure to sitagliptin, and some of the remaining animals showed marked acinar to ductal metaplasia, a potentially premalignant change characteristic of chronic pancreatitis (7). Gier et al. (8) noted that the pancreatic duct gland (PDG) compartment of the pancreas is particularly responsive to the proliferative actions of GLP-1 and confirmed that GLP-1 simulates proliferative signaling in human pancreatic ductal epithelium. Two short-term studies were subsequently performed at the request of the FDA. These studies were carried out with exenatide and liraglutide in the ZDF rat model of diabetes and were reassuring with respect to possible adverse effects of GLP-1 mimetic therapy on the exocrine pancreas. Notwithstanding, pancreatic enzymes rose in both studies: one of twelve animals treated with exenatide died of massive pancreatic necrosis, and pathological findings in treated animals included acinar to ductal metaplasia and foci of ductal hyperplasia (9,10).

Some of the relevant preclinical studies are summarized in Table 1 (5–13). In aggregate, they offer a plausible mechanism for the occurrence of acute pancreatitis in patients exposed to GLP-1–based therapies since duct proliferation might

lead to duct occlusion (particularly in the setting of existing dysplastic lesions), occlusion would generate back pressure, and back pressure would stress acinar cells thereby activating and releasing the digestive enzymes that they contain—a well-established causal mechanism for pancreatitis.

Human pancreatitis revisited

—Animal studies do not necessarily reflect the experience in humans, but the identification of a plausible mechanism is an important step toward establishing a potential hazard and indicates a need for more detailed analysis in humans. Observational and pharmacoepidemiologic studies have suggested that acute pancreatitis is more common than expected in the diabetic population and is not increased by exenatide relative to other therapies (2–4). Although space does not permit detailed consideration here, there are some anomalies. For example, Dore et al. (2) examined the frequency of pancreatitis in a claims database comprising 25,700 patients on exenatide (past or present users) as compared with 234,500 patients on other antihyperglycemic therapies. Overall, there were more cases of confirmed pancreatitis in past or present exenatide users as compared with other therapies (40/25,719 vs. 254/234,536 = 1.56/1,000 vs. 1.08/1,000 users). The study found a reduced frequency of pancreatitis in present users of exenatide, but a propensity adjusted RR (relative risk) of 2.8 (CI 1.6–4.7) for past use. The latter observation was discounted because those being studied were no longer taking exenatide at the time of the episode, but the exclusion would not be valid if exenatide had been stopped because of premonitory symptoms of abdominal pain or if the proposed mechanism persisted in those no longer taking the drug. Garg et al. (14) found no evidence of an increased risk of pancreatitis with exenatide, but concede that “the limitations of this observational claims-based analysis cannot exclude the possibility of an increased risk.” A recent case-control study addressed many of the limitations of previous reports, including inadequate power, and found that current and recent (1 month–2 years) users of GLP-1–based therapies had a twofold risk of acute pancreatitis (adjusted odds ratio 2.24 [95% CI 1.36–3.68] for current use and 2.01 [1.27–3.18] for recent use) (15).

Studies conducted by the manufacturer under the eyes of the regulators may

provide reliable information. A recent review identified 11 such reports in studies conducted by Novo Nordisk, the manufacturer of liraglutide. Seven occurred in the LEAD (Liraglutide Effect and Action in Diabetes) studies (16), two in other studies, and two in postmarketing reports. Adverse events from the FDA Serious Adverse Event (SAE) reports were not considered. The findings were considered to “implicate liraglutide as the cause in at least some of these cases” (17).

Further cause for concern comes from FDA MedWatch data. An excess of acute pancreatitis was already evident for exenatide within 1 year of launch (1), and an updated analysis in 2011 found that, as compared with other non-GLP-1–based diabetes therapies, the reporting rate for acute pancreatitis with exenatide was dramatically increased ($P < 2 \times 10^{-4}$) (18). This easily checked analysis has not been seriously challenged.

The FDA alert system was designed to identify potential safety problems, not to confirm them. Notwithstanding its limitations, to our knowledge there is no single instance in which a strong sustained signal has turned out to be entirely spurious. When Elashoff et al. (18) was published, there were 971 reported pancreatitis events for exenatide and 131 for sitagliptin. The corresponding numbers are now 2,327 and 718 (Table 2). Recognition of an adverse event undoubtedly increases the reporting frequency, but there was a signal for exenatide long before the first FDA alert was issued, and there was no reason to anticipate a similar problem with sitagliptin. Furthermore, there are now 888 reported pancreatitis events for liraglutide, 125 for saxagliptin, and 43 for linagliptin (Table 2). Every GLP-1–based therapy with sufficient market exposure has generated a signal for pancreatitis, and no other diabetes medication has done so.

We conclude that the balance of evidence does suggest an association between widely used GLP-1–based therapies and acute pancreatitis, suggesting a class effect, and that this is underpinned by a plausible mechanism.

What are the implications?—

The major concern is not pancreatitis, unpleasant though this is. The concern is that acute events may be no more than the tip of an iceberg, and that these agents might cause subclinical duct proliferation, acinar to ductal metaplasia, and subclinical pancreatitis in a much higher proportion of individuals. Pancreatitis,

Table 1—Animal studies of GLP-1–based therapy on the exocrine pancreas

Reference	Species/age	Treatment/day duration#	Pancreas weight	Pancreas enzymes	Histology	Replication/method
Perfetti et al., 2000 (5)	Wistar rat 22 months	GLP-1 1.5 pmol/kg · min, 5 days	↑	→	NR	↑ Ducts and acinar cells PCNA
Koehler et al., 2009 (6)	Mice 9–12 weeks	Exenatide 48 nmol/kg, 4 wks	↑	→	NR	NR
	Mice 9–12 weeks	Liraglutide 75 µg/kg, 1 wk	↑	→	NR	NR
Matveyenko et al., 2009 (7)	HIP rats 2 months	Sitagliptin 200 mg/kg, 12 weeks	↑	NR	Pancreatitis (1/8) and acinar to ductal metaplasia (3/16)	↑ Ducts, Ki67
Nachnani et al., 2010 (12)	Rats 8 weeks	Exenatide 10 µg/kg, 11 weeks	NR	↑ Amylase	Exocrine inflammation	NR
Tatarkiewicz et al., 2010 (11)	Mice 10 weeks	Exenatide 7.2 nmol/kg, 4 weeks	→	→	No pancreatitis	→ Ducts Ki67
Vrang et al., 2012 (9)	ZDF rats 7 weeks	Exenatide 0.25 mg/kg, 13 weeks	→	↑ Amylase	1/12 death pancreatic necrosis; focal acinar hyperplasia;	→ Ducts Ki67*
		Liraglutide 1.0 mg/kg, 13 weeks	→	→	3/12 death by overdose, unexplained; increased ductal proliferation and acinar to ductal metaplasia	→ Ducts Ki67*
Nyborg et al., 2012 (13)	Cynomolgus monkeys age NR	Liraglutide 5 mg/kg, 87 weeks	NR	NR	Normal	NR
	Rats age NR	Liraglutide 1 mg/kg, 26 weeks	NR	NR	Normal	NR
	Mice age NR	Liraglutide 3 mg/kg, 104 weeks	NR	NR	Normal	NR
Gier et al., 2012 (8)	Rats 10 weeks	Exenatide 10 µg/kg, 12 weeks	↑	→	PDG hyperplasia; chronic pancreatitis and advanced PanINs	↑ PDG and ducts Ki67
	Pdx-1 Kras mice 6 weeks	Exenatide 5 nmol/kg, 12 weeks	↑	↑ Lipase		↑ Ducts Ki67
Tatarkiewicz et al., 2012 (10)	ZDF rats 8 wks	Exenatide 250 µg/kg, 12 weeks	→	↑ Amylase	Normal	→ Ducts Ki67*

Preclinical animal studies reporting the effects of GLP-1–based therapies on the exocrine pancreas. *Indicates pancreas fixed in formaldehyde for 24 h or more, typically denaturing proteins to the extent that measurement of cellular replication by Ki67 is unreliable. #Maximal dose and duration of GLP-1–based therapy included in each study is shown in the summary. NR, not recorded.

whether clinical or subclinical, is well known to predispose to pancreatic cancer, and there is a signal for cancer of the pancreas for exenatide in both the FDA and German regulatory databases and for sitagliptin in the FDA database (18,19). The signal has grown stronger with 258 pancreatic cancers reported for exenatide, 63 for liraglutide, 81 for sitagliptin, 18 for saxagliptin, and 1 for linagliptin (Table 2).

Low-grade asymptomatic chronic pancreatitis with associated proliferative changes is not uncommon in the middle-aged target population for this drug class (20), making it likely that the proproliferative actions of GLP-1 therapy will at times be superimposed upon low-grade

pancreatitis and its associated dysplastic changes. Some insight into this possibility was gained in the chronic pancreatitis-prone Kras^{G12D} mouse model in which exenatide therapy accelerated formation and growth of dysplastic intraepithelial neoplasia (PanIN) lesions as well as pancreatitis (8). To date, this is the only study of the actions of incretin treatment in a model of chronic pancreatitis (Fig. 1). In contrast, two studies of short-term GLP-1 exposure superimposed on acute toxin-induced pancreatitis were reported to show a protective effect, but such studies do not address the mechanism of relevance (6,11).

The incidence of pancreatic cancer, as of pancreatitis, is increased in type 2

diabetes (21). Work over the past decade has established that premalignant changes known as pancreatic intraepithelial (PanIN) lesions precede and predict the onset of pancreatic cancer. PanINs are present in up to 50% of the middle-aged population, although relatively few actually progress to cancer (20). Both PanINs and pancreatic cancer express the GLP-1 receptor in humans (8). Since progression of PanINs to pancreatic cancer is via the accumulation of additional somatic mutations, any driver of increased cellular replication in PanINs is likely to increase that probability. This theoretical risk was illustrated by the progression of PanINs in the exenatide-treated

Table 2—FDA adverse event reports for GLP-1-based drugs

Exenatide and sitagliptin vs. controls (04Q1 to 12Q2)

Drug	Pancreatitis events	Control events	OR	95% CI	P value
Exenatide	2,327	1,660	19.17	(16.41–22.50)	<2.2e-16
Sitagliptin	718	411	23.89	(19.76–28.93)	<2.2e-16
Controls	207	2,832			
Drug	Pancreatic cancer events	Control events	OR	95% CI	P value
Exenatide	258	1,660	2.99	(2.41–3.73)	<2.2e-16
Sitagliptin	81	411	3.80	(2.80–5.11)	<2.2e-16
Controls	147	2,832			
Drug	Thyroid cancer events	Control events	OR	95% CI	P value
Exenatide	74	1,660	3.94	(2.56–6.20)	1.67e-11
Sitagliptin	5	411	1.08	(0.33–2.81)	0.80
Controls	32	2,832			

Liraglutide vs. controls (10Q2 to 12Q2)

Drug	Pancreatitis events	Control events	OR	95% CI	P value
Liraglutide	888	259	56.81	(43.52–74.71)	<2.2e-16
Controls	84	1,393			
Drug	Pancreatic cancer events	Control events	OR	95% CI	P value
Liraglutide	63	259	5.64	(3.80–8.38)	<2.2e-16
Controls	60	1,393			
Drug	Thyroid cancer events	Control events	OR	95% CI	P value
Liraglutide	57	259	17.99	(10.12–33.56)	<2.2e-16
Controls	17	1,393			

Saxagliptin vs. controls (09Q4 to 12Q2)

Drug	Pancreatitis events	Control events	OR	95% CI	P value
Saxagliptin	125	65	30.96	(21.33–45.35)	<2.2e-16
Controls	100	1,618			
Drug	Pancreatic cancer events	Control events	OR	95% CI	P value
Saxagliptin	18	65	6.04	(3.21–10.95)	6.85e-8
Controls	74	1,618			
Drug	Thyroid cancer events	Control events	OR	95% CI	P value
Saxagliptin	0	65	0	(0.00–5.48)	>0.99
Controls	19	1,618			

Linagliptin vs. controls (11Q3 to 12Q2)

Drug	Pancreatitis events	Control events	OR	95% CI	P value
Linagliptin	43	14	42.36	(20.86–90.82)	<2.2e-16
Controls	43	601			
Drug	Pancreatic cancer events	Control events	OR	95% CI	P value
Linagliptin	1	14	1.79	(0.04–12.72)	0.45
Controls	24	601			
Drug	Thyroid cancer events	Control events	OR	95% CI	P value
Linagliptin	0	14	0	(0–27.80)	>0.99
Controls	8	601			

The updated adverse event reports from Elashoff et al. (18) to include most recent available quarters and GLP-1 drugs launched since the original Elashoff report. Since rosiglitazone (Avandia) is now rarely used in the U.S., the control drugs have been increased to include insulin preparations available in the U.S. The pattern of findings is comparable with or without these added controls. Control drugs include "AVANDIA", "ROSIGLITAZONE", "STARLIX", "NATEGLINIDE", "PRANDIN", "REPAGLINIDE", "NOVONORM", "GLIPIZIDE", "GLUCOTROL", "INSULIN DETEMIR", "LEVEMIR", "INSULIN ASPART", "NOVOLOG", "HUMULIN N", "HUMULIN R", "INSULIN LISPRO", "HUMALOG", "INSULIN GLARGINE", "LANTUS", "HUMULIN 70/30", and "NOVOLOG MIX 70/30". Pancreatitis events include "PANCREATITIS". Pancreatic cancer events include "PANCREATIC MASS", "PANCREATIC NEOPLASM", "ADENOCARCINOMA PANCREAS", and "PANCREATIC CARCINOMA". Thyroid cancer events include "THYROID CANCER", "THYROID GLAND CANCER", "THYROID NEOPLASM", and "THYROID MASS". Control events include "BACK PAIN", "CHEST PAIN", "COUGH", "SYNCOPE", and "URINARY TRACT INFECTION".

Kras^{G12D} mouse model (8). It is worth noting here that such a potential link between GLP-1 therapy and risk for pancreatic cancer is analogous to estrogen therapy and

breast cancer. Estrogen does not initiate breast cancer, but in individuals with pre-malignant dysplastic ductal changes that bear estrogen receptors, estrogen accelerates

growth and malignant conversion in some individuals (22). Likewise, the very high concentration of insulin delivered to the bronchial tree with inhaled insulin was as-

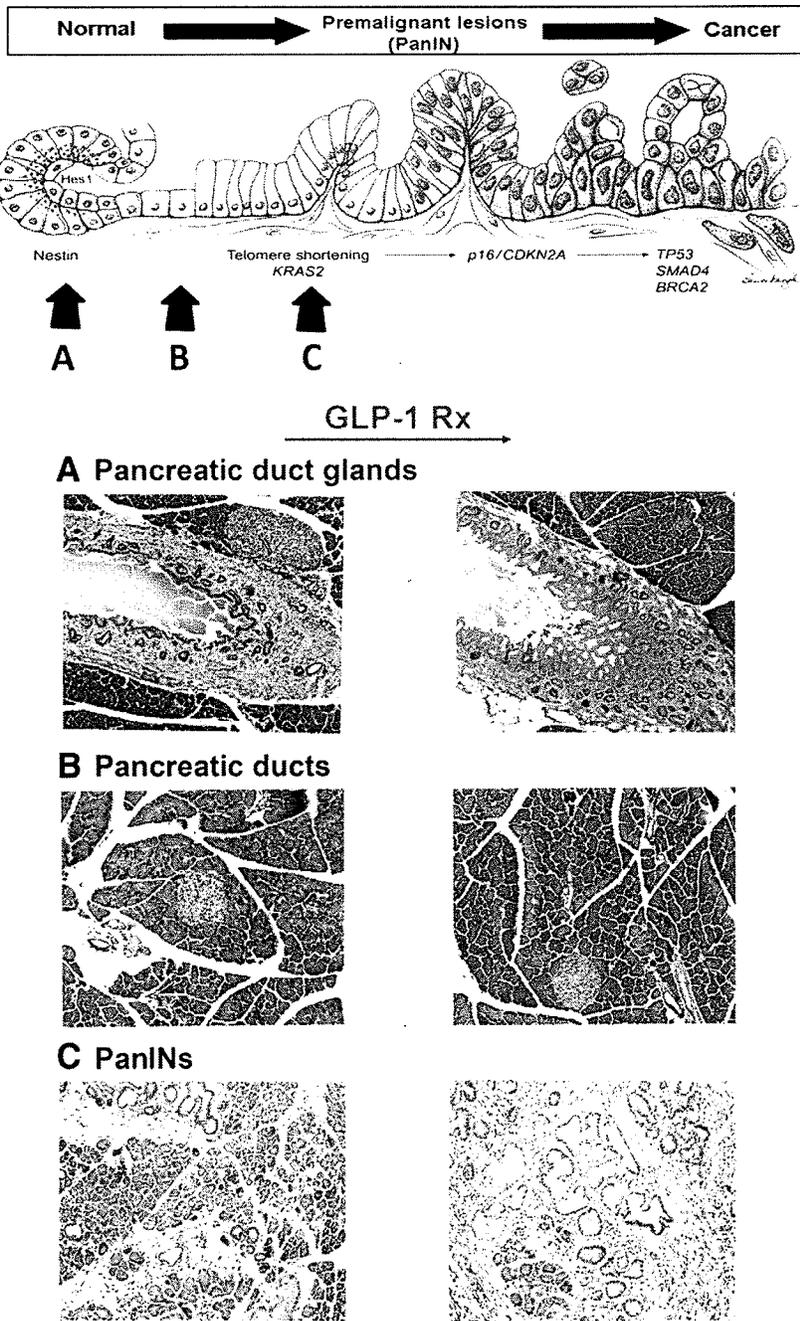


Figure 1—GLP-1 actions on exocrine pancreas in animal studies depend on compartment studied and pancreas health. The histological characteristics of the transition from normal pancreas to premalignant changes (PanINs) typically present in the progression from asymptomatic chronic pancreatitis to cancer and, as established by human pathological and mouse genetic studies (top panel, modified from Maitra and Hruban [31]). In nondiabetic animal studies, exposure of pancreas to GLP-1 therapies has minimal discernible impact except in the pancreatic duct gland compartment where marked proliferation generates intraductal papillary projections (A: Pancreatic duct glands are markedly expanded in nondiabetic rats treated with exenatide 10 $\mu\text{g}/\text{kg}$ daily for 12 weeks). However the pancreatic ducts show no obvious abnormalities in the same animals. B: In contrast, GLP-1 therapy accelerates pancreatitis and neoplasia in mice prone to chronic pancreatitis. C: Formation of PanINs and pancreatitis are markedly accelerated in the *Pdx1-Cre; LSL-Kras^{G12D}* mouse model treated with exenatide 5 nmol/kg for 12 weeks. A, B, and C used with permission from Gier et al. (8).

sociated with an increased incidence of lung cancer (23). Are estrogen, insulin, or GLP-1 carcinogens? No, but all three can serve as growth factors, and when pharmacological stimulation of growth is imposed on dysplastic lesions, accelerated declaration of cancer is not unexpected.

Where do we go from here?—

The regulatory reflex, when presented with a safety concern, is to request further descriptive data from the manufacturers. Our view is that the request for further epidemiologic analysis misses the real point of concern and wastes valuable time. The answer lies in the human pancreas, and (until this answer is known) there are more relevant questions to ask.

One question is this: If subclinical pancreatitis is common (consistent with the episodes of abdominal pain or discomfort described by many users), we might anticipate subclinical increases in pancreatic enzymes. Anecdotally, many clinicians already know this to be the case, but there is only one published case series (24). We accept that pancreatic enzyme levels fluctuate in people with diabetes and that confirmation of increased levels in people exposed to GLP-1-based therapies does not in itself constitute evidence of subclinical pancreatitis, but if a signal is there, we need to know.

The debate has been conducted in the absence of a single report from the pancreas of a human exposed to GLP-1 therapies. This is where the answer lies (25). Most recently, the first data have become available from human pancreas following a year or more of incretin therapy; 7 individuals treated by sitagliptin and 1 by exenatide compared to 12 individuals with type 2 diabetes treated with other agents and nondiabetes (26). The pancreas was 40% enlarged with increased exocrine pancreas proliferation in incretin-treated individuals. Moreover, there was an increase in the number of PanIN (pre-malignant) lesions after prior incretin treatment, consistent with the findings in the *Kras^{G12D}* mouse model (8). A striking finding in the human pancreas after incretin treatment was marked α -cell hyperplasia with glucagon-expressing microadenomas in 3 of the 8 individuals, and a glucagon-expressing neuroendocrine tumor in 1 of the 8. Given the heavily promoted action of incretin therapy to suppress glucagon secretion, and the prior reports of α -cell hyperplasia and risk for progression to pancreatic neuroendocrine tumors (26), this finding,

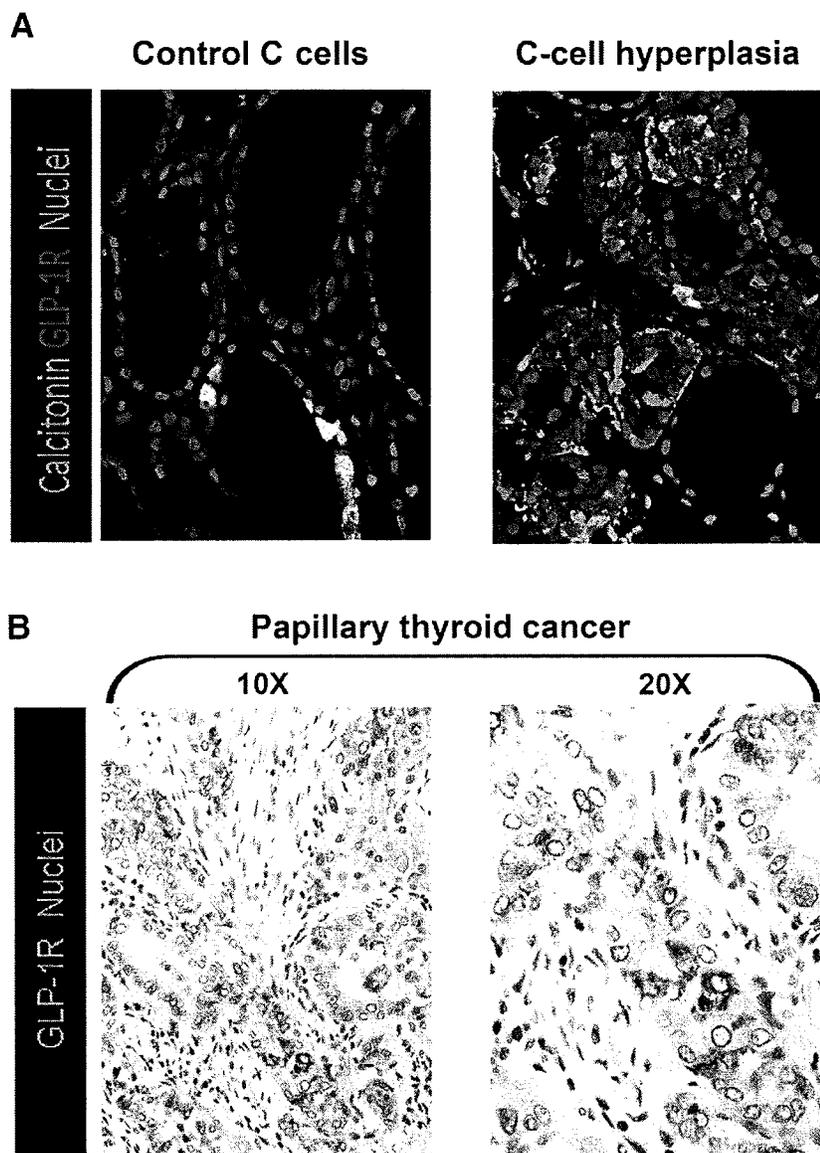


Figure 2—GLP-1 receptors (GLP-1R) are expressed in premalignant lesions in human thyroid. A: Human thyroid immunostained by immunofluorescence for calcitonin (green), GLP-1 receptor (red), and nuclei (blue) in a normal thyroid (left) and in C-cell hyperplasia (right). Yellow color indicates GLP-1 receptor expression in C cells, which is present occasionally in normal thyroid and frequently in C-cell hyperplasia. B: Human thyroid from papillary thyroid cancer (left and right panels) stained by immunohistochemistry for GLP-1 receptor (GLP-1R) (brown). GLP-1 receptor expression is present in ~20% of papillary thyroid cancers and most medullary thyroid cancers. Used with permission from Gier et al. (28).

while of concern, is perhaps not unexpected. No changes were reported in the exocrine pancreata of 10 monkeys that were treated with liraglutide for 87 weeks (13). Treatment was discontinued 2 weeks before the pancreata were obtained. The weight of the pancreata was not however reported (this would have been expected to increase). Long-term treatment of nondiabetic human primates with exenatide

has not been published. The concerns raised in this article go well beyond the scope of routine histologic analysis conducted for regulatory purposes, and a full review by independent experts in pancreatic pathology would now seem justified (25).

In summary, a plausible mechanism links GLP-1–based therapy with acute pancreatitis—and a potential risk of pancreatic

cancer—in individuals with type 2 diabetes. The model proposes acceleration of pancreatic dysplasia in the setting of low-grade chronic pancreatitis leading to sufficient ductal obstruction in a minority of individuals to provoke an episode of acute pancreatitis. Subclinical changes would be expected in a larger proportion of those exposed. The absence of pancreatitis or pancreatic dysplasia in nondiabetic models or short-term treatment of models of diabetes does not exclude the proposed mechanism. GLP-1 treatment, like estrogen in breast cancer, might promote development of pancreatic cancer in some individuals. Alternatively, periductal α -cell hyperplasia may cause duct obstruction and potentially progress to neuroendocrine neoplasia.

GLP-1 and thyroid cancer: Now you see it, now you don't

Preclinical registration studies of liraglutide found an increased number of C-cell tumors of the thyroid in rodents. Studies sponsored by the manufacturers have suggested that C cells in humans do not express the GLP-1 receptor; that humans exposed to liraglutide have, in aggregate, little or no rise in calcitonin levels; and that nonhuman primates exposed to liraglutide do not develop thyroid tumors (27). In contrast, analysis of a much larger sample of human thyroid glands and C cells established that a subpopulation of C cells in humans does indeed express the GLP-1 receptor (28) (Fig. 2). It was further established that GLP-1 receptor expression was more abundant in C-cell hyperplasia, a potential precursor of medullary thyroid cancer. Moreover, GLP-1 receptor expression is also present in 20% of those with papillary thyroid cancer, a much more common tumor for which calcitonin levels would be irrelevant. While medullary thyroid cancer is rare (29), a relatively high proportion of the population has apparently quiescent micro foci of papillary thyroid cancer (30).

Once again we must ask whether relatively short-term negative studies of GLP-1 mimetic therapy and thyroid cancer in normal monkeys provide adequate reassurance against the risk of malignancy in humans. As in the pancreas, the concern is that proliferative actions of GLP-1 superimposed on premalignant lesions (C-cell hyperplasia or micropapillary thyroid cancer) may accelerate the progression of these lesions toward cancer. And, once again, adverse event reporting shows a clear excess of reported thyroid cancer on both exenatide (74

thyroid cancer events) and liraglutide (57 thyroid cancer events), although there is currently no similar signal for the DPP-4 inhibitors (Table 2).

Conclusions: Déjà vu all over again?

The story is familiar. A new class of antidiabetic agents is rushed to market and widely promoted in the absence of any evidence of long-term beneficial outcomes. Evidence of harm accumulates, but is vigorously discounted. The regulators allow years to pass before they act. The manufacturers are expected—quite unrealistically—to monitor the safety of their own product. We should be thankful that those responsible for aircraft safety do not operate on the assumption that the absence of evidence is evidence of absence.

The safety of the GLP-1 therapies can no longer be assumed, and there will be rapid developments in this area. Drug safety can never be assumed, and the legal principle of “innocent until proved guilty” does not apply. The case presented here does not prove that these agents are unsafe, but it does suggest that the burden of proof now rests with those who wish to convince us of their safety.

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See accompanying articles.

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