

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

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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  $\beta$ -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  $\beta$ -cell mass. However, sitagliptin also increases  $\beta$ -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  $\beta$ -cell toxicity. Conversely, metformin treatment decreases islet amyloid formation by decreasing  $\beta$ -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  $\beta$ -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,  $\beta$ -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  $\beta$ -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  $\beta$ -cell secretion without increasing amyloid formation and protects against amyloid-induced  $\beta$ -cell loss. This suggests a novel effect of sitagliptin to protect the  $\beta$ -cell in type 2 diabetes that appears to occur without adverse effects on the exocrine pancreas.

DPP-4 inhibitor; IAPP;  $\beta$ -cell mass; amyloid; exocrine pancreas pathology

DEFICITS IN  $\beta$ -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  $\beta$ -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  $\beta$ -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  $\beta$ -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  $\beta$ -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  $\beta$ -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 HSS 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 ( $\dot{V}O_2$ ) 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 ( $K_g$ ), 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  $\beta$ -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  $\beta$ -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,  $\beta$ -cell, and amyloid areas were determined. Amyloid severity was calculated as percent amyloid-positive area/section area.  $\beta$ -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  $\chi^2$  test or analysis of variance (ANOVA) with least significant difference post hoc analysis. Exocrine tissue pathology data were compared using  $\chi^2$  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<sup>-1</sup>·day<sup>-1</sup> sitagliptin, 0.6 g·kg<sup>-1</sup>·day<sup>-1</sup> 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  $\mu$ M/day sitagliptin alone, 122  $\pm$  8  $\mu$ 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  $\mu$ M/day metformin alone, 1,222  $\pm$  162  $\mu$ 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  $\dot{V}O_2$  (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 <sup>2</sup> ) region of necrosis	2 small (<0.1 mm <sup>2</sup> ) or 1 large (>0.1 mm <sup>2</sup> ) region of necrosis	>2 small (<0.1 mm <sup>2</sup> ) or >1 large (>0.1 mm <sup>2</sup> ) region of necrosis



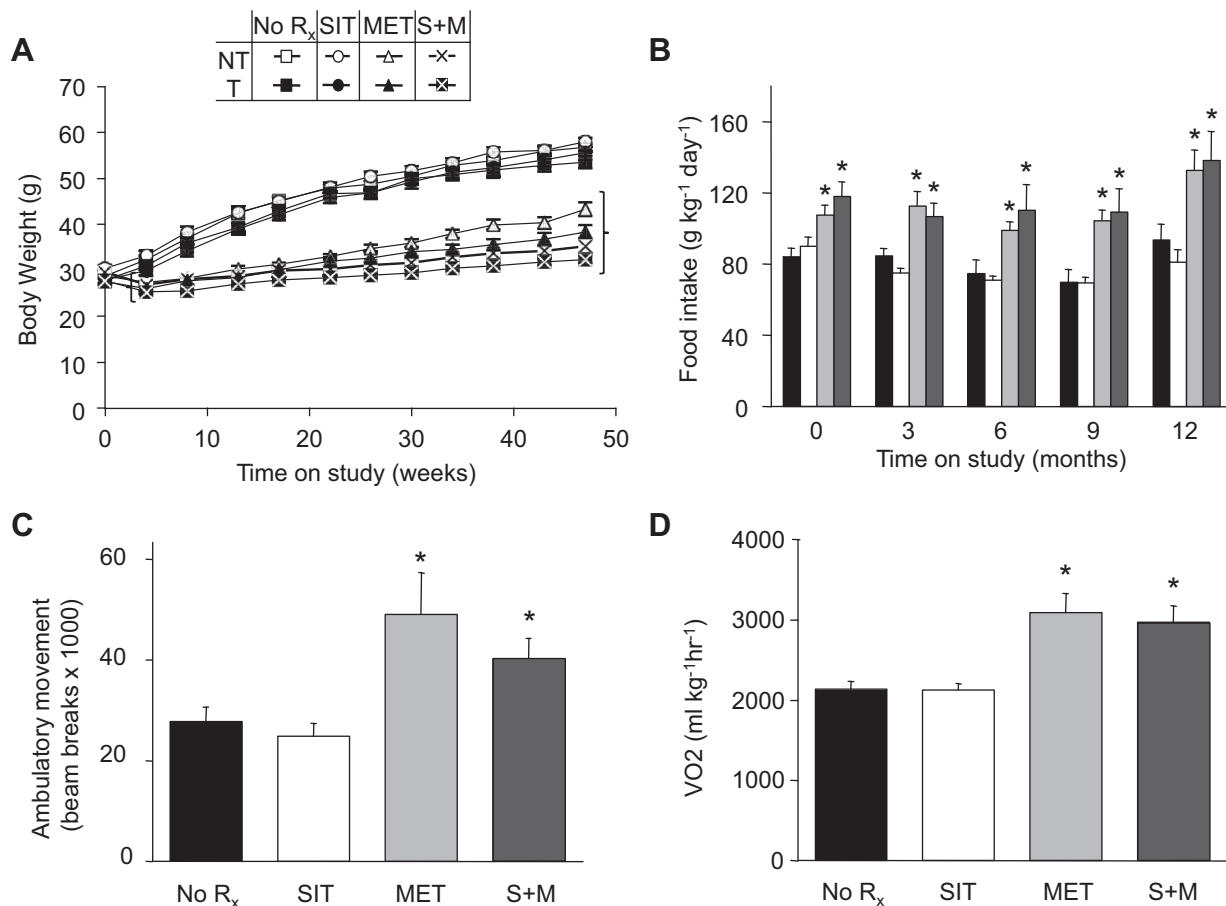


Fig. 1. Body weight (A;  $n = 18-24$ ) throughout the study in human islet amyloid polypeptide (*hIAPP*) transgenic (T; filled symbols) and nontransgenic (NT; open symbols) mice following 1 yr of no treatment (No Rx; squares) or treatment with sitagliptin (SIT; circles), metformin (MET; triangles), or 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).

**$\beta$ -Cell mass.** Untreated *hIAPP* transgenic mice had decreased  $\beta$ -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  $\beta$ -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.

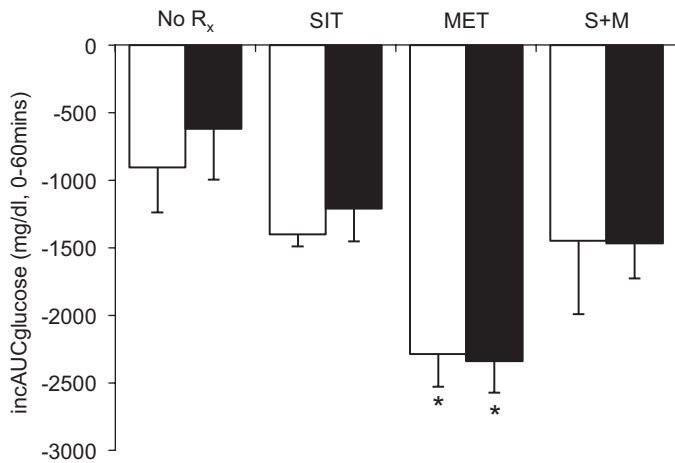


Fig. 2. Inverse incremental AUC glucose during an ip insulin tolerance test (ANOVA  $P < 0.01$ ) in *hIAPP* transgenic (filled bars) and nontransgenic mice (open bars). Mice were No  $R_x$  or treated with SIT, MET, or S+M for 1 yr;  $n = 4-7$ . \* $P < 0.05$  vs. No  $R_x$ .

nontransgenic mice (Fig. 4C). In contrast, *hIAPP* transgenic mice treated with sitagliptin had increased  $\beta$ -cell mass compared with untreated *hIAPP* transgenic mice; there was no difference in  $\beta$ -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  $\beta$ -cell mass than untreated mice (Fig. 4C). Sitagliptin plus metformin treatment also resulted in significantly reduced  $\beta$ -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% Ki67-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.

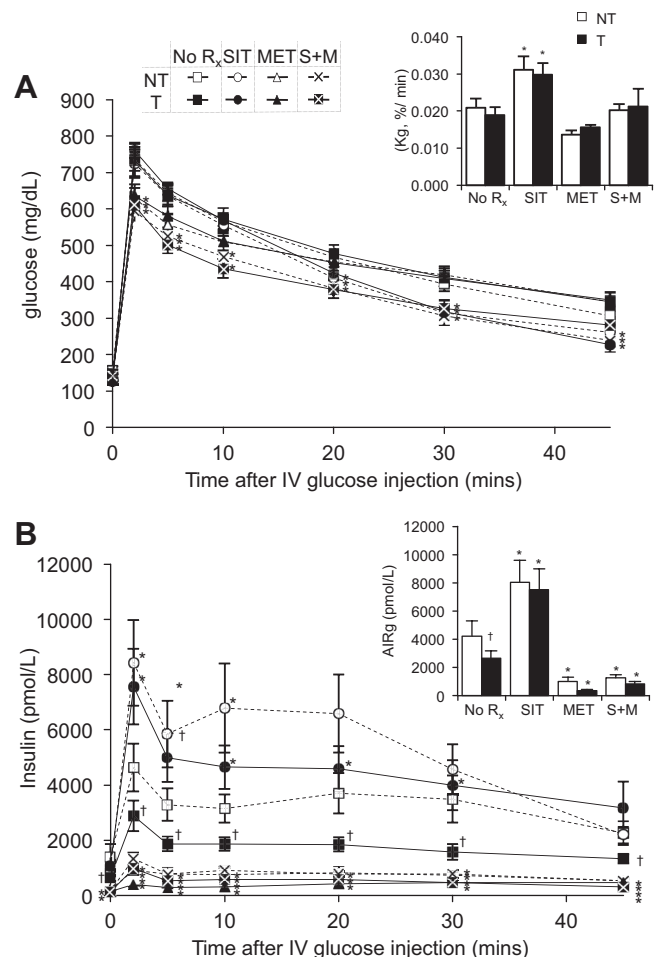


Fig. 3. Plasma glucose levels (A), rate of glucose disappearance ( $K_g$ ; inset in A, ANOVA  $P < 0.001$ ), plasma insulin levels (B), and the acute insulin response to glucose (AIRg; inset in B, ANOVA  $P < 0.001$ ) during an iv glucose tolerance test in *hIAPP* transgenic (T; filled symbols) and nontransgenic 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.

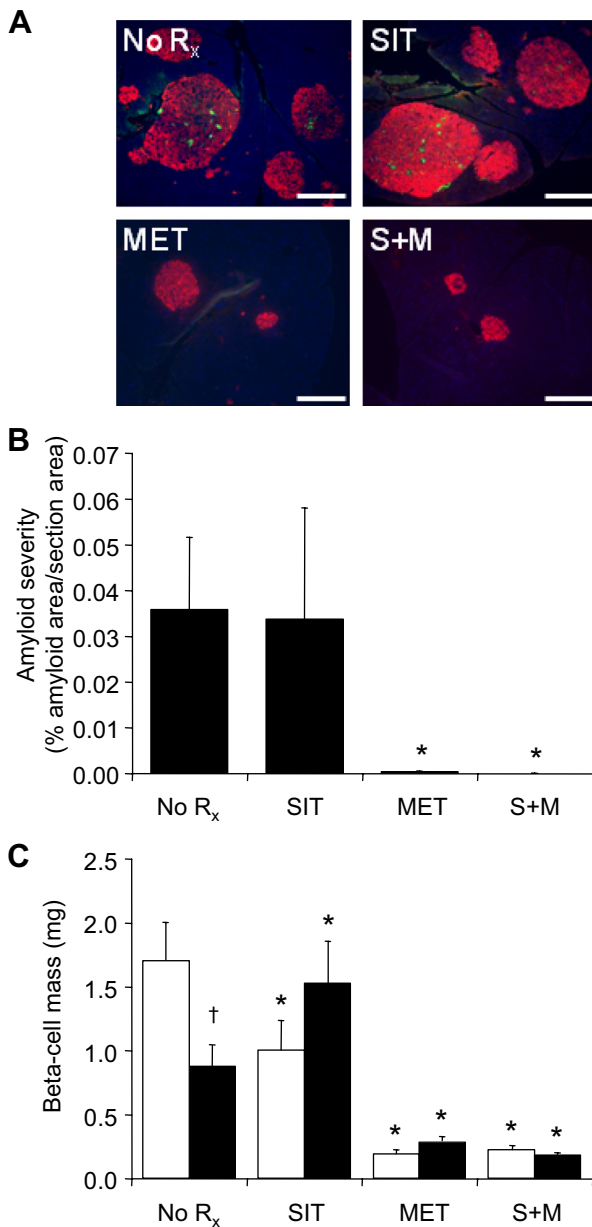


Fig. 4. *A*: representative photomicrographs of pancreas sections showing amyloid deposits (green) and insulin immunostaining (red). *B*: amyloid deposition in *hiAPP* transgenic mice (ANOVA  $P = 0.01$ ). Nontransgenic mice did not develop any islet amyloid, as expected; thus, amyloid data are not shown for those groups. *C*:  $\beta$ -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<sub>x</sub> or treatment with SIT, MET, or S+M;  $n = 10$ –21. Scale bar, 100  $\mu$ m. \* $P < 0.05$  vs. No R<sub>x</sub>; † $P < 0.05$  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  $\beta$ -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

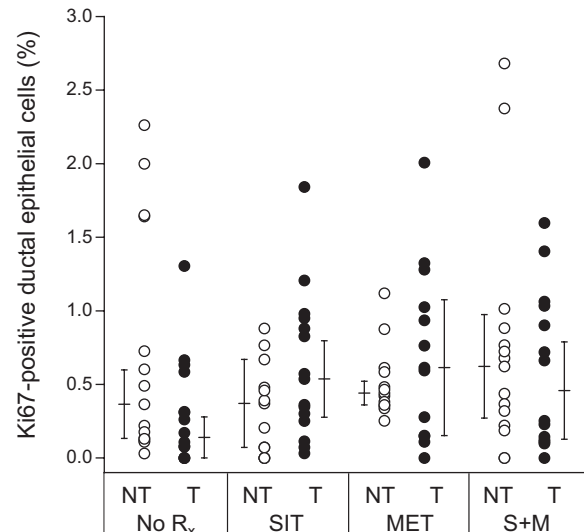


Fig. 5. Ductal proliferation in *hiAPP* transgenic (filled symbols) and nontransgenic mice (open symbols). Mice were No R<sub>x</sub> or treated with SIT, MET, or S+M for 1 yr;  $n = 10$ –15, ANOVA  $P = 0.18$ . Individual points (circles) represent data for each mouse, with mean  $\pm$  interquartile 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  $\beta$ -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.

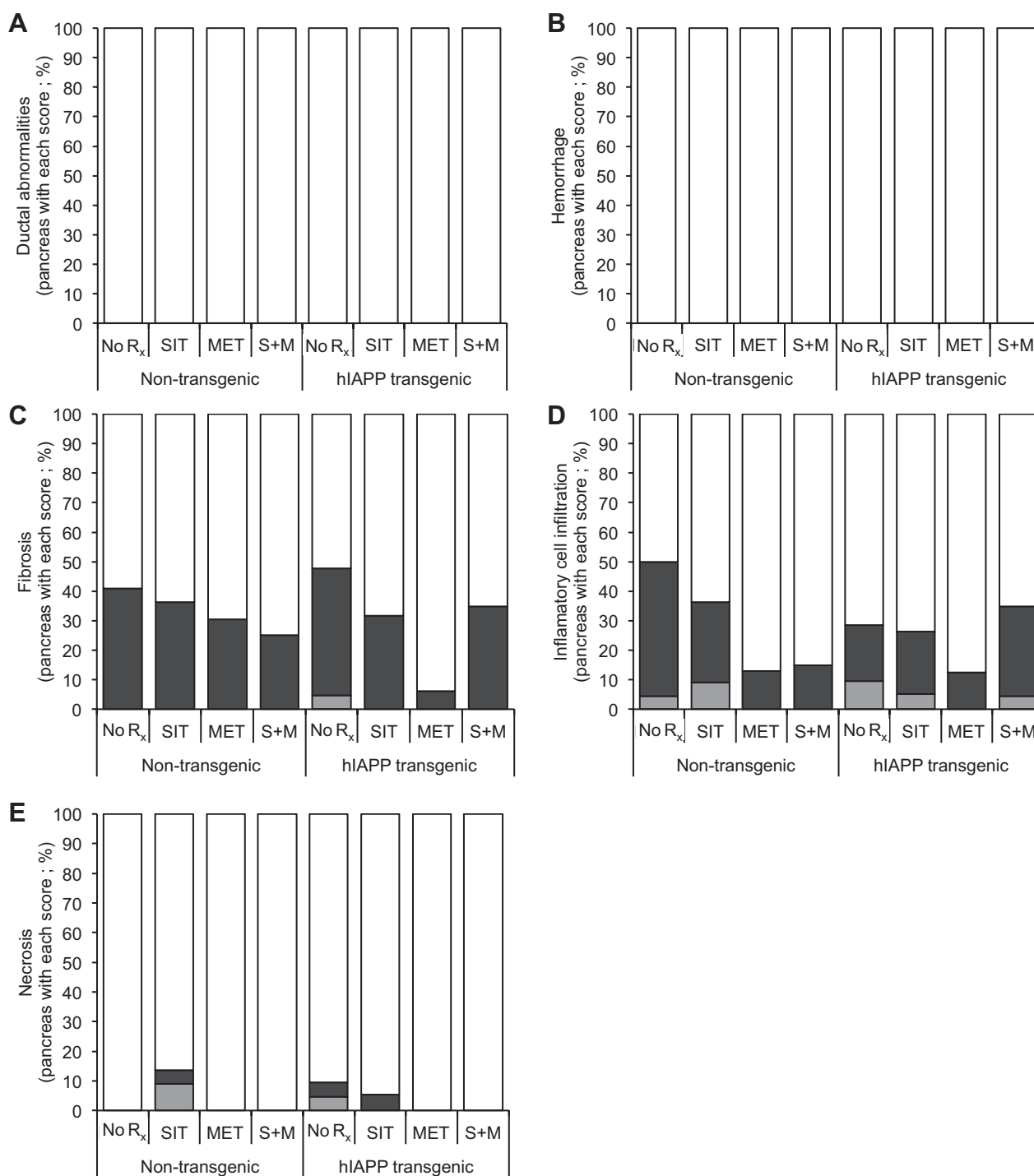


Fig. 6. Percentage of mice with exocrine pancreas pathology, assessed as ductal abnormalities (A), hemorrhage (B), fibrosis (C,  $P = 0.4$ ), inflammatory cell infiltration (D,  $P = 0.3$ ), and necrosis (E,  $P = 0.4$ ) in *hIAPP* transgenic and nontransgenic mice. Mice were No  $R_x$  or treated with 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  $\beta$ -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  $\beta$ -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  $\beta$ -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  $\beta$ -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  $\beta$ -cell mass following metformin treatment reflects islet pathology. Rather, we believe this is an appropriate response to the prevailing  $\beta$ -cell secretory demand. Previously, we (21) reported that long-term high-fat feeding in rodents stimulated  $\beta$ -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  $\beta$ -cell mass. The  $\beta$ -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  $\beta$ -cell secretory demand relative to high-fat-fed mice.

In *hIAPP* transgenic mice, sitagliptin treatment resulted in increased  $\beta$ -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  $\beta$ -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  $\beta$ -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  $\beta$ -cell mass (32, 34–36, 42). Rather, in the present study sitagliptin treatment resulted in decreased  $\beta$ -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  $\beta$ -cell adaptation to increased secretory demand with high-fat feeding. As mentioned, long-term high-fat feeding stimulates  $\beta$ -cell expansion in mice (21). In the present study, sitagliptin treatment stimulated insulin release pharmacologically, which we believe in turn decreased the need for  $\beta$ -cell expansion in order to meet secretory demand. Consistent with our findings, a recent study

showed that sitagliptin treatment, while increasing  $\beta$ -cell mass in mice fed a low-fat diet, actually decreased  $\beta$ -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  $\beta$ -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  $\beta$ -cell loss. This suggests a novel mechanism by which sitagliptin may protect the  $\beta$ -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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