

The Human GLP-1 Analog Liraglutide and the Pancreas

Evidence for the Absence of Structural Pancreatic Changes in Three Species

Niels C.B. Nyborg,¹ Anne-Marie Mølck,² Lars W. Madsen,³ and Lotte Bjerre Knudsen⁴

Glucagon-like peptide (GLP)-1 analogs have been implicated as a risk factor for pancreatitis in humans. We investigated whether liraglutide, the once-daily human GLP-1 analog, induces pancreatitis in rats, mice, and monkeys. Pancreata from mice, rats, and nonhuman primates were examined macro- and microscopically. Evaluation of preneoplastic proliferative lesions in the pancreata from nonhuman primates was performed. After 2 years of treatment, 3 of 79 male mice in the control group and 2, 1, 1, and 1 mice in the different liraglutide groups (of 67–79 mice per group) had pancreatitis based on microscopic criteria. For females, the numbers were 0 of 79 mice in the control group and 3 mice in all the liraglutide groups (of 66–76 mice per group). Pancreatitis was not the cause of death in any animals. There were no cases of pancreatitis, macroscopically or microscopically, in 400 rats. Neither pancreatitis nor preneoplastic proliferative lesions was found in monkeys dosed for 87 weeks, with plasma liraglutide exposure 60-fold higher than that observed in humans at the maximal clinical dose. In conclusion, liraglutide did not induce pancreatitis in mice, rats, or monkeys when dosed for up to 2 years and at exposure levels up to 60 times higher than in humans. *Diabetes* 61:1243–1249, 2012

Glucagon-like peptide (GLP)-1 receptor agonists or incretin mimetics provide several advantages over other diabetes therapies because of their ability to effectively lower blood glucose with a low risk of hypoglycemia and the additional benefit of weight loss (1). Exenatide was the first marketed GLP-1 receptor agonist, introduced in 2005 as a twice-daily injection, to be dosed with the morning and evening meals. Postmarketing surveillance has suggested a potential association between exenatide and the development of acute pancreatitis (2,3). The number of reported cases, however, is too low to evaluate whether there is a casual relationship between the use of exenatide and the development of pancreatitis. Of importance, a recent pharmacovigilance study using a large health care database has not demonstrated evidence for an association between exenatide and pancreatitis and found no increase in pancreatitis associated with exenatide compared with metformin or glyburide,

agents not historically linked to the development of pancreatitis (4). In support of a relationship between exenatide and pancreatitis, one preclinical study demonstrated evidence that exenatide caused pancreatic acinar inflammation in a juvenile rat model, whereas another study actually demonstrated an anti-inflammatory response of exenatide in mice (5,6). Also, exenatide has been tested in a model of chemically induced pancreatitis in mice, without evidence of a facilitating effect on pancreatitis (6). Liraglutide was marketed in 2009 in Europe and in 2010 in the U.S. Liraglutide is a once-daily human GLP-1 analog with a higher homology to human GLP-1 (97%) than exenatide (52%) (7,8). The clinical efficacy for liraglutide has been demonstrated in seven large randomized studies (9–14). The number of cases with pancreatitis following exposure to liraglutide has been very low, and it has not been possible to establish whether liraglutide is associated with pancreatitis.

An extensive preclinical development program for liraglutide has allowed us to investigate whether lifelong (2-year) dosing of liraglutide in rats and mice up to 36 times the exposure levels achieved in humans induces changes in pancreas morphology suggestive or diagnostic of pancreatitis. These studies were further supplemented by dosing liraglutide to nonhuman primates for 87 weeks. The current study evaluates the macroscopic and microscopic pancreatic findings in mice, rats, and nonhuman primates. Furthermore, pancreatic acinar cell proliferation was assessed in the pancreata from rats treated with liraglutide for 26 weeks, and specific histological evaluation for pancreatic intraepithelial neoplasia (PanINs) was performed in the high-dose group of nonhuman primates. This study is the first to report on lifelong dosing of normal animals with high doses of an incretin and should provide some insights into the potential risk for pancreatitis.

RESEARCH DESIGN AND METHODS

All animals were purpose bred and obtained from certified and approved breeders. All studies were carried out under good laboratory practice (15,16) and applicable national laws regarding the use of animals for biomedical research. Animals were housed in climate-controlled rooms under a 12-h light/dark cycle and fed standard laboratory animal diets for the respective species with free access to water. All animals were acclimatized for at least 7 days before dosing was initiated.

The studies presented below were part of the nonclinical development program for liraglutide supporting chronic administration in humans. The study duration ranged from 4 weeks to 2 years. The studies were conducted according to current International Conference of Harmonization guidelines. Pharmacological responsive species were studied, i.e., rats (Sprague-Dawley [CrI: CD(SD) IGS BR]) and nonhuman primates (Cynomolgus Monkey, *Macaca fascicularis*) were selected as the rodent and nonrodent species, respectively. CD-1 mice (CrI: CD-1(ICR)BR) were selected as the second rodent species for the 2-year carcinogenicity studies.

In rats, 4-, 13-, and 26-week repeated-dose toxicity studies were performed. Doses of 0, 0.1, 0.25, and 1 mg/kg/day were administered to groups of 10 males

From the ¹Department of Nonclinical Development Management, Novo Nordisk, Bagsværd, Denmark; the ²Department of Diabetes Toxicology and Safety Pharmacology, Novo Nordisk, Bagsværd, Denmark; the ³Department of Regulatory Affairs–New Diabetes and Obesity Projects, Novo Nordisk, Bagsværd, Denmark; and the ⁴Department of Diabetes and Pharmacology Management, Novo Nordisk, Bagsværd, Denmark.

Corresponding author: Lotte Bjerre Knudsen, lbkn@novonordisk.com.

Received 5 July 2011 and accepted 17 October 2011.

DOI: 10.2337/db11-0936

© 2012 by the American Diabetes Association. Readers may use this article as long as the work is properly cited, the use is educational and not for profit, and the work is not altered. See <http://creativecommons.org/licenses/by-nc-nd/3.0/> for details.

See accompanying articles, pp. 986, 989, and 1250.

and 10 females or 15 males and 15 females in the 26-week study. In mice, 4- and 13-week repeated-dose studies were performed. In the 4-week study, doses of 0, 0.1, 0.5, 1.0, and 5.0 mg/kg/day and in the 13-week study doses of 0, 0.2, 1.0, and 5.0 mg/kg/day were administered to groups of 10 males and 10 females. Dose selection for the 2-year carcinogenicity studies in rats and mice were discussed and agreed with the U.S. Food and Drug Administration Carcinogenicity Assessment Committee. In the 104-week rat study, doses of 0, 0.075, 0.25, and 0.75 mg/kg/day were administered to groups of 50 males and 50 females. In the 104-week mouse study, doses of 0, 0.03, 0.2, 1.0, and 3.0 mg/kg/day were subcutaneously administered to groups of 67–79 males and 67–79 females. In monkeys, 4-, 13-, 52-, and 87-week studies were performed. In the 4-, 13-, and 52-week studies, the doses were 0, 0.05, 0.5, and 5.0 mg/kg/day and in the 87-week study 0, 0.25, and 5.0 mg/kg/day. The group sizes were three males and three females in the 4-week study, four males and four females in the 13- and 26-week studies, and five males and five females in the 87-week study. In the 13-week study, an additional two males and two females were included as “recovery” animals in each of the control and high-dose groups. These animals had treatment discontinued for 2 weeks before necropsy.

Treatment. All animals received a single daily subcutaneous injection of liraglutide (Novo Nordisk). A 6 mg/mL liraglutide solution (Victoza) was diluted with the vehicle. The solutions were prepared weekly and stored at 2–8°C. Dose volume was 1–5 mL/kg, depending on the species. During treatment, all animals were observed daily by trained personnel for clinical signs of adverse effects, and once each week all animals received a detailed clinical examination, including appearance, movement, and behavior patterns; skin and hair condition; eyes and mucous membranes; respiration; and excreta. At the end of the treatment, mice and rats were killed by exposure to carbon dioxide. Nonhuman primates received an intravenous injection of an overdose of sodium pentobarbitone. Each animal was weighed and subsequently exsanguinated.

Evaluation of pancreas. Clinical signs recorded during the *in vivo* phase were reviewed at necropsy, and a macroscopic examination of the entire pancreas was performed in all animals. Any macroscopically abnormal pancreatic tissue was sampled and microscopically examined. The pancreata were fixed in 10% neutral buffered formalin for at least 48 h before they were trimmed, dehydrated, and paraffin embedded according to standard histological procedures. Sections at a nominal thickness of 4–5 μ m were sampled systematically as a homogeneous transverse section from the midpart of the pancreas representing the pancreatic body, stained with hematoxylin and eosin and examined under the light microscope. In the microscopic evaluation of rodent studies up to 26 weeks' duration, only the control and high-dose animals were examined. In all other studies of longer duration in rodents and in all nonhuman primate studies, all animals were examined microscopically. In total, pancreata from 1,483 animals were microscopically examined. The sections were evaluated by a trained toxicological pathologist. In accordance with standard practice and to increase the chance of identifying subtle differences between control and dosed animals, the sections were read unblinded to treatment (17–19). From each group and sex, at least 10% randomly selected sections were peer reviewed by another pathologist to confirm the diagnosis and to ensure consistency between studies. In addition, all pancreatic sections of mice dosed with liraglutide for 104 weeks were peer reviewed by a third toxicological pathologist. Internationally accepted diagnostic criteria were followed for nonneoplastic lesions, including inflammatory, regenerative, and degenerative conditions, as well as neoplastic lesions (20–23). In short, macroscopic pathological pancreatic findings were described according to visual features (e.g., size, color [dark, pale, reddened, or discolored], and consistency [soft or gelatinous]). Microscopically, pancreatitis is a severe and complex lesion and has been defined either as acute (focal or diffuse), chronic, or a mix of both. In the acute phase, varying degrees of necrosis, inflammation, infiltration, hemorrhage, and edema are present. Fat cell necrosis, mineralization, and arteritis also can be present. In the more chronic stage, granulomatous inflammation, fibrosis with loss of acinar tissue, and replacement by simplified glands or fat are observed. Islets often are unaffected until later stages of the condition. Focal inflammatory cell infiltration, which is a common background finding, is defined as a minimal to mild focal accumulation of inflammatory cells, often mononuclear cells. The histological findings were graded by the pathologist as either absent, minimal, mild, moderate, marked, or severe. Neoplastic lesions were classified as either benign or malignant.

Pancreata from all nonhuman primates in the high-dose group were investigated for PanINs to assess treatment-induced preneoplastic lesions (24,25). The criteria for PanINs are developed by the Pancreas Think Tank, sponsored by the National Cancer Institute, and involve the evaluation of small-caliber ducts instead of the main pancreatic duct (25). Cell proliferation in the pancreas in the 26-week rat study was qualitatively assessed in all control and high-dose group animals by proliferating cell nuclear antigen (PCNA) staining using a mouse anti-PCNA enhanced polymer one-step conjugate (code U7032; DakoCytomation, Ely, Cambridgeshire, U.K.).

Statistical analysis. In the rodent studies up to and including 26 weeks' duration and nonhuman primate studies, the incidence of the microscopic

changes seen in groups were compared with the control group by a Fisher exact test. Carcinogenicity studies are designed with the focus on overall survival (i.e., on providing a sufficient number of animals for a meaningful evaluation of any organ changes). As outlined by regulatory agencies, the number of animals to survive should preferably be at least 25 in each group and sex. During the 104-week mouse and rat studies, the incidence of all the nonneoplastic changes seen in the groups dosed with liraglutide was compared with the control group by a two-sided Fisher exact test. For neoplastic lesions, the data were analyzed using Peto time-adjusted methods, and the incidences in the groups dosed with liraglutide were compared with the control group. Each sex was analyzed separately, and in all tests a *P* value <0.05 was considered statistically significant.

RESULTS

Mouse and rat repeat-dose toxicity studies. Macroscopic examination of the pancreas did not reveal any signs of treatment-induced pancreatitis in the 4- and 13-week mouse studies or in the 4-, 13-, and 26-week rat studies. In the 4- and 13-week mouse studies, no histopathological abnormalities were detected in the high-dose animals. In the 4-week study in rats, one to two animals in the control and high-dose groups were diagnosed with focal minimal acinar cell atrophy and minimal basophilic focus. In the 13-week rat study, inflammatory cell foci of minimal grade were observed in the exocrine pancreata in four control group animals but in only one dosed animal in the treatment recovery group (i.e., formerly dosed with liraglutide). In the 26-week rat study, the incidence of animals with histological findings tended to increase, but there was no significant difference (*P* > 0.05) between dosed animals and control animals (Table 1). There were no histological signs of pancreatitis in any repeat-dose toxicity studies in rats. PCNA staining indicated cell proliferation within the normal range in animals treated with high doses of liraglutide, compared with animals dosed with the vehicle.

Mouse carcinogenicity study. At necropsy, no overt macroscopic signs of pancreatitis were noted in mice following 2 years of liraglutide exposure. The most frequent finding was a pale pancreas in female mice, with no difference between control and dosed animals. One low middle-dose liraglutide female mouse had pancreatic masses identified at necropsy as a result of the infiltration of malignant lymphoma cells. The microscopic examination of pancreata from all animals revealed pancreatitis in few animals in all dose groups except in the female control group (Table 2). Of these, two cases of pancreatitis were associated with either metastatic leiomyosarcoma or mesothelioma infiltrating the pancreas. There was no overall dose relationship between treatment groups. There was an equal distribution of the histological pancreatitis into focal, diffuse, and chronic (active) pancreatitis, with zero

TABLE 1
Incidences of exocrine pancreata histological findings in the 26-week rat toxicity study with liraglutide

Liraglutide dose (mg/kg/day)	Male rats		Female rats	
	0	1.0	0	1.0
Animals examined	15	15	15	15
Animals with no abnormalities detected	7	8	14	10
Microscopic pancreatitis	0	0	0	0
Acinar cell atrophy	2	5	0	2
Focal inflammation of exocrine pancreas	6	3	1	4
Inflammatory cell infiltration periductal	2	3	0	1
Eosinophilic focus	0	1	0	0

One animal may have more than one diagnosis.

TABLE 2

Incidences of microscopically identified pancreatitis in mice and rats dosed with liraglutide for 2 years and monkeys dosed for 87 weeks

	Male					Female				
Mice										
Liraglutide (mg/kg/day)	0	0.03	0.2	1.0	3.0	0	0.03	0.2	1.0	3.0
Animals examined	79	67	67	67	79	79	67	66	66	76
Animals with microscopic pancreatitis	3	2	1	1	1	0	3	3	3	3
Focal pancreatitis										
Minimal	1	0	0	0	0	0	0	1	0	1
Mild	0	1	1	0	0	0	1	0	1	0
Diffuse pancreatitis										
Minimal	0	1	0	0	0	0	0	0	0	1
Mild	2	0	0	0	0	0	0	1	1	0
Moderate	0	0	0	0	0	0	1	0	1*	0
Marked	0	0	0	0	0	0	0	0	0	1
Chronic/chronic-active										
Minimal	0	0	0	0	0	0	1	0	0	0
Mild	0	0	0	0	0	0	0	1	0	0
Moderate	0	0	0	1†	1	0	0	0	0	0
Rat										
Liraglutide (mg/kg/day)	0	0.075	0.25	0.75		0	0.075	0.25	0.75	
Animals examined	50	50	49	50		50	50	50	50	
Animals with microscopic pancreatitis	0	0	0	0		0	0	0	0	
Monkey										
Group (mg/kg/day)	0	0.25	5			0	0.25	5		
Animals examined	5	5	5			5	4	5		
Animals with microscopic pancreatitis	0	0	0			0	0	0		

The doses of liraglutide correspond to 0.2-, 1.6-, 13-, and 36-fold the maximum recommended human dose at 1.8 mg/day based on area under the curve (0–24 h) for mice; 0.5-, 2.4-, and 8.1-fold for rats; and 9- and 63-fold for monkeys. No animals had macroscopically identified pancreatitis. The histological diagnosis of pancreatitis was categorized as focal or diffuse, as chronic or chronic-active, and graded into minimal, mild, moderate, or marked based on the severity. *One female mouse had histological-verified pancreatitis associated with a metastatic leiomyosarcoma originating from the cecum. †One male mouse had histological-verified pancreatitis associated with a mesothelioma infiltrating the pancreas.

to two animals in each category. Figure 1 shows the histological features of a normal exocrine pancreas, a pancreas with minimal inflammatory cell infiltration, and mild or moderate pancreatitis, in both the control and liraglutide-dosed animals, respectively. The histological examination of the exocrine and endocrine pancreata revealed several common histological changes (Table 3). The most frequent diagnoses were mild to moderate focal inflammatory cell infiltration, diffuse edema, acinar cell degeneration, and vacuolation. There was no significant difference in incidences between groups in either sex ($P > 0.05$). A few animals in all groups had islet cell hyperplasia with no significant difference in incidences between treatment groups in either sex ($P > 0.05$). Two male mice, one control animal and one low middle-dose animal, had islet adenoma. No islet cell carcinomas were observed.

Rat carcinogenicity study. Necropsy findings were few, and the most frequent finding was pale pancreas. No macroscopic or microscopic signs of pancreatitis were noted in rats dosed for 2 years with liraglutide (Table 2). The histological examination of the pancreas for neoplastic and nonneoplastic histological changes (Table 4) showed several common findings in the exocrine pancreas, with no evidence of treatment-related effects ($P > 0.05$). The most common finding was that of pancreatic atrophy being observed at a higher rate in males than females but with no significant difference between groups in either sex ($P > 0.05$). Additional examination of the endocrine pancreas did not show any treatment-related effects concerning islet cell hyperplasia and benign adenomas.

Nonhuman primate repeat-dose toxicity studies. There were no macroscopic observations compatible with pancreatitis in the 4-, 13-, and 52-week studies. There were no microscopic signs of inflammatory responses in the exocrine pancreas in the 4-week study. In the 13-week study, minimal focal inflammatory cell infiltration in the exocrine pancreas was seen in one control female and one high-dose liraglutide male (of six monkeys at each dose level and sex). After a recovery period of 2 weeks following the 13-week treatment, minimal inflammatory cell foci were noted in one high-dose recovery male monkey. In the 52-week study, minimal focal inflammatory cell infiltration of the exocrine pancreas was seen in one male in the high-dose group, one female in the low-dose group, and in one control male monkey. There were no histological signs of pancreatitis or signs of proliferative or preneoplastic lesions in the exocrine ductal epithelium (PanINs) in nonhuman primates dosed for up to 52 weeks. In the 87-week nonhuman primate study, there were no histological signs of pancreatitis (Table 2). Focal inflammatory cell infiltration in the pancreas was observed in a few animals in the high-liraglutide dose group (Table 5). No proliferative or preneoplastic lesions were found in exocrine ductal epithelium (PanINs), when examining all the high-dose group animals.

DISCUSSION

The diagnosis of pancreatitis in humans relies on clinical symptoms of severe abdominal pain and three key laboratory findings: 1) increased amylase, 2) increased lipase,

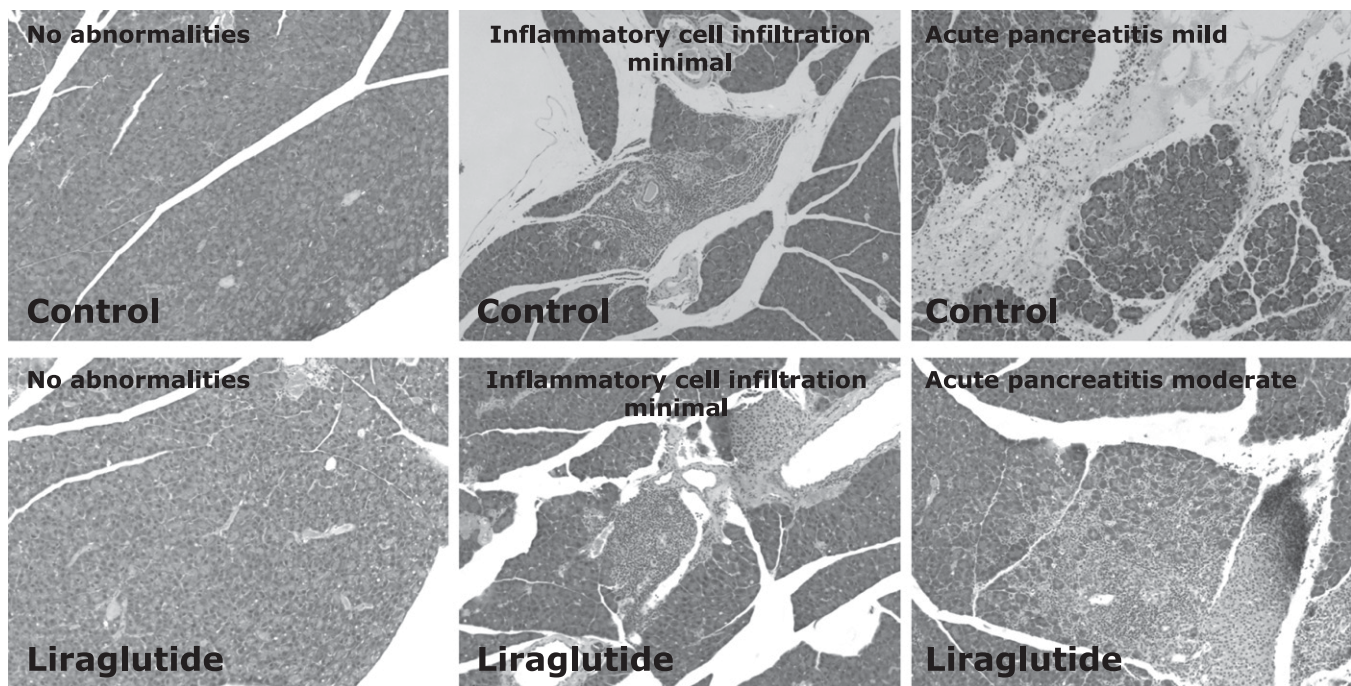


FIG. 1. Photomicrographs of exocrine pancreata sections obtained from male control mice (*upper panel*) and male high-dose liraglutide mice (*lower panel*; 3 mg/kg/day dose), from the 2-year carcinogenicity study in mice. The *left column* shows pancreata with no abnormal findings. The *middle column* shows pancreata with minimal inflammatory cell infiltration. The *right column* shows pancreata with acute pancreatitis. Magnification is $\times 10$ objective.

and 3) radiographic findings showing an edematous pancreas (26). Macroscopic examination of an acute inflamed pancreas in humans reveals edematous areas with hemorrhage and necrosis (27). Repeated attacks of acute pancreatitis eventually leads to fibrous scarring, thinning of the exocrine glands that makes the pancreas look smaller and fibrous at necropsy (28,29). In principle, the diagnosis of pancreatitis in animals relies on the same diagnostic criteria as that in humans (i.e., on clinical observations suggesting abdominal pain or discomfort and increases in amylases and lipase). In long-term dosing studies such as those reported here, pancreatitis in animals may be diagnosed when the animal is killed for animal welfare reasons, when the animal dies during the study, or when the animal is killed as scheduled at the end of the experimental phase and subjected to necropsy and microscopic examination of the pancreas (29).

No signs of pancreatitis, acute or chronic, were noted by the trained personnel performing the macroscopic examination of the animals. In the mouse carcinogenicity study, in which the animals were treated for most of their lifetime, 20 animals were diagnosed with pancreatitis following microscopic examination. These animals were equally distributed between treatment groups. Eight of these animals were killed as scheduled and 12 were prematurely killed or found dead, and in none of these was pancreatitis given as cause of death. Peritonitis was ascribed as the cause of death in two animals, and this could have resulted from acute pancreatitis. However, the microscopic evaluation revealed pathological changes of such low magnitude that pancreatitis could not be assumed to be the cause of death. One previously published study evaluated pancreatitis in mice dosed with liraglutide and exenatide. This study showed that both exenatide and liraglutide increased anti-inflammatory signals in the pancreas, PAP, and RegIIIa. The article suggests that PAP could be a protective signal for the

pancreas (6). The article also reports testing exenatide in a model of pancreatitis, induced with the cholecystokinin (CCK) agonist cerulein. Exenatide did not worsen symptoms and did not increase amylase levels more than cerulein alone. Again, PAP was increased. The most important differences between the previously published studies and ours are the number of animals, as well as our use of diagnostic criteria in accordance with current internationally accepted guidelines (20–25) and confirmation of the diagnosis by peer review. In our studies, although there were a variety of additional findings in the exocrine and endocrine pancreata in the mice treated for 104 weeks with liraglutide, most of these were infrequent and inconsistent. There was a small increase in a few findings in the highest-dose group with liraglutide (e.g., diffuse edema, inflammatory cell infiltration, and vacuolation) (Table 3). The incidences were, however, not statistically significantly different from those observed in the control group, and, furthermore, the exposure levels in the mouse at this dose level is 36-fold higher than that in humans at the maximal recommended dose level of liraglutide of 1.8 mg/day. Our data show that acute pancreatitis does occur in both control and liraglutide-dosed mice but in very few animals. Also, minimal to mild focal inflammatory cell infiltration is a common finding in several organs in rodents and is not related to pancreatitis (29).

The literature on GLP-1 reports a possible effect on ductal cell differentiation into an endocrine phenotype (30), and this has contributed to the discussion that there may be undesirable effects of GLP-1 on ductal cells (31). In our 26-week rat study, proliferation seemed to be similar in control and dosed animals. Thus, liraglutide did not seem to change cellular proliferation in the ductal or acinar epithelium of the exocrine pancreas. Our rat data are in contrast to a study that reported on exenatide (5). That study reports an increase in lipase levels, acinar inflammation, and number of

TABLE 3

Incidences of all neoplastic and nonneoplastic histological findings in the exocrine and endocrine pancreata in the 2-year mouse study, other than pancreatitis

Liraglutide (mg/kg/day)	Male mice					Female mice				
	0	0.03	0.2	1.0	3.0	0	0.03	0.2	1.0	3.0
Exocrine pancreas										
Animals examined	79	67	67	67	79	79	67	66	66	76
Animals with no abnormalities detected	60	52	54	53	56	48	35	41	34	36
Exocrine pancreas										
Mesothelioma (malign), metastasizing	0	0	0	1	0	0	0	0	0	0
Hemangioma (benign)	0	0	0	0	0	1	0	0	0	0
Metastasis from primary tumor in skin	0	0	0	0	0	0	0	1	0	0
Metastasis from primary tumor in alimentary tract	0	0	0	0	0	0	0	0	1	0
Metastasis from primary tumor in muscle	0	0	0	0	0	0	0	0	0	1
Infiltration by lymphoma cells	4	3	0	4	5	11	4	4	8	11
Infiltration by leukemia cells	1	1	0	0	0	0	0	1	0	0
Infiltration by histiocytic sarcoma	0	0	0	0	0	3	2	1	2	1
Peritonitis	1	1	0	0	0	1	1	0	0	2
Arteritis/periarteritis	0	0	0	0	0	0	2	0	0	0
Fat necrosis	0	0	0	0	0	0	1	0	0	0
Inflammatory cell infiltration	2	4	3	3	9	4	8	7	3	9
Pigmented macrophages with focal inflammation	0	0	0	0	0	0	0	0	1	0
Diffuse edema	2	3	2	1	6	10	13	9	13	5
Acinar cell hypertrophy	1	1	1	1	1	2	1	1	0	3
Focal basophilic alteration	1	0	0	0	0	0	0	0	0	0
Acinar cell degranulation	2	2	2	3	0	4	2	0	2	8
Focal vacuolar degeneration	0	0	0	0	0	0	0	0	1	0
Vacuolation	2	1	2	1	4	2	0	3	4	1
Focal mineralization	0	0	1	0	0	0	0	0	0	0
Atrophy	0	0	1	0	1	4	0	1	1	0
Duct ectasia	0	0	0	0	0	0	0	1	0	0
Hemopoiesis, extramedullary, mesenteric	0	0	0	0	0	0	0	0	1	0
Endocrine pancreas										
Animals examined	79	67	66	67	78	78	64	65	66	74
Animals with no abnormalities detected	74	65	63	66	75	73	61	64	60	71
Islet cell adenoma (benign)	1	0	1	0	0	0	0	0	0	0
Infiltration by lymphoma cells	2	0	0	0	0	2	0	1	3	1
Islet cell hyperplasia	2	2	2	1	3	3	3	0	3	2

The incidence of pancreatitis is shown in Table 2. One animal may have more than one diagnosis.

pyknotic nuclei in 10 male and 10 female rats treated for 75 days with exendin-4. No similar findings were made in our rat studies, even with much longer duration, very high doses, and a considerably higher number of animals. We did find pathological changes (e.g., acinar cell atrophy) (Table 4). However, this was common in the control groups as well and was not related to the dose of liraglutide. None of the other sporadic findings are associated to pancreatitis or early stages hereof. Also, literature using acinar cells or cell lines with a good amylase secretion response to CCK-8 has shown only a very small potentiation by exendin-4 on CCK-8-induced amylase release (32) and no effect of GLP-1 on either amylase release alone or potentiation of CCK-8-induced release (33,34). Our data confirm this lack of a direct adverse effect of GLP-1 on acinar cells.

The current studies were conducted before the potential association between GLP-1 agonists and pancreatitis in humans was described; therefore, we do not have data on plasma markers of pancreatitis. However, a recent article on exenatide, reporting on data in numerous diabetic animal models with up to 4 weeks of dosing, shows no increases in amylase, lipase, or triglyceride plasma levels (35). One additional study has been quoted as showing evidence of GLP-1-induced pancreatitis in rodents. The actual study uses the human islet amyloid polypeptide transgenic rat and

reports pancreatitis in one of eight animals dosed with the dipeptidyl peptidase-4 inhibitor sitagliptin, as well as an increase in ductal metaplasia and proliferation. Although dipeptidyl peptidase-4 inhibitors inhibit degradation of endogenous GLP-1 in humans, the data from animals are much less clear, and the study does not include measurements of GLP-1 levels following sitagliptin dosing, nor does it include data on a GLP-1 analog (36). The commentary that accompanied the publication of the article suggested a concern that long-term, asymptomatic pancreatitis may lead to pancreatic cancer (31). Although such a concern is valid, because of the lack of long-term human studies, our studies do not support a direct link between pancreatitis or pancreatic cancer and GLP-1 receptor activation, even with continuous exposure to high levels of a human GLP-1 analog.

In the nonhuman primates, focal inflammatory cell infiltration was seen. This inflammatory cell infiltration, a common finding in nonhuman primates, consisted of small clusters of a few mononuclear cells surrounded by gland cells with normal appearance, and they had no histological resemblance to pancreatitis (37). Improved diagnostic classification of the early stages of pancreatic cancer has been developed over the last decades (24,25). Using this histological classification system, reevaluation of pancreata sections from the high-dose group of nonhuman primates

TABLE 4
Incidences of all neoplastic and nonneoplastic histological findings in the 2-year rat study, in the exocrine and endocrine pancreas

Liraglutide dose (mg/kg/day)	Male rats				Female rats			
	0	0.075	0.25	0.75	0	0.075	0.25	0.75
Exocrine pancreas								
Animals examined	50	50	49	50	50	50	50	50
Animals with no abnormalities detected	32	26	30	36	46	41	39	42
Acinar cell adenoma (benign)	1	1	0	0	0	0	0	0
Focal acinar cell hypertrophy	2	4	1	1	0	0	2	0
Atrophy	13	20	19	12	4	8	8	7
Periarteritis	1	0	0	0	0	1	0	0
Lymphocytic infiltration	0	0	0	0	0	1	0	0
Infiltration by lymphoma cells	0	0	1	0	0	0	1	0
Infiltration by histiocytic sarcoma	0	0	0	0	0	0	0	1
Infiltration by leukemia cells	1	0	0	0	0	0	0	0
Diffuse acinar cell atrophy	0	0	0	1	0	0	0	0
Pigment deposits	1	0	0	0	0	0	0	0
Endocrine pancreas								
Animals examined	50	50	49	50	50	50	50	49
Animals with no abnormalities detected	44	49	44	46	50	50	50	48
Islet cell adenoma (benign)	6	1	2	1	0	0	0	1
Mixed acinar/islet cell adenoma (benign)	0	0	0	1	0	0	0	0
Islet cell hyperplasia, focal	0	0	3	2	0	0	0	0

One animal may have more than one diagnosis.

dosed for 87 weeks revealed no indication of early pancreatic intraductal proliferation at exposures of liraglutide higher than 60-fold that in humans.

Our studies underline several important parameters to take into consideration when designing studies to look for histological diagnosis of rare findings such as pancreatitis. The number of animals has to be very large, and the use of trained pathologists using classified diagnostic criteria to ensure the consistency of the findings is important. When a novel drug has to be approved for use in diabetes, lifetime (2-year) carcinogenicity studies in rodents have to be conducted. Such studies are designed with a focus on securing the overall survival of preferably at least 25 animals per group per sex through the 2-year dosing period. Even with 67–79 mice in each group per sex, we only found pancreatitis in a maximum of 3 mice in each group per sex. In group sizes of 50 rats, no animals were found to have pancreatitis. With such low incidences, statistical analyses often may not have sufficient power to show a statistically

significant outcome. For example, in female mice, in which the incidence in the control animals was 0 of 79, an incidence of 6 of 79 would be needed to result in a *P* value of <0.05. Therefore, it is important to evaluate the findings qualitatively if there are dose-dependent effects, even if they are not statistically significant. Dose dependency is a strong indicator of a true drug-induced adverse effect. However, no such effects were seen in the present studies.

In conclusion, animal safety studies are used as a tool to identify target organs and potential signals of special concern for human safety. On the other hand, studies in animals can never rule out adverse findings in humans. Potential limitations to our studies are that we used only healthy, nondiabetic animals and that none of the common risk factors for pancreatitis in humans were present. Nevertheless, our studies do support that a simple pharmacological induction of pancreatitis by liraglutide is unlikely.

TABLE 5
Incidences of exocrine and endocrine pancreatic nonneoplastic histological changes in the 87-week monkey study

Liraglutide dose (mg/kg/day)	Male monkeys			Female monkeys		
	0	0.25	5	0	0.25	5
Exocrine pancreas						
Animals examined	5	5	5	5	4	5
Animals with no abnormalities detected	5	5	2	5	4	4
PanINs in the ductal epithelium	NE	NE	0	NE	NE	0
Minimal focal lobular atrophy	0	0	1	0	0	1
Minimal perivascular inflammatory cell infiltration foci	0	0	1	0	0	0
Minimal ductal inflammatory cell infiltration foci	0	0	1	0	0	0
Minimal parachymal inflammatory cell foci	0	0	1	0	0	0
Endocrine pancreas						
Animals examined	5	5	5	5	4	5
Animals with no abnormalities detected	4	5	5	5	4	5
Mild focal hemagiectasis in islets	1	0	0	0	0	0

NE, not examined.

ACKNOWLEDGMENTS

All authors are full-time employees of Novo Nordisk, which developed liraglutide for the treatment of diabetes. All authors hold minor stock portions as part of an employee offering program. No other potential conflicts of interest relevant to this article were reported.

N.C.B.N. took part in drawing conclusions from the results of the study and wrote parts of the RESEARCH DESIGN AND METHODS and RESULTS sections. A.-M.M. took part in drawing conclusions from the results of the study, wrote parts of the RESEARCH DESIGN AND METHODS and RESULTS sections, and collected and reviewed the study data. L.W.M. took part in drawing conclusions from the results of the study. L.B.K. took part in drawing conclusions from the results of the study and wrote the abstract and the major part of the DISCUSSION. L.B.K. is the guarantor of the article and takes full responsibility for the results and the conclusions. All authors took part in reviewing the manuscript.

Parts of this study were presented in abstract form at the 70th Scientific Sessions of the American Diabetes Association, Orlando, Florida, 25–29 June 2010, and at the 46th Annual Meeting of the European Association for the Study of Diabetes, Stockholm, Sweden, 20–24 September 2010.

REFERENCES

- Drucker DJ, Sherman SI, Gorelick FS, Bergenstal RM, Sherwin RS, Buse JB. Incretin-based therapies for the treatment of type 2 diabetes: evaluation of the risks and benefits. *Diabetes Care* 2010;33:428–433
- Denker PS, Dimarco PE. Exenatide (exendin-4)-induced pancreatitis: a case report. *Diabetes Care* 2006;29:471
- Ahmad SR, Swann J. Exenatide and rare adverse events. *N Engl J Med* 2008;358:1970–1971; discussion 1971–1972
- Dore DD, Seeger JD, Arnold Chan K. Use of a claims-based active drug safety surveillance system to assess the risk of acute pancreatitis with exenatide or sitagliptin compared to metformin or glyburide. *Curr Med Res Opin* 2009;25:1019–1027
- Nachnani JS, Bulchandani DG, Nookala A, et al. Biochemical and histological effects of exendin-4 (exenatide) on the rat pancreas. *Diabetologia* 2010;53:153–159
- Koehler JA, Baggio LL, Lamont BJ, Ali S, Drucker DJ. Glucagon-like peptide-1 receptor activation modulates pancreatitis-associated gene expression but does not modify the susceptibility to experimental pancreatitis in mice. *Diabetes* 2009;58:2148–2161
- Knudsen LB, Nielsen PF, Huusfeldt PO, et al. Potent derivatives of glucagon-like peptide-1 with pharmacokinetic properties suitable for once daily administration. *J Med Chem* 2000;43:1664–1669
- Drucker DJ, Dritselis A, Kirkpatrick P. Liraglutide. *Nat Rev Drug Discov* 2010;9:267–268
- Marre M, Shaw J, Brändle M, et al.; LEAD-1 SU Study Group. Liraglutide, a once-daily human GLP-1 analogue, added to a sulphonylurea over 26 weeks produces greater improvements in glycaemic and weight control compared with adding rosiglitazone or placebo in subjects with type 2 diabetes (LEAD-1 SU). *Diabet Med* 2009;26:268–278
- Nauck M, Frid A, Hermansen K, et al.; LEAD-2 Study Group. Efficacy and safety comparison of liraglutide, glimepiride, and placebo, all in combination with metformin, in type 2 diabetes: the LEAD (liraglutide effect and action in diabetes)-2 study. *Diabetes Care* 2009;32:84–90
- Garber A, Henry R, Ratner R, et al.; LEAD-3 (Mono) Study Group. Liraglutide versus glimepiride monotherapy for type 2 diabetes (LEAD-3 Mono): a randomised, 52-week, phase III, double-blind, parallel-treatment trial. *Lancet* 2009; 373:473–481 [North American Edition]
- Zinman B, Gerich J, Buse JB, et al. Efficacy and safety of the human glucagon-like peptide-1 analog liraglutide in combination with metformin and thiazolidinedione in patients with type 2 diabetes (LEAD-4 Met+TZD). *Diabetes Care* 2009;32:1224–1230 [Erratum in *Diabetes Care* 2010;33:692]
- Russell-Jones D, Vaag A, Schmitz O, et al.; Liraglutide Effect and Action in Diabetes 5 (LEAD-5) Met+SU Study Group. Liraglutide vs insulin glargine and placebo in combination with metformin and sulphonylurea therapy in type 2 diabetes mellitus (LEAD-5 met+SU): a randomised controlled trial. *Diabetologia* 2009;52:2046–2055
- Buse JB, Rosenstock J, Sesti G, et al.; LEAD-6 Study Group. Liraglutide once a day versus exenatide twice a day for type 2 diabetes: a 26-week randomised, parallel-group, multinational, open-label trial (LEAD-6). *Lancet* 2009;374:39–47
- Organisation for Economic Co-operation and Development. OECD series on principles of good laboratory practice and compliance monitoring [article online], 1998. Available from [http://www.oecd.org/officialdocuments/displaydocumentpdf/?cote=env/mc/chem\(98\)17&doclanguage=en](http://www.oecd.org/officialdocuments/displaydocumentpdf/?cote=env/mc/chem(98)17&doclanguage=en). Accessed 1 June 2011
- Medicines and Healthcare Products Regulatory Agency. Guide to UK GLP regulations [article online], 1999. Available from <http://www.mhra.gov.uk/howweregulate/medicines/inspectionandstandards/goodlaboratorypractice/guidance/index.htm#11>. Accessed 1 June 2011
- Crissman JW, Goodman DG, Hildebrandt PK, et al. Best practices guideline: toxicologic histopathology. *Toxicol Pathol* 2004;32:126–131
- Faccini JM, Butler WR, Friedmann JC, et al. IFSTP guidelines for the design and interpretation of the chronic rodent carcinogenicity bioassay. *Exp Toxicol Pathol* 1992;44:443–456
- Holland T, Holland C. Unbiased histological examinations in toxicological experiments (or, the informed leading the blinded examination). *Toxicol Pathol* 2011;39:711–714
- Eustis SL, Boorman GA, Hayashi Y. Pathology of the Fisher rat: reference and atlas. In *Exocrine Pancreas*. Boorman GA, Eustis SL, Elwell MR, Montgomery JR, Eds. San Diego, CA, Academic Press, 1990, p. 95–108
- Hansen JF, Ross PE, Makovec GT, Eustis SL, Sigler RE. Proliferative and other selected lesions of the exocrine pancreas in rats. In *Guides for Toxicologic Pathology*. Vol. GI-6. Washington, DC, Society of Toxicologic Pathologist, American Registry of Pathology, and Armed Forces Institute of Pathology, 1995, p. 2–7
- Mohr U (Ed.). *International Classification of Rodent Tumours: Part 1: The Rat Fascicle No. 10: Digestive System*. Lyon, France, International Agency for Research on Cancer, 1997, p. 85–91
- Denda A, Tsutsumi M, Konishi Y. *Exocrine Pancreas in Pathobiology of the Aging Rat*. Vol 2. Mohr U, Dungworth DL, Capen CC, Eds. Washington, DC, ILSI Press, 1994, p. 351–360
- Koorstra JB, Feldmann G, Habbe N, Maitra A. Morphogenesis of pancreatic cancer: role of pancreatic intraepithelial neoplasia (PanINs). *Langenbecks Arch Surg* 2008;393:561–570
- Hruban RH, Adsay NV, Albores-Saavedra J, et al. Pancreatic intraepithelial neoplasia: a new nomenclature and classification system for pancreatic duct lesions. *Am J Surg Pathol* 2001;25:579–586
- Working Party of the British Society of Gastroenterology; Association of Surgeons of Great Britain and Ireland; Pancreatic Society of Great Britain and Ireland; Association of Upper GI Surgeons of Great Britain and Ireland. UK guidelines for the management of acute pancreatitis. *Gut* 2005;54 (Suppl. 3):iii1–iii9
- Imrie CW. Acute pancreatitis: section 14.18.3.1. In *Oxford Textbook of Medicines*. Vol. 2, 4th ed. Warrel DA, Cox TM, Firth JD, Benz EJ, Eds. Oxford, U.K., Oxford University Press, 2003, p. 679–687
- Imrie CW. Chronic pancreatitis: section 14.18.3.2. In *Oxford Textbook of Medicines*. Vol. 2, 4th ed. Warrel DA, Cox TM, Firth JD, Benz EJ, Eds. Oxford, U.K., Oxford University Press, 2003, p. 687–696
- Greaves P. *Histopathology of preclinical toxicity Studies*. 3rd ed. Greaves P, Ed. Amsterdam, NL, Academic Press, Elsevier, 2007, p. 515–527
- Xu G, Kaneto H, Lopez-Avalos MD, Weir GC, Bonner-Weir S. GLP-1/exendin-4 facilitates beta-cell neogenesis in rat and human pancreatic ducts. *Diabetes Res Clin Pract* 2006;73:107–110
- Butler PC, Matveyenko AV, Dry S, Bhushan A, Elashoff R. Glucagon-like peptide-1 therapy and the exocrine pancreas: innocent bystander or friendly fire? *Diabetologia* 2010;53:1–6
- Malhotra R, Singh L, Eng J, Raufman JP. Exendin-4, a new peptide from Heloderma suspectum venom, potentiates cholecystokinin-induced amylase release from rat pancreatic acini. *Regul Pept* 1992;41:149–156
- Fehmamm HC, Göke B, Weber V, et al. Interaction of glucagon-like peptide-1 (7–36)amide and cholecystokinin-8 in the endocrine and exocrine rat pancreas. *Pancreas* 1990;5:361–365
- Zhou J, Montrose-Rafizadeh C, Janczewski AM, et al. Glucagon-like peptide-1 does not mediate amylase release from AR42J cells. *J Cell Physiol* 1999;181: 470–478
- Tatarkiewicz K, Smith PA, Sablan EJ, et al. Exenatide does not evoke pancreatitis and attenuates chemically induced pancreatitis in normal and diabetic rodents. *Am J Physiol Endocrinol Metab* 2010;299:E1076–E1086
- Matveyenko AV, Dry S, Cox HI, et al. Beneficial endocrine but adverse exocrine effects of sitagliptin in the human islet amyloid polypeptide transgenic rat model of type 2 diabetes: interactions with metformin. *Diabetes* 2009;58:1604–1615
- McClure HM, Chandler FW. A survey of pancreatic lesions in nonhuman primates. *Vet Pathol Suppl* 1982;19(Suppl. 7):193–209