The effects of 13 weeks of liraglutide treatment on endocrine and exocrine pancreas in male and female ZDF-rats
– A quantitative and qualitative analysis revealing no evidence of drug induced pancreatitis

Running head: Liraglutide does not induce pancreatitis in the ZDF rat


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Abbreviations

BID  Twice daily  
cAMP  Cyclic adenosine monophosphate  
CCK8  Cholecystokinin 8  
CK20  Cytokeratin 20  
DAB  Diaminobenzidine 4%  
DPP-IV  Dipeptidyl peptidase IV  
FDA  Food and Drug administration  
GLP-1  Glucagon like peptide 1  
HRP  Horse radish peroxidase  
P-amylase  Pancreatic amylase  
QD  Once daily  
TBS-T  Tris phosphate buffer + tween 20  
ZDF  Zucker diabetic fatty rat  

Disclosures:

LS, IT, HS and LBKN are full time employees of Novo Nordisk who has developed liraglutide for the treatment of diabetes. All these authors hold minor stock portions, as part of an employee offering programme. JJ and NV are founders and owners of gubra—a contract research organization working with diabetes and obesity research. AEJ is a full time employee at gubra with no ownership. NV is a consultant on a scientific committee at Novo Nordisk. JJ, NV and AEJ have no stock portions in Novo Nordisk.
Author Contributions

NV, JJ, LS, LBKn designed the study. NV was responsible for the in vivo phase. JJ was responsible for the stereology. LS was overall responsible for data quality. IT, HS designed and was responsible for the histopathology. NV, LS, LBKn wrote the paper.
Abstract

Background and Aim A possible association between glucagon-like peptide-1 (GLP-1) analogues and incidences of pancreatitis has been suggested based on clinical studies. In male and female diabetic Zucker diabetic fatty (ZDF) rats we investigated the effects of continuous administration of liraglutide and exenatide on biochemical (lipase, pancreatic amylase (P-amylase)) and histopathological markers of pancreatitis.

Methods 13 weeks of liraglutide (0.4mg/kg/day or 1.0mg/kg/day, s.c. once daily) or exenatide (0.25mg/kg/day, s,c, Alzet osmotic mini-pumps) treatment to male and female ZDF rats. P-amylase and lipase plasma activity was measured, and an extended histopathological and stereological (specific cell-mass and proliferation rate) evaluation of the exocrine and the endocrine pancreas performed.

Results Expectedly, liraglutide and exenatide lowered blood glucose and HbA1c in male and female ZDF rats, while beta cell mass and proliferation rate was increased with greatly improved blood glucose control. Whereas neither analogue affected lipase activity, small increases in P-amylase activity were observed in animals treated with liraglutide and exenatide. However, concurrent or permanent increases in lipase and P-amylase activity were never observed. Triglycerides were lowered by both GLP-1 analogues. The qualitative histopathological findings did not reveal adverse effects of liraglutide. The findings were mainly minimal in severity and focal in distribution. Similarly, the quantitative stereological analyses revealed no effects of liraglutide and exenatide on overall pancreas weight, or exocrine and duct cell mass or proliferation.

Conclusions The present study demonstrates that in overtly diabetic male and female ZDF-rats prolonged exposure to GLP-1 receptor agonists does not affect biochemical
or histopathological markers of pancreatitis and while both exenatide and liraglutide
increases beta-cell mass they have no effect on the exocrine pancreas. However,
clinical outcome studies and studies using primate tissues and/or studies in non-
human primates are needed to further assess human risk.

**Keywords** GLP-1 receptor agonists; exenatide; beta-cell mass; stereology
**Introduction**

Glucagon-like-peptide 1 (GLP-1) receptor agonists are emerging as an important drug class for the treatment of type 2 diabetes (15). While the GLP-1 receptor agonists are generally well tolerated (8, 16), post-marketing case reports of possible exenatide associated acute pancreatitis (5-6, 12, 44) led the FDA to issue post-marketing safety warnings to exenatide and liraglutide, and the DPP-IV inhibitor sitagliptin (1-3).

Because of the low number of documented pancreatitis events even in epidemiological studies it has been difficult to confirm a causal relationship between exenatide treatment and pancreatitis.

Acute pancreatitis is a clinical condition with a variable severity ranging from mild to life-threatening. The diagnosis is based on a number of clinical signs and laboratory findings, where abdominal pain coupled with increases in lipase and pancreatic amylase (P-amylase) are among the most common findings (21). It is believed that the pancreatic acinar cells play an important role in the pathogenesis of acute pancreatitis and that auto-digestion and efflux of digestive enzymes into the interstitial space are the primary events leading to exaggerated inflammation, oedema and necrosis (27).

Recent epidemiological studies have demonstrated that while both obesity and diabetes increase the risk of acute pancreatitis (9, 22) exenatide treatment does not appear to increase the risk of acute pancreatitis in these patient populations(13-14, 22). However, a paper analyzing the FDA AERS database showed a significantly increased incidence of pancreatitis with exenatide and sitagliptin (17). It should be noted that the individual reports in the database are unvalidated.
Preclinical studies have indicated the presence of a functional GLP-1 receptor on acinar cells (19, 30, 36, 38), and on pancreatic ductal cells (45-46). Koehler and co-workers (28) demonstrated an increased pancreas weight but also increased expression of pancreatic anti-inflammatory proteins in mice treated with exendin-4 or liraglutide, hereby indicating a GLP-1 receptor mediated proliferation of the pancreas. No effects of exendin-4 on chemically (caerulein) induced pancreatitis was observed (28), in agreement with other recent studies (42). In contrast to these studies chronic treatment with exendin-4 has been reported to increase the signs of pancreatitis in rats with increased scores of inflammation and pyknotic nuclei, and increased lipase (but not amylase) (33). Similarly, it has been suggested that elevated GLP-1 levels were responsible for sitagliptin associated ductal metaplasia and increased duct cell proliferation in a transgenic rat model of type 2 diabetes (32). However, in the latter study treatment with a GLP-1 receptor agonist was not investigated, nor were plasma levels of GLP-1 measured (32).

Since GLP-1 receptor mediated beta-cell neogenesis has been observed in diabetic rodents (45-46) and since pancreatitis is more frequently encountered in diabetic than in healthy humans we used the diabetic ZDF rat as a model to uncover potential side-effects of exenatide and liraglutide. Further, while a number of papers have used stereological methods to quantify changes in endocrine pancreas mass (beta/non-beta cell mass) following GLP-1 receptor stimulation (23, 37, 40), none of the available papers investigating effects of GLP-1 based drugs on the exocrine pancreas have used stereological quantification (28, 32-33, 42). We therefore applied a combination of histopathological and stereological methods to describe not only qualitative but also
quantitative changes in the endocrine and exocrine pancreas following 13 weeks
dosing with exenatide or liraglutide to diabetic ZDF rats.

Methods

Animals

144 obese fa/fa (72 males, 72 females) and 24 lean Fa/? (12 males, 12 females) ZDF
rats were purchased from Charles River, Germany. Female rats (5-7 weeks of age at
arrival) and male rats (6-7 weeks on arrival) were housed 2 per cage, at target
temperature 22 ± 2°C, relative humidity 50 ± 10%, light/dark cycle: 12/12. Animals
were provided with bedding, nesting and a hide for environmental enrichment.
Animals had free access to diet and tap water. Male rats were fed Purina 5008
(Brogaarden, Denmark) during the entire study period, while female rats were fed the
high-fat diet RD12468 (Research Diets, New Jersey, USA) for 6 weeks, and then
switched to Purina 5008 for the remainder of the study. All animal experiments were
conducted in accordance with the internationally accepted Principles of Laboratory
Animal Care (NIH publication no. 85–23, revised 1985), and in compliance with
personal animal licenses for Jacob Jelsing (2008/561-1565) issued by the Danish
Committee for Animal Research.

Compounds

6mg/ml liraglutide (Victoza®, Novo Nordisk, Denmark) was diluted in isotonic
buffered solution. Exenatide mini-pump phase dose formulations were prepared by
dissolving exendin-4 (Bachem, Switzerland) in sodium acetate buffer, pH 4.5 (50mM
sodium acetate, 0.05% tween 80, 2% glycerol). During the subcutaneous dose-
titration period exenatide dose formulations were diluted in PBS pH 7.4 (Invitrogen,
Denmark), containing 0.1% BSA (Sigma-Aldrich, Denmark). For all s.c.
administrations, the dose volume was 1ml/kg.

**Experimental design**

The main design was agreed with the FDA, as the study was part of the post-
marketing requirements for liraglutide. Both male and female diabetic animals were
randomly allocated into 6 groups of 12 animals. Groups were: 1) Vehicle s.c., QD, 2)
Liraglutide 0.4mg/kg/day s.c. QD, 3) Liraglutide 1.0mg/kg/day s.c. QD, 4) Pump
vehicle s.c. via Alzet osmotic mini-pumps (2ML4, Alzet, CA, USA), 5) Exenatide
0.25mg/kg/day, s.c. via Alzet osmotic mini-pumps, 6) Untreated diabetic baseline
group terminated at the time of initiation of treatment. Group 7) Lean rats receiving
vehicle s.c., QD. Liraglutide and exenatide treated animals were dose-titrated during
the first 7-10 days (liraglutide QD, exenatide BID) to avoid adipsia-induced
dehydration.

Morning fed blood glucose levels were measured bi-weekly prior to compound
administration using a BIOSEN c-Line glucose meter (EKF-diagnostics, Germany).
Non-fasted HbA1c levels (every second week) and pancreatic-amylase, lipase and
triglyceride levels (once monthly) were measured using a Hitachi 912 and kits
according to the manufacturers instructions (Roche Diagnostics, Germany).

**Termination and pancreas histology**

All animals were anesthetized with CO2 before decapitation. Trunk blood was
collected and the animals were subjected to a macroscopic examination. The pancreas
was removed, mounted on filter paper and immersion fixed in 4% buffered
formaldehyde. Four pancreatic sub-regions were identified and isolated according to
the definitions by Elayat et al. (18). Each sub-region was divided into two parts of equal size; one part (alternating left or right) was processed for histopathological examination, the other for stereological analyses. For histopathology, each sub-region was embedded in blocks of paraffin, sections of 5µm were cut using a Microm HM340E (ThermoScientific) and stained with hematoxylin-eosin (Sigma Aldrich). For stereology the sub-regional tissue samples selected were processed using systematic uniform sampling as previously described (35).

Three separate double immunohistochemical labelling procedures were performed. Beta- and non-beta-cells were identified by use of Guinea pig-anti-Insulin (A0564, DAKO, Denmark) and an antibody cocktail consisting of Mouse-anti–Glucagon (H-028-02, Phoenix Pharmaceuticals, USA), Rabbit-anti-Somatostatin (A566, DAKO, Copenhagen, Denmark) and Rabbit-anti-Pancreatic Polypeptide (B32-1, Eurodiagnostica, Sweden), as previously described (35). Beta-cell (insulin) and Ki67 immunohistochemistry were performed according to the same procedure substituting the non-beta-cell antibody-cocktail with a rabbit anti-Ki67 (ab16667, ABCAM) diluted 1:800. Following incubation with the Ki67 antibody, sections were rinsed 3x3 min in TBS-T, incubated with a goat anti-rabbit MACH2 HRP polymer (Biocare Medical) and visualized in a staining solution containing DAB and Nickel sulphate. Ki67. Duct cell (CK20) immunohistochemistry were performed over two separate days. After Ki67 staining was performed (visualized using DAB-NI) sections were stored overnight in PBS, then subjected to repeated antigen retrieval and peroxidase-treatment (eliminating the first Ki67 primary antibody and remaining HRP-enzyme) and reprocessed using a rabbit anti-CK20 (ab76126, Abcam) diluted 1:200. CK20 was visualized using DAB as a chromagen. The repeated antigen/peroxidase treatment and the fact that pancreatic duct cells (cytoplasm staining of CK20) were clearly
distinguishable from Ki67 positive nuclei made the use of two primary rabbit antibodies possible. Sections were stained with a Mayer solution for recognition of the pancreatic morphology.

Stereological analyses

Estimates of total mass were determined using the Cavalieri principle of uniform random systematic sectioning in combination with point counting (25). Exocrine and endocrine cell populations were identified by use of immunohistochemical and histochemical staining procedures (Figure 1). CK20, an epithelial marker which is known to be expressed in rat pancreatic cells of the ductal system from centroacinar cells to main ducts (7) were used to identify intercalated duct cells from exocrine acinar cells (Figure 1). No differentiation was made between centroacinar cells and main ducts as the latter contribute little to the overall duct cell mass (Figure 1). The stereological estimations were performed using the newCAST system (Visiopharm, Copenhagen, Denmark) on digital slides scanned with a 20x objective on a Hamamatsu Nanozoomer 2.0 HT. Cell masses were estimated by point counting using a grid system where all points hitting the structure of interest were counted (Figure 1). The estimation of proliferating cells was based on a modification of the unbiased 3-dimensional counting rules. Hence, potential changes in specific cell proliferation were assessed by counting Ki67 labelled profiles using a 2D unbiased counting frame hereby providing an estimate of Ki67 labelled nuclei per area reference volume (Figure 1). The Ki67 ratio was calculated as the Ki67 labelled nuclei per area reference volume multiplied by specific cell mass estimates to compensate for difference in total cell mass between experimental groups.
Statistical analysis

In-life data were analysed by one-way ANOVA models with change from baseline value as response and with baseline value as a covariate. In addition to the normal active drug to vehicle group comparisons, the s.c. diabetic vehicle group was compared to the lean vehicle group and the diabetic baseline group, respectively. Statistical analyses were performed using SAS 9.2. Each gender was analyzed separately and p<0.05 was considered statistically significant.
Results

Body weight, food- and water intake

Both liraglutide and exenatide treatment initially reduced body weight gain (Figure 2A, B). In females, liraglutide treated animals had increased body weight towards the end of the treatment period, whereas the body weight of the exenatide group was unchanged (Figure 2B). Both liraglutide and exenatide markedly reduced food- and water intake (Figure 2C-F).

Blood glucose, HbA1c and triglyceride levels

Blood glucose was lowered significantly in all treated animals (Figure 3A, B). In females fed the high-fat diet, mean blood glucose levels were around 17mmol/l at the time of initiation of treatment. Blood glucose levels continued to rise in vehicle dosed animals to around 25mmol/l while on the high fat diet. When animals were switched back to chow diet (Purina 5008) blood glucose levels dropped to mean stable blood glucose level around 20mmol/l (Figure 3B). Both liraglutide and exenatide treatment caused a greater drop in blood glucose in females than in males (Figure 3A vs. 3B). Exenatide treatment reduced blood glucose to a greater extent than liraglutide in males (Figure 3A), whereas liraglutide treatment was more efficacious in females (Figure 3B). In both male and female rats, the effect of exenatide on blood glucose was not fully apparent until the continuous dosing regime was initiated (Figure 3A, B).

HbA1c levels were significantly improved in both liraglutide and exenatide treated animals at the end of the treatment period (Table 1, Figure 3C, D).
Triglyceride levels in male animals were significantly reduced in the low and the high dose liraglutide groups, while no significant effect of exenatide treatment were observed (Table 1). In females, exenatide treatment significantly reduced triglyceride levels while no significant effect of liraglutide treatment was observed (Table 1).

**Biochemical markers of pancreatitis**

Neither liraglutide nor exenatide treatment increased plasma activity of lipase (Figure 4A, B). Actually, the lipase activity in liraglutide and exenatide treated animals tended to be lower than in vehicle animals (Figure 4A, B). In females treated with both low and high dose liraglutide, lipase activity was significantly lower than in vehicle animals at the end of treatment (Table 1). In male rats, P-amylase activity remained constant throughout the study period (Figure 4C). However, when adjusting for baseline, end of treatment P-amylase activities were in all treated groups significantly higher than in the corresponding vehicle groups (Table 1, Figure 4C). In female rats, P-amylase activity was increased in treated rats (Figure 4D), and as for males, end of treatment baseline adjusted values were significantly higher (Table 1). The increase in P-amylase in female rats was most pronounced on study day 29 immediately following the diet change on day 28 (Figure 4D). No animals displayed permanent elevations of lipase or P-amylase, and more importantly no animals displayed simultaneous elevations of lipase and P-amylase (Figures 4E, F).

**Mortality**

A total of nine rats (four males and five females) died or were terminated prematurely. The four male animals were from the pump vehicle group and three deaths were related to a single erroneous dosing of liraglutide (0.5mg/kg), which would be...
expected to lead to dehydration, as GLP-1 acutely has powerful effects on water intake and diuresis (41). The death of the fourth male animal was related to a persistent pump infection. Two female animals terminated prematurely were from the pump vehicle group, while three female animals were from the exenatide group. Three of these deaths were related to infections around the pump or associated with pump change, while two terminations (one pump vehicle- and one exenatide treated animal) were due to general lethargy and weight-loss.

Qualitative histopathological evaluation of pancreas

In one prematurely killed female dosed with exenatide, moderate apoptosis-like necrosis, minimal inflammatory infiltration and slight haemorrhage/oedema, were observed in the pancreas. This correlated with an oedematous and hemorrhagic pancreas seen at necropsy. Furthermore, at necropsy, the distal intestines appeared dark and haemorrhagic in line with the clinical observations prior to termination, that this animal suffered from acute generalised disease.

The main histopathological pancreas findings from the terminally killed animals are compiled in Table 2. In general, all histopathological changes were low in incidence and severity (Figure 5). Most of the findings were given the score minimal and were focal in distribution. The highest incidences of pathological lesions were observed in the lean vehicle group (males) and the diabetic baseline groups (both sexes), with only focal acinar cell hyperplasia being more frequently observed in the exenatide treated male rats. In females, minimal “acinar cell atrophy/metaplasia/duct proliferation” was seen with slightly higher incidence in the liraglutide treated groups compared with the liraglutide vehicle group. However, the incidences were similar to or below those seen in female lean and diabetic baseline animals (Figure 5, Table 2).
Quantitative stereological evaluation of pancreas and pancreas weight

Liraglutide and exenatide treatment did not affect pancreas weight in male ZDF rats (Figure 6A, B). In females, liraglutide significantly decreased pancreas weight, while no effect was observed of exenatide (Figure 6B). The stereologically quantified masses of exocrine acinar and duct cell mass in males and females are shown in Figure 6C, D, E, F. Neither duct nor acinar cell mass was significantly altered by treatment. In contrast, a significant effect of both phenotype and age was apparent when comparing the control groups (Figure 6C, D, E, F). The assessment of specific cell proliferation in males showed no significant changes in duct and acinar cell proliferation in liraglutide treated rats, whereas the acinar cell proliferation ratio was slightly increased in the exenatide group (Figure 7A, C). In females, no significant effects of treatment were seen in duct or acinar cell proliferation (Figure 7B, D). Significant alterations in cellular proliferation rates were related to control groups.

Stereological estimates of beta-cell and non-beta-cell mass are shown in Figure 8. In males, exenatide treatment led to a significantly higher beta-cell mass, while no effect on beta-cell mass of liraglutide treatment was observed (Figure 8A). A significantly higher beta-cell mass was observed in male lean ZDF rats and in the diabetic rats terminated at baseline compared with the s.c. vehicle dosed diabetic rats (Figure 8A). In females, both liraglutide and exenatide treated rats had a significantly higher beta-cell mass (Figure 8B). As seen in males, beta-cell mass in the baseline group was high (Figure 8B). In both males and females, beta-cell proliferation ratios were higher in groups with a high beta-cell mass (Figure 8C, D). The quantitative analyses of endocrine non-beta-cell mass revealed no effects of treatment (Figure 8E, F).
Discussion

The data from the current study are the first reporting long-term anti-diabetic efficacy data in the female ZDF-rat. The glucose-lowering effects of both liraglutide and exenatide were more potent in females than in males. In addition, the female study revealed a dose-response effect of liraglutide which was not apparent in the males. One possible explanation for this apparent discrepancy relates to the differential diabetes syndromes in male and female ZDF rats. While males spontaneously develop diabetes on the Purina 5008 diet, females only become diabetic if fed a specialized high-fat diet (43). Pilot studies performed in our laboratory (data not shown) revealed an increased mortality when female ZDF rats were fed the high-fat diet (RD12468) for more than 8 weeks – probably related to high blood glucose levels (above 30mM), kidney stones and nephritis. We therefore induced diabetes by feeding the female ZDF rats the RD12468 diet for 6 weeks, then switched to Purina 5008.

Mean lipase activities of liraglutide and exenatide treated rats were relatively stable during the study period, except for an increase in exenatide treated female rats at the last time-point. Notably, there was a general tendency for lipase activity to be significantly lower than the corresponding vehicle groups. P-amylase activities were generally higher in the liraglutide and exenatide treated animals. In males, P-amylase did not change markedly during the study period, whereas in females P-amylase were increased in liraglutide and exenatide treated groups, most markedly at day 29, immediately following the diet change to Purina 5008 on day 28. One possible explanation for the selective elevation of P-amylase activity could be a differential excretion and re-absorption of lipase and amylase in the kidneys. In rats, both lipase
and amylase are excreted by the kidneys, but the re-absorption of lipase is higher than that of amylase (26, 31). Furthermore, it has been shown that urinary excretions of amylase in rats with normal kidney function is directly proportional to the glomerular filtration rate (20). Hence, although a certain degree of tubular re-absorption occurs, osmotic diuresis can increase amylase excretion (20). As support of the explanation for the paradoxical elevations in P-amylase, lipase and amylase were never simultaneously elevated and no treatment-related pathological or stereological indications of acute pancreatitis were identified in the post-mortem pancreatic analyses. Finally, it should be noted that the elevations in P-amylase observed here (maximally 50%) were of a considerable lower magnitude than that observed in rat models of chemically induced pancreatitis where 200-300 % elevations in P-amylase and lipase were seen (42). Our data are in agreement with a study using shorter treatment period (42) as well as data from healthy SPD rats treated for 12 weeks with exenatide (24). Exenatide has actually been shown to exert a slight but significant protection against chemically induced pancreatitis in diabetic ob/ob mice, Sprague-Dawley and ZDF rats (42). In one study, exendin-4 administration for 75 days to normoglycaemic male Sprague-Dawley rats induced minor elevations (64%) in lipase activity but a non-significant decrease in amylase (33).

There was no indication from the histopathological or stereological evaluation of the exocrine pancreas that 13 weeks of liraglutide or exenatide treatment induced pancreatitis in the ZDF rat. Neither exenatide nor liraglutide increased pancreas weight. In the current study, the histopathological analysis focused on morphological changes in the exocrine pancreas. In one decedent female dosed with exenatide necrosis in the
pancreas was observed. This necrosis was described as apoptosis-like due to the condensed acinar cells and the minimal inflammatory cell infiltration. The mild degree of inflammatory cell infiltration and the apoptosis-like necrosis were not considered to support the diagnosis of an acute pancreatitis. Furthermore, macroscopic findings in the intestine indicated that this animal suffered from acute generalised disease. In all other animals, the histopathological evaluation of the exocrine pancreas showed that the lean vehicle and baseline groups had the highest incidences of "acinar cell atrophy/metaplasia/duct proliferation", “acinar cell atrophy and inflammatory cell infiltration”. Slightly higher incidences of acinar cell atrophy/metaplasia/duct proliferation were seen in female ZDF rats treated with liraglutide, compared to the vehicle group. However, the incidences were similar to or below those seen in the lean vehicle group and the diabetic baseline group. A similar difference was not seen in males where the highest incidence of “acinar cell atrophy/metaplasia/duct cell proliferation” was seen in the lean vehicle group. Thus, this finding is not likely to be related to liraglutide treatment. While our histopathological findings are in good agreement with pancreatic histopathological data recently reported from a 2-year rat carcinogenicity study conducted as part of the development program for liraglutide (34) and studies examining effects of exenatide on caerulein induced pancreatitis (1, 4, 42), it should be noted that one study reported increased inflammation and pyknotic nuclei scores in the pancreas following exenatide administration for 75 days (33). Unfortunately, the article did not report the number of cases from their vehicle and exenatide treated Sprague-Dawley rats displaying morphological changes, but rather group-based scores of pyknotic nuclei and inflammation making direct comparisons between that study and other reports difficult (33). Our data indicating that prolonged GLP-1 receptor stimulation does not
induce pancreatitis is also in agreement with pancreas histopathological findings in healthy male SPD rats treated once daily (10µg/kg) for 12 weeks (24). However, Gier et al (24) also examined duct cell proliferation with a particular emphasis on the recently identified so called periductal glands (39). Interestingly, exenatide treatment appeared to increase not only the number of periductal glands but also the proliferation of duct cells in these glands and in glands around the large collecting ducts (24). Although the physiological and pathophysiological role of these mucin producing glands is still rather speculative, gland number has been reported increased during chronic pancreatic in mice and it has been speculated that this mucinous metaplasia could be the precursor of pancreatic epithelial neoplasia (39). In support of these findings in rats Gier et al (24) found increased chronic pancreatitis and pancreatic epithelial neoplasia in a chronic pancreatitis/pancreas cancer progression mouse model (PDX-1/Kras). It should be noted, however, that although Gier et al (24) counted a lot of cells they did not apply stereological sampling and counting principles. This could potentially introduce sampling bias, which is critical when hyperplastic/hypertrophic structures (like periductal glands, (39)) are to be quantified. Despite the methodological issues the findings by Gier et al (24) are intriguing and it will be important, not only to further explore the role of the periductal glands in pancreas physiology/pathology, but also to investigate in greater detail - and with stereological methods - the effects of GLP-1 receptor agonists on periductal gland mass and proliferation in healthy and diabetic animal models. It should be noted that in the current study we found no evidence for an increased proliferation in duct cells following either exenatide or liraglutide. On the other hand, as the mass of the collecting ducts (including the periductal glands which also express CK20) only
makes up a fraction of total duct cell mass we cannot exclude a potential increased proliferation in this particular cell type.

In the current study, using quantitative stereological analyses of the exocrine pancreas, we found no clear treatment effects on exocrine acinar or duct cell mass or proliferation. This is interesting in view of some papers suggested that increased duct cell proliferation may be one of the mechanisms for GLP-1 induced pancreatitis and/or pancreas cancer (10, 24). This study is the first to provide true quantitative estimates of acinar and duct cell mass and proliferation using stereology. From the current data there is therefore no indication that exenatide, dosed continuously, or liraglutide should increase exocrine cell proliferation or exocrine cell mass.

Our finding that neither exenatide nor liraglutide increase duct cell proliferation is interesting in light of the proposed role of duct cells as beta-cell precursors. While previous studies have indicated that GLP-1 and exenatide increase duct cell proliferation (possibly giving rise to novel beta-cells of ductal origin) (45-46) we failed to show this association in the current study. In males we found significant long-term effects of liraglutide and exenatide on glucose homeostasis, but only in exenatide treated rats was a higher beta-cell mass observed. In females both liraglutide and exenatide led to a higher beta-cell mass. While these data could indicate that GLP-1 receptor mediated beta-cell mass increase, the strong coupling in both males and females between the effect on blood glucose and beta-cell mass could indicate that better glucose control rather than GLP-1 receptor stimulation per se is what regulates beta-cell mass in the long run. Interestingly, Ki67 immunostaining revealed that the highest degree of proliferation in both duct and beta-cells was found
in the diabetic baseline group terminated at a time where beta-cell mass is rapidly
expanding. This could indicate that new beta-cells could be of both beta- and duct cell
origin in line with previous reports (11, 29, 45-46)

In conclusion, the present study showed that continuous exposure to the GLP-1
receptor agonists, liraglutide (dosed once-daily) or exenatide (dosed continuously by
pump) for 13 weeks to male and female diabetic ZDF rats did not induce pancreatitis.
Plasma markers that often increases in connection with pancreatitis, P-amylase and
lipase, did not indicate any signs of changes. The histopathological evaluation did not
indicate any adverse treatment-related lesions in the pancreas. The quantitative
stereology revealed no treatment induced increases in overall pancreas mass, exocrine
cell mass or exocrine cell proliferation rate. Even though these data indicate that there
is not a straightforward pharmacological effect of GLP-1 receptor agonists on rat duct
cells or acinar cells they cannot rule out a human risk. The outcome studies for the
GLP-1 analogues and DPP-4 inhibitors will be important, but also more studies are
needed with primate tissue, including human, and/or in vivo studies in non-human
primates.
Figure legends

Figure 1
Stereological estimates of mass and proliferation. (A) The pancreata was cut into 4-5 slabs and mounted in paraffin blocks. From each block two sections were cut with an interval of 0.5 mm, representing in total a systematic uniform random sample of the whole pancreas. (B) Endocrine beta-cells (arrow) and non-beta-cells (alpha, delta and gamma cells; double-arrow) were identified using specific immunohistochemical stainings. Point-counting was used to assess changes in total cell mass. (C) Duct cell proliferation was estimated in 2D counting frames on sections double stained for cytokeratin-20 and Ki67. (D) High magnification images demonstrating Ki67 labelled duct cells (arrow) and acinar cells (double arrow). Duct cell mass and acinar cell mass was estimated by point counting.

Figure 2
Body weight (A, B), food intake (C, D) and water intake (E, F) in male (A, C, E) and female (B, D, F) ZDF rats during the 13-week treatment period. Exenatide pumps were changed every four weeks, at the times indicated in the graphs. The shift from high-fat diet to Purina diet of female ZDF-rats is likewise indicated in the graphs. Data are means and n=8-12

Figure 3
Blood glucose (A, B) and HbA1c (C, D) in male (A, C) and female (B, D) ZDF rats during the 13-week treatment period. Exenatide pumps were changed every four
weeks, at the times indicated in the graphs. The shift from high-fat diet to Purina diet of female ZDF-rats is likewise indicated in the graphs. Data are means and n=8-12.

**Figure 4**

Lipase (A, D) and pancreatic-amylase (B, E) activity in male (A, B) and female (D, E) ZDF rats during the 13-week treatment period. Exenatide pumps were changed every four weeks, at the times indicated in the graphs. The shift from high-fat diet to Purina diet of female ZDF-rats is likewise indicated in the graphs. Data are means and n=8-12. C and F are scatter plots of lipase and P-amylase in male (C) and female (F) animals from all groups at the end of the treatment period, illustrating that values are not simultaneously increased.

**Figure 5**

Pancreas histopathology. Photomicrographs from a male (A) and female (C) ZDF rat terminated at baseline and a male (B) and female (D) ZDF rat treated with liraglutide for 13 weeks (1mg/kg/day). Small areas of focal acinar cell atrophy/metaplasia/ductal proliferation are evident in both groups (magnification of insert shown in lower right corner).

**Figure 6**

Absolute pancreas mass (A, B), duct cell mass (C, D), and acinar cell mass (E, F) in male (A, C, E) and female (B, D, F) ZDF rats. Bars represent mean±SEM, n=8-12. *p<0.05 compared with the corresponding vehicle group. The diabetic baseline group is compared to the s.c. vehicle group.

**Figure 7**
Duct cell proliferation (A, B) and acinar cell proliferation (C, D) in male (A, C) and female (B, D) ZDF-rats. Bars represent mean±SEM, n=8-12. *p<0.05 compared with the corresponding vehicle group. The diabetic baseline group is compared to the s.c. vehicle group.

Figure 8
Beta cell mass (A, B), beta cell proliferation (C, D) and non-beta cell mass (E, F) in male (A, C, E) and female (B, D, F) ZDF-rats. Bars represent mean±SEM, n=8-12. *p<0.05 compared with the corresponding vehicle group. The diabetic baseline group is compared to the s.c. vehicle group.

Table legends
Table 1
Mean±SD; all statistical estimates of treatment differences are performed with baseline as covariate. Treatment differences are estimated as compared to the corresponding vehicle group (s.c./pump).
*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Table 2
# The lesions were mostly of minimal grade and focal in distribution
§Acinar cell atrophy/metaplasia/duct proliferation: areas showing reduced numbers of acinar cells and increased numbers of duct cells due to acinar cell atrophy, acinar cell metaplasia and/or duct proliferation
*Acinar cell atrophy: areas where only the acinar cells are affected
The inflammatory cells were located in the interstitium between the acini, and in most cases the acinar cells were unaffected. In some cases, the inflammatory cells were seen to exist concurrently with acinar cell atrophy/metaplasia/duct proliferation and acinar cell atrophy.
Acknowledgements

We thank Søren Andersen for statistical analysis. We thank Farida Sahebzadeh, Lotte Handgaard Jørgensen, Hanne Jensen-Holm and Majbrit Balle for excellent technical assistance.
References


Figure 1
Vrang et al
Figure 2
Vrang et al
Figure 3

Vrang et al
Figure 4
Vrang et al
Figure 5
Vrang et al
Figure 6
Vrang et al
Figure 7
Vrang et al
Figure 8
Vrang et al
<table>
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<th></th>
<th>Vehicle s.c.</th>
<th>Liraglutide 0.4mg/kg</th>
<th>Liraglutide 1.0mg/kg</th>
<th>Vehicle pump</th>
<th>Exenatide 0.25mg/kg</th>
<th>Lean vehicle s.c.</th>
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<td><strong>HbA1c males [%]</strong></td>
<td>8.1±0.3</td>
<td>7.3±0.5</td>
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Mean±SD; all statistical estimates of treatment differences are performed with baseline as covariate. Treatment differences are estimated as compared to the corresponding vehicle group (s.c./pump).

*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001
### Table 2: Summary of selected histopathological findings in the exocrine pancreas in terminally killed animals

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<tr>
<th>Lesion#, males</th>
<th>Vehicle s.c.</th>
<th>Liraglutide 0.4mg/kg</th>
<th>Liraglutide 1.0mg/kg</th>
<th>Vehicle pump</th>
<th>Exenatide 0.25mg/kg</th>
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</table>

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