The human GLP-1 analogs liraglutide and semaglutide: Absence of histopathological effects on the pancreas in nonhuman primates.

Running title: GLP-1 receptor agonists and the pancreas


Novo Nordisk, Novo Nordisk Park, DK-2760 Måløv, Denmark.

Corresponding author: Lotte Bjerre Knudsen, Novo Nordisk, Novo Nordisk Park, DK-2760 Måløv, Denmark. lbkn@novonordisk.com. phone +45 30754788.

Word count abstract: 191

Word count manuscript: 3983

Number of tables: 3

Number of figures: 5
Abstract

Increased pancreas mass and glucagon-positive adenomas have been suggested to be a risk associated with sitagliptin or exenatide therapy in humans. Novo Nordisk has conducted extensive toxicology studies including data on pancreas weight and histology in *Cynomolgus* monkeys dosed with two different human GLP-1 receptor agonists. In a 52-week study with liraglutide, a dose-related increase in absolute pancreas weight, in female monkeys only, was observed. Such dose-related increase was not found in studies of 4, 13 or 87 weeks’ duration. No treatment-related histopathological abnormalities were observed in any of the studies. Quantitative histology of the pancreas from the 52-week study showed an increase in the exocrine cell mass in liraglutide-dosed animals, with normal composition of both endocrine and exocrine cellular compartments. Proliferation rate of the exocrine tissue was low and comparable between groups. Endocrine cell mass and proliferation rate were unaltered by liraglutide treatment. Semaglutide showed no increase in pancreas weight and no treatment related histopathological findings in the pancreas after 13 or 52 weeks’ dosing. Overall, based on 138 nonhuman primates, there were no histopathological changes in the pancreas associated with liraglutide or semaglutide, two structurally different GLP-1 receptor agonists.
Introduction

Pancreas safety has become a subject of much debate concerning dipeptidylpeptidase-4 (DPP-4) inhibitors (DPP-4i’s) and glucagon-like peptide-1 (GLP-1) receptor agonists (GLP-1RA’s). This concern is based in part on the well described effect of GLP-1 to induce growth of pancreatic beta-cells (1). Both drug classes increase effective GLP-1 levels, but to a different degree, and have different mode of actions, thus it is important to differentiate between them, particularly when considering mechanistic hypotheses being considered for potential safety concerns or signals. GLP-1RA’s mediate their effects directly through the GLP-1R (2, 3). While increased levels of GLP-1 and GIP are considered important parts of the mechanism of action of DPP-4i’s (4), many other hormones are also known to be degraded by DPP-4 (5). GLP-1, GIP and PYY have been shown to be increased by DPP-4 inhibitors (6, 7). This complicates the understanding of both desired and potentially undesired effects of this class of compounds. Within GLP-1RA’s differences exist in safety related parameters: One sub-group is the exendin-4 based drugs with exenatide and lixisenatide, which are structurally distinct from human GLP-1. Due to the low amino acid homology to native human GLP-1, these medications are associated with an increased number of immune-reactions which are however all of a relatively mild form, i.e. mostly antibody development, injection site nodules and loss of efficacy (8, 9). The other sub-group is based on human GLP-1, and contains liraglutide, taspoglutide and larger covalently conjugated molecules like albiglutide and dulaglutide. Clinical development of taspoglutide was stopped due to severe immune-related side effects with cases of anaphylactic shock, possibly caused by the formulation (8, 10). No such findings have been reported with other GLP-1 based analogs.
Semaglutide is a once-weekly analog of GLP-1 that is in phase 3 clinical development (11). Where liraglutide is acylated with a palmitic acid and has an extra amino acid as spacer between the palmitic acid and the Lys26 where the fatty acid is attached, semaglutide is acylated with a stearic diacid also at Lys26 but also has a much larger synthetic spacer, and is furthermore modified for DPP-4 stability in position 8, where the amino acid alpha-aminobutyric acid has been introduced.

In support of drug development and regulatory approval for treatment of chronic diseases, repeat dose toxicity and carcinogenicity studies are conducted. These studies are carried out at different dosing durations to support the different phases of clinical development, and with doses aiming to obtain exposure several multiples higher than the clinically relevant exposure with the aim to identify potential drug related organ toxicity and carcinogenicity. Repeat dose toxicity studies are typically conducted in a rodent and a non-rodent species. For liraglutide and semaglutide Cynomolgus monkeys were chosen as the non-rodent species. Repeat dose toxicity studies in nonhuman primates are designed to screen for potential hazards and not designed or statistically powered for identification of differences in the incidence or severity of individual organ changes. The number of nonhuman primates per group is limited to 3-5 for ethical reasons (12). Because of the statistical limitations, standardization of the examinations are critical: If pathological findings are identified at a frequency or severity exceeding those in the in-study control group, they are often compared to historical control data to assist interpreting the significance of the finding. This principle also applies to organ weights. Histopathological findings in the pancreas from the repeat dose studies in rodents and nonhuman primates and carcinogenicity studies in rodents with liraglutide have been published previously (13). Liraglutide was not found to have a causal relationship to any histopathological findings.
Some studies in rats and mice have shown an increased pancreas weight induced by DPP-4i’s or GLP-1RA’s (14, 15), and a recent *ex vivo* study with human pancreata suggested an increase in glucagonomas as well as increased pancreas weight (16). Here, pancreas weight in *Cynomolgus* monkeys is reported for liraglutide in toxicology studies with 4, 13, 52, and 87 weeks’ dosing, and for semaglutide in toxicology studies with 13 and 52 weeks’ dosing, as well as a full histopathological evaluation of these same studies except the 87 week’s study which has been reported previously (13). For liraglutide also a full quantitative histological assessment of the endocrine as well as the exocrine pancreas was performed in the 52 weeks’ study.

Research design and methods

Research design and methods for liraglutide studies in *Cynomolgus* monkeys have been described previously (13). All animals were examined daily for clinical signs in the in-life phase. Studies with semaglutide were in general performed similarly, and both following international guidelines provided by International Conference of Harmonisation. Dose levels, duration and group sizes for all studies are described in Figure 1. Compounds were administered as s.c. injections.

Body weight and pancreas weight:

Terminal body weight was obtained in sedated animals immediately before sacrifice. The entire pancreas of each animal was examined macroscopically for any abnormalities, excised, cleaned of fat and connective tissue, and weighed.

Tissue preparation:

A transverse section from the mid-part of pancreas from all animals was sampled and both this section and the rest of the pancreata were fixed in 10% neutral buffered formalin for at least 48
hours. The sections were then dehydrated and paraffin embedded according to standard histopathological procedures. According to international standard practices one section per animal was cut at a nominal thickness of 4-5 µm and stained with hematoxylin and eosin (HE) (17). The slides were read un-blinded as this is recommended by experts in toxicopathology as a way to increase the chance of separating subtle changes from normal background changes (18). Further details of the methodology are previously described (13). Furthermore, the pancreas specimens from animals sacrificed at termination of dosing in the 52 week liraglutide study were evaluated by quantitative histology (all groups for alpha-cells and Ki67, only vehicle and high-dose groups for beta-, delta-, pancreatic polypeptide-, (PP) ductal-, and acinar cells). The cranial and caudal remnants were sectioned longitudinally, cut into ~40 pieces and distributed to four capsules, with one fourth in each, according to the smooth fractionator principle (19, 20). Processing and staining for detection of beta-, alpha-, delta-, and PP-cells was carried out as previously described (21). The reactivity of the primary antibodies to insulin, glucagon, somatostatin and pancreatic poly-peptide in monkey pancreas had been tested individually to validate the method. Co-staining for duct plus acinar cells (using mouse anti-CK-7 (DAKO, Glostrup, Denmark) and rabbit anti-a-amylase (Calbiochem, Darmstadt, Germany)) plus Ki-67 (using polyclonal rabbit anti-Ki-67 (Novus Europe, Cambridge, UK)) followed the same principles as above. Stained slides were scanned in a Hamamatsu NanoZoomer 2.0HT (Hamamatsu, Hamamatsu City, Japan). Images were subsequently analyzed automatically in an image analysis program (Visiopharm Integrator System; Visiopharm A/S, Hørsholm, Denmark).

Calculations:

Historical control data: Data on pancreas weights were collected from the contract laboratories where the study of interest was conducted to ensure matching for origin, age-range and
environmental conditions to the highest possible extent. Historical control data on pancreas weights from animals used as vehicle controls in other studies of 4, 13 and 39-52 weeks’ duration was used for comparison to liraglutide: 4-week (n=29 males and 28 females as historical controls), 13-week (n=19 males and 19 females as historic controls) and 52-week (n=22 males and 17 females as historical controls). No historical control data were available for comparison to the 87-week study, as this is not a standard length toxicology study.

Quantitative histology: Measurements were carried out on samples obtained by systematic uniform random sampling technique. The mean value for each animal was calculated relative to the total tissue area counted for each estimate. Volume fractions were measured in % of the total pancreas volume. Total cell mass in mg was calculated by multiplying the volume fraction with the total pancreas weight.

Statistics:
The liraglutide data on pancreas weight were per-study protocol analyzed by an ANCOVA on body weights and pancreas weights combining both sexes and using day 0 body weight and terminal body weight, respectively, as covariates. These pre-specified models were slightly different between studies, as they were data-driven. Using this analysis, only the 52-week study reported an increase in pancreas weight (22). In order to obtain consistency across studies and thus enhance comparisons, post-hoc modeling was performed using the same models in all studies. In the pre-specified analyses terminal bodyweight was taken into consideration in the statistical models. However as liraglutide lowers body weight that is problematic. The post-hoc analysis is thus a one-way ANOVA of the pancreas weight, for each study and sex separately. In case of variance inhomogeneity, measured by means of Bartlett’s and Brown-Forsythe tests, a Kruskal-Wallis test
was performed to evaluate overall group effects. As statistical significant effects of liraglutide were seen in some studies for some of the doses, a one-way ANOVA was also performed for the relative pancreas weight to further examine a possible effect. Dunnett’s multiple comparison test was used as post-test after ANOVA in cases where the overall group effect was significant. Parameters from quantitative histology were analyzed by Student’s t-test for each sex except for alpha-cell mass where one-way ANOVA was used. p<0.05 was considered statistically significant. Data are presented as mean±SEM unless otherwise stated.

Results

Liraglutide:

Pancreas weight:

Comparison to in-study control groups:

Pancreatic weight data from liraglutide studies are shown in Figure 1. A significant increase that was apparently not dose-related was found in the 4-week study in male animals (p=0.036 by ANOVA) whereas no increase was seen in females in the same study. No statistically significant differences were found for any dose level in male or female animals after 13 or 87 weeks of dosing. Similarly, in the 52-week study, no statistically significant differences were found for the male animals but a significantly higher pancreas weight, apparently dose-related, was found in the female high-dose group compared to the in-study control group (p=0.007 by ANOVA).

Comparison to historical control data:

This comparison did not show any statistically significant differences in pancreas weight for any dose level in males or females from the 4-, and 13-week studies or males in the 52-week study. When comparing against historical controls in females from the 52-week study, a statistically
significant difference across groups was found (p=0.005 by ANOVA), but post-test showed that only the in-study controls exhibited a statistically significant different (lower) pancreas weight than the historical controls (p=0.04, n=4 and 17, respectively).

Comparison of pancreas weight adjusted for body weight:

An ANOVA analysis of the relative pancreas weight showed a significant increase compared to in-study controls in the male mid-, and high-dose groups and in the female high-dose group in the 52-week liraglutide study (data not shown). There were no statistically significant differences in the 4-, 13-, or 87-week liraglutide studies.

Recovery animals:

Additional animals were included in order to explore reversibility of potential findings (n=2 males and 2 females, in the control and high-dose groups). These additional animals were dosed for the same duration as all other animals in the study, but kept for additional 4 weeks after end of treatment before sacrifice. In the 52 weeks’ study, after the 4-week treatment-free period, there was no longer any difference in pancreas weight vs. controls (data not shown).

Histology:

Histological examination of the pancreas from the 4, 13, and 52 week studies are shown in Table 2. Representative histological sections of the different compartments of pancreas after 52 or 87 weeks of dosing are illustrated in the Figures 2-3. The histological examination did not reveal treatment-related differences between dosed and control animals at any time point. The endocrine pancreas revealed well demarcated islets with normal pale islet cells both after 52 and 87 weeks of dosing (Figure 2). In the exocrine pancreas, all ducts appeared normal, both the large main duct with the high columnar epithelium and abundant surrounding connective tissue (shown in Figure 3, left side panels, and in higher magnification in online supplementary Figure 1) and the medium sized interlobular ducts with the lower cuboidal epithelium and less surrounding connective tissue (Figure
3, right side panels). The small intercalated ducts with flat epithelium and no or sparse surrounding connective tissue also appeared normal (not shown in Figures). The acinar cell parenchyma consisted of normal pyramid-shaped cells where the apical part was filled with eosinophilic zymogen granules and the basophilic basal part contained the nucleus as shown in Figure 3 (and in online supplementary Figure 2).

Quantitative histology of pancreas in 52-week liraglutide study:

A quantitative histological assessment of the pancreas in the 52-week liraglutide study was conducted to evaluate if there were changes in the mass of pancreatic cell types which could not be identified by the qualitative histological analysis. Table 1 shows absolute mass of beta-, alpha-, delta-, PP-, duct-, and acinar cells. Liraglutide dosed monkeys showed no significant differences in any of these measures except for absolute duct cell and acinar cell mass which was significantly increased in the female high-dose group. When evaluating proportion of cells per volume of pancreas, no changes were found for ductal cell volume in males (liraglutide 6.14±0.59 vs. control 6.71±0.62%, p=0.53) or females (7.40±0.63 vs 6.55±1.29%, p=0.57) or for acinar cell volume in males (91.4±0.9 vs 88.7±0.8%, p=0.06) or females (90.3±0.4 vs 90.0±0.7%, p=0.71) high-dose compared to vehicle. Thus, the increased pancreas weight is a balanced increase of the exocrine pancreas with no apparent change in the ratio of ductal to acinar tissue. To further evaluate if alpha-cell mass specifically was changed by liraglutide, the low-, and medium-dose liraglutide groups were also evaluated quantitatively. Alpha-cell mass was not changed by liraglutide in males in the low-, or middle-dose groups (30.0±8.4, 24.2±3.3, respectively, vs. 29.9±3.6mg for controls, from Table 1) or females (16.0±1.6, 20.4±2.0, respectively, vs. 22.6±3.3mg for controls, from Table 1), p=0.74 and p=0.22 for males and females across groups by ANOVA.
Examples of beta and non-beta cell staining (Figure 4) and alpha-cell and proliferation (Ki67) (Figure 5, and higher magnification in online supplementary Figure 3) in representative sections of pancreas from males and females in the vehicle and the liraglutide high-dose group of the 52-week dosing study are shown. In control and high-dose animals, glucagon staining showed a high and variable number of alpha-cells in islets (typically around 50% of non-beta cells were alpha-cells). Small numbers of single cells or small clusters of glucagon positive cells associated with ducts were seen in the exocrine pancreas (Figure 5). Very few cells in the endocrine and exocrine pancreas were positive for Ki-67 and there was no apparent difference between liraglutide and control groups (data not shown). As a positive control for proliferation, lymph nodes, present in 15 out of the total of 32 pancreata showed strong labeling of many cells in germinal centers, and also some single cells (shown as inserts in Figure 5).

Semaglutide:

Pancreas weights from semaglutide studies are shown in Figure 1. In the 13-week study there was no statistically significant treatment related effect on pancreas weight in males whereas a significant difference was seen across groups in the females (p=0.02 by ANOVA). However, by post-test, no significant difference was found between treated groups and the control group, and no apparent dose-related effects were seen. In the 52-week study, no statistically significant differences across study groups were observed for pancreas weight; the highest pancreas weights were found in the control groups.

Histological examination of the pancreas from the 13 and 52 weeks’ studies revealed common background findings of minimal to mild severity and with a focal distribution. There were no signs of treatment-related effects. The data are shown in Table 2.
Discussion

Reported here are further data from nonhuman primate studies conducted with liraglutide as a supplement to previously published data on pancreas histology in mice, rats and nonhuman primates (13). An apparent dose-related increase in absolute pancreas weight was found in females in one out of 4 monkey studies with liraglutide and in none of 2 monkey studies with semaglutide, and an increase that did not appear to be dose-related was found in a 4-week liraglutide study, in males only (Table 3). A comparison to historical control data was made. This is a common way of setting toxicological data into perspective, and may be especially useful in nonhuman primate studies where the number of animals is low due to ethical reasons. The histological analysis of the pancreas from both liraglutide and semaglutide studies did not reveal any potentially adverse findings which could be related to treatment (e.g. pancreatitis, inflammatory cell infiltrations or hyperplasia). Overall, this led to the conclusion that an increase in pancreas weight cannot be ruled out, but no consistent dose-related increases in pancreas weight were seen across the liraglutide and semaglutide studies in monkeys. In combination with the lack of treatment related histopathological changes, the data showed no adverse effects on the pancreas by liraglutide dosing of up to 60-times the clinically relevant exposure for up to 87 weeks or semaglutide dosing for up to 52 weeks in monkeys.

The studies reported here have a high relevance for humans since the pancreas of nonhuman primates are closely related to humans both anatomically and physiologically. Additionally the morphology of nonhuman primate islets is like that seen in humans and different from rodents (23). Quantitative histology was used to assess changes in the mass of different pancreatic tissue
components taking the three-dimensional structure of the organ into consideration (24). An inherent weakness of these studies is the relatively limited number of nonhuman primates per group as number of animals is constrained for ethical reasons. However, this paper is based on data from 90 and 48 animals dosed liraglutide and semaglutide, respectively. Another limitation is that only one transverse section was examined per animal. As a consequence thereof, the statistical power is low. Three out of the four studies that have described adverse effect of DPP-4i’s or GLP-1RA’s on the pancreas were performed in rodents (15, 25, 26). Thus, at the current point in time with the very few studies available in nonhuman primates, the cumulative data in these studies have a strong relevance for the assessment of adverse pancreas effects in humans, despite the relatively limited number of animals per group.

A recent study with human pancreas from patients previously treated with sitagliptin or exenatide (7 sitagliptin, 1 exenatide) reported an increased pancreas weight (16). There are a number of important considerations with the study design that may have affected the results: the groups are unbalanced with an 18 year age difference between the groups and no attempt to control for type of diabetes, weight, age or sex. Pancreas weight depends on body weight, stage of diabetes, age and gender (27, 28). These methodological problems are clearly elucidated in two related commentaries/reviews (29, 30).

The data presented here do not show an increase in cell replication or number of alpha-cells caused by liraglutide treatment. Quantitative histology of the pancreas from the 52-week study with liraglutide demonstrated an increase of exocrine pancreas tissue with an apparently unchanged ratio between acinar and ductal cells and with normal tissue architecture. Cell proliferation in the pancreas measured by Ki-67 appeared unchanged by liraglutide; very low proliferation rates were
found in all animals. There appeared to be no change of beta-, delta-, or PP-cell mass by 52 weeks’
of liraglutide treatment in the high-dose group and there was apparently no change in alpha-cell
mass or indication of proliferation of alpha-cells in any of the three liraglutide dosed groups in the
52-week study. The islets from both the control and the liraglutide groups in the 52-week study had
around 50% non-beta endocrine cells, with a substantial portion of those being alpha-cells (25-30% of
docrine cell mass), less delta-cells (around 15%) and only a few PP-cells (4-7%). The fraction
of the four endocrine cell types in our study is in agreement with data from human pancreas and a
descriptive study in Cynomolgus monkeys (31-34). The intra-islet organization of the beta-, and
non-beta cells was random with no clear rodent-like mantle and core but with a more complex sub-
unit structure of mantles and cores, as characteristic for nonhuman primates and humans (23, 35,
36). In all groups, including the controls, the distribution of alpha-cells was identical in islets and
islets-like structures of variable size. Both control and liraglutide dosed groups showed a number of
single glucagon positive cells in the exocrine area, single cells and small clusters of glucagon
positive cells associated to the epithelial lining of both main and smaller ducts. This finding of
small clusters and single endocrine cells is normal in Cynomolgus monkeys (34). A similar pattern
with single cells in the exocrine areas and associated to ductal structures was observed for beta-cells
and less frequently with delta-, and PP-cells, with no differences between groups. An apparently
dose-related increase in absolute pancreas weight was found in one study out of four, and only in
one sex, in nonhuman primate studies with liraglutide. Despite this apparently dose-related increase
in pancreas weight, no histopathology was associated thereto, and there were no PanIN lesions (13).

The four studies that have suggested adverse effect of DPP-4i’s or GLP-1RA’s on the pancreas
have described or discussed increased risk for pancreatitis, metaplasia or inflammation, pancreatic
adenocarcinomas and glucagonomas (15, 16, 25, 26). In contrast, hundreds of other studies have
investigated effects of these drugs on the pancreas but have not reported adverse effects; a few are referenced here for liraglutide (37-39). A study, performed after potential adverse findings had been reported by others, confirmed the absence of pathology in diabetic rats, and also did not show any regional differences in the pancreas induced by liraglutide when the pancreas was divided into four regions and examined by stereology (40). A recent publication uses a human islet amyloid polypeptide (hIAPP) transgenic model similar to one of the earlier studies, just in mice instead of rats, treats the animals for 1 year instead of 12 weeks, uses 20-25 animals in each group instead of 8, and finds no pancreas pathology associated with sitagliptin (25, 41). The Food and Drug Administration recently published that they have re-assessed data from fifty GLP-1 based therapeutics, and found no changes indicating pancreatic injury (42). To understand if there are any potential adverse effects of GLP-1RA’s on the human pancreas, the expression pattern of GLP-1R may be important. It has recently been recognized that most studies measuring the GLP-1R may be invalid as the antibodies used are not specific for GLP-1R (30, 43, 44). G-protein coupled receptors (GPCRs) are notoriously known for this problem (45-47). Some scientific journals have provided new guidance for validation experiments that must be available for reliable documentation of expression of a GPCR (48). However, there is valid studies available documenting GLP-1R expression (49). These studies measure receptor expression by ligand binding and they show that pancreatic adenocarcinomas do not express GLP-1R (49, 50). Thus, from a molecular target point of view it seems unlikely that GLP-1R agonism should directly worsen or induce pancreatic adenocarcinomas when such tumors do not express GLP-1R.

Based on the totality of information available to Novo Nordisk, there is insufficient information available to confirm or exclude an association between liraglutide and pancreatitis and there is no evidence that it increases the risk of pancreatic cancer in patients with type 2 diabetes. The
LEADER® cardiovascular outcome study (NCT01179048) will prospectively evaluate the overall safety of liraglutide. The trial has enrolled 9340 patients with type 2 diabetes and a high cardiovascular risk profile; patients are randomised 1:1 in a double blind study design to liraglutide or placebo and will be followed for a minimum of 42 months for the primary endpoint of adjudicated macrovascular events including non-fatal myocardial infarction, stroke, or cardiovascular death. Adjudication of all adverse reactions related to pancreatitis and any neoplasm is an integral part of the protocol throughout the duration of the LEADER® study. Randomized, controlled, long duration trials with independent adjudication are the only way to evaluate rare side effects, as also recently mentioned by Kahn and Drucker (29, 30). The LEADER® study will report in 2016.

Lotte Bjerre Knudsen is the guarantor of this manuscript, and takes full responsibility for the content.

Contributions:
CG performed the quantitative histology. IT reviewed the histopathological data and selected the pictures. AMM collected and reviewed parts of the data, and took part in drawing conclusions from the liraglutide studies. NCBN took part in drawing conclusions from the liraglutide studies. ZS designed the semaglutide studies, and concluded the results for those. LBK took part in drawing conclusions from the results of the studies and wrote the major part of abstract, introduction and discussion. ML wrote the major part of the methods and results section, and some parts of the
abstract and discussion, and performed the statistical analysis. All authors took part in reviewing the manuscript.

Conflicts of interest: Novo Nordisk markets liraglutide for the treatment of diabetes, and has semaglutide in phase 3 clinical development. All authors are full time employees of Novo Nordisk, and hold minor share portions as part of their employment.

Acknowledgements: Charles Pyke, Novo Nordisk, is thanked for careful reading of the manuscript and interpretations of the methodology used.
References


2. Campbell J, Drucker D: Pharmacology, physiology, and mechanisms of incretin hormone action. *Cell Metabolism.*


8. Buse JB, Garber A, Rosenstock J, Schmidt WE, Brett JH, Videbaek N, Holst J, Nauck M: Liraglutide treatment is associated with a low frequency and magnitude of antibody formation with no apparent impact on glycemic response or increased frequency of adverse events:
Results from the liraglutide effect and action in diabetes (LEAD) trials. *J Clin Endocrinol Metab.* 96:1695-1702, 2011


12. WHO guidelines on the quality, safety, and efficacy of biotherapeutic products prepared by recombinant DNA technology.


16. Butler AE, Campbell-Thompson M, Gurlo T, Dawson DW, Atkinson M, Butler PC: Marked expansion of exocrine and endocrine pancreas with incretin therapy in humans with increased
exocrine pancreas dysplasia and the potential for glucagon-producing neuroendocrine tumors.

_Diabetes._ , 2013


22. Novo Nordisk: Liraglutide (injection) for the treatment of patients with type 2 diabetes. NDA 22-341.


Tables

Table 1: Absolute mass (mg) for endocrine cell types, duct-, and acinar cells (mean±SEM) in pancreas from monkeys dosed with vehicle or liraglutide for 52 weeks.

<table>
<thead>
<tr>
<th>Group</th>
<th>Beta-cells (mg)</th>
<th>Alpha-cells (mg)</th>
<th>Delta-cells (mg)</th>
<th>PP-cells (mg)</th>
<th>Duct cells (mg)</th>
<th>Acinar cells (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle control, male</td>
<td>49.1±4.4</td>
<td>29.9±3.6</td>
<td>17.3±2.5</td>
<td>3.73±1.06</td>
<td>251±36</td>
<td>3319±454</td>
</tr>
<tr>
<td>Liraglutide 5 mg/kg/day, male</td>
<td>43.4±9.1, p=0.59</td>
<td>4.0±3.5, p=0.28</td>
<td>13.8±3.5, p=0.45</td>
<td>6.30±2.02, p=0.30</td>
<td>312±55, p=0.39</td>
<td>4563±482, p=0.11</td>
</tr>
<tr>
<td>Vehicle control, female</td>
<td>45.4±10.3</td>
<td>22.6±3.3</td>
<td>10.1±3.1</td>
<td>3.32±2.26</td>
<td>199±53</td>
<td>2694±450</td>
</tr>
<tr>
<td>Liraglutide 5 mg/kg/day, female</td>
<td>51.8±10.1, p=0.67</td>
<td>24.0±3.3, p=0.78</td>
<td>12.3±0.9, p=0.52</td>
<td>6.00±1.50, p=0.36</td>
<td>439±64, p=0.03</td>
<td>5325±469, p=0.007</td>
</tr>
</tbody>
</table>

n=4 per group. p-values compared to control group same sex.
Table 2: Summary of histopathological findings in 4, 13 and 52 weeks liraglutide studies and 13 and 52 weeks semaglutide studies.

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 week liraglutide (mg/kg/day)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Animals examined</td>
<td>3 3 3 3 3 3 3 3</td>
<td></td>
</tr>
<tr>
<td>Endocrine pancreas</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No abnormality detected</td>
<td>3 3 3 3 3 3 3 3</td>
<td></td>
</tr>
<tr>
<td>Exocrine pancreas</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No abnormality detected</td>
<td>3 3 3 3 3 3 3 3</td>
<td></td>
</tr>
</tbody>
</table>

| 13 week liraglutide (mg/kg/day) | 0 | 0.05 | 0.5 | 5 | 0 | 0.05 | 0.5 | 5 |
| Animals examined            | 4 | 4    | 4    | 4 | 4 | 4    | 4    | 4 |
| Endocrine pancreas          | 4 | 4    | 3    | 3 | 2 | 3    | 4    | 4 |
| No abnormality detected     | 4 | 4    | 3    | 3 | 2 | 3    | 4    | 4 |
| Prominent islets            | 0 | 0    | 1    | 1 | 1 | 0    | 0    | 0 |
| Fat infiltration, minimal   | 0 | 0    | 0    | 0 | 1 | 1    | 0    | 0 |

<p>| Exocrine pancreas          |                     |
| No abnormality detected    | 4 | 4    | 4    | 4 | 3 | 4    | 4    | 4 |
| Inflammatory cell infiltration, minimal | 0 | 0    | 0    | 0 | 1 | 0    | 0    | 0 |</p>
<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>52 week liraglutide (mg/kg/day)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 mg/kg/day</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>0.05 mg/kg/day</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>0.5 mg/kg/day</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>5.0 mg/kg/day</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Animals examined</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Endocrine pancreas</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No abnormality detected</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Exocrine pancreas</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No abnormality detected</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Fibrosis, focal, minimal</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>
| Inflammatory cell infiltration, focal, minimal | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0
| Ectopic splenic tissue       | 0     | 0       |

| 13 week semaglutide (mg/kg twice weekly) |       |         |
| 0 mg/kg twice weekly             | 4     | 4       |
| 0.004 mg/kg twice weekly         | 4     | 4       |
| 0.086 mg/kg twice weekly         | 4     | 4       |
| 0.47 mg/kg twice weekly          | 4     | 4       |
| Animals examined                 | 4     | 4       |
| Endocrine pancreas               |       |         |
| No abnormality detected          | 4     | 4       |
| Islet atrophy                    | 0     | 0       |
| Exocrine pancreas                |       |         |
| No abnormality detected          | 4     | 3       |
| Chronic focal inflammation, mild | 0     | 0       |
| Inflammatory cell foci, minimal  | 0     | 0       |

*4*
<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th></th>
<th></th>
<th></th>
<th>Females</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Focal brown pigment, minimal</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ectopic splenic tissue</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>52 week semaglutide (mg/kg twice weekly)</th>
<th>0</th>
<th>0.01</th>
<th>0.06</th>
<th>0.36</th>
<th>0</th>
<th>0.01</th>
<th>0.06</th>
<th>0.36</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animals examined</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

**Endocrine pancreas**

No abnormality detected 4 4 4 4 4 4 4 4

**Exocrine pancreas**

No abnormality detected 3 3 4 4 3 1 3 3

Focal arteritis/periarteritis, minimal 0 0 0 0 0 1 0 0

Focal interstitial inflammatory cell infiltration, minimal 1 1 0 0 2 1 1 1

Focal periductal inflammatory cell infiltration, slight 0 0 0 0 1 0 0 0

*: two animals were terminated after 36 days, see legend for figure 1 for details.
Table 3: Summary of dose related tendencies for absolute pancreas weight (g) across studies with liraglutide and semaglutide.

<table>
<thead>
<tr>
<th>Pancreas weight, tendency for dose related change?</th>
<th>Males</th>
<th>Females</th>
<th>Consistent in both sexes</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-week liraglutide</td>
<td>→*</td>
<td>→</td>
<td>No</td>
</tr>
<tr>
<td>13-week liraglutide</td>
<td>→</td>
<td>→</td>
<td>Yes</td>
</tr>
<tr>
<td>52-week liraglutide</td>
<td>→</td>
<td>→ →**</td>
<td>No</td>
</tr>
<tr>
<td>87-week liraglutide</td>
<td>→</td>
<td>→</td>
<td>Yes</td>
</tr>
<tr>
<td>13-week semaglutide</td>
<td>→</td>
<td>→</td>
<td>Yes</td>
</tr>
<tr>
<td>52-week semaglutide</td>
<td>→</td>
<td>→</td>
<td>Yes</td>
</tr>
</tbody>
</table>

*: p<0.05 for a statistically significant increase in absolute pancreas weight, but not apparently dose-related. **: p<0.01 for an apparently dose-related increase in absolute pancreas weight.
Figure legends

Figure 1: Absolute pancreas weight (g) in male and female control and liraglutide (s.c. once daily, A-D) or semaglutide (s.c. twice weekly, E and F) dosed nonhuman primates (horizontal line indicates group mean in each data set). A: 4 weeks’ liraglutide dosing, B: 13 weeks’ liraglutide dosing, C: 52 weeks’ liraglutide dosing, D: 87 weeks’ liraglutide dosing, E: 13 weeks’ semaglutide dosing and F: 52 weeks’ semaglutide dosing. M: male, F: female. Con M and Con F are historical control animals in panel A-C (from other studies of the same duration, run in the same facility). 0M and 0F are vehicle dosed animals on all graphs. A-C: 1M/1F were dosed with 0.05 mg/kg day liraglutide, 2M/2F with 0.5 mg/kg/day and 3M/3F with 5.0 mg/kg day. D: 1M/1F were dosed with 0.25 mg/kg/day liraglutide, 2M/2F with 5 mg/kg/day. E: 1M/1F were dosed with 0.004 mg/kg, 2M/2F with 0.086 mg/kg and 3M/3F with 0.47 mg/kg semaglutide twice weekly. F: 1M/1F were dosed with 0.01 mg/kg, 2M/2F with 0.06 mg/kg and 3M/3F with 5.0 mg/kg semaglutide twice weekly.

A: Males: \( p=0.036 \) by ANOVA across M0, M1, M2 and M3, \( p=0.054 \) vs 0M for 1M by post-test, \( p=0.18 \) by ANOVA across all groups including Ctrl M. Females: \( p=0.38 \) by ANOVA across F0, F1, F2 and F3, \( p=0.10 \) by ANOVA across all groups.

B: Males: \( p=0.73 \) by Kruskal-Wallis test across M0, M1, M2 and M3, \( p=0.66 \) by Kruskal-Wallis test across all groups. Females: \( p=0.93 \) by ANOVA across F0, F1, F2 and F3, \( p=0.92 \) by ANOVA across all groups.

C: Males: \( p=0.37 \) by ANOVA across M0, M1, M2 and M3, \( p=0.86 \) by ANOVA across all groups. Females: \( p=0.76 \) and \( p=0.07 \) vs 0F for 1F and 2F by post-test, \( p=0.005 \) by ANOVA across all groups, \( p=0.29 \), \( p=0.98 \) and \( p=0.08 \) for F1, F2 and F3 respectively compared to Con F by post-test.

D: Males: \( p=0.68 \) by ANOVA across all groups. Females: \( p=0.70 \) by ANOVA across all groups.
E: Males: p=0.36 by Kruskal-Wallis test across all groups. Females: p=0.025 by ANOVA across all
groups, p=0.30, p=0.09 and p=0.34 vs.F0 for F1, F2 and F3 by post-test.

F: Males: p=0.10 by ANOVA across all groups. Females: p=0.19 by ANOVA across all groups.

§: Animal terminated at 63 weeks’ on humane grounds. In the 13-week semaglutide study, the high
dose was reduced from 0.98 to 0.47 mg/kg after 6 weeks due to severe dehydration in 2 female
animals indicating that 0.98 mg/kg was not a tolerated dose. These two animals were terminated
after 36 days and not included in the analysis of pancreas weight, leading to a group size of 2.

Figure 2: Liraglutide studies in nonhuman primates, HE staining. Endocrine pancreatic islets from
males from 52 weeks’ study (left) and females from 87 weeks’ study (right) from control (upper
row) or liraglutide high-dose group (lower row). Well demarcated islets with normal looking pale
islet cells. Liraglutide dosed animals look similar to what is seen in control animals. 200X
magnification; bar indicates 100 µm.

Figure 3: Liraglutide studies in nonhuman primates, HE staining. Top row: 52 weeks’ study control
males. Second row: 87 weeks’ study control females, Third row: 52 weeks study high-dose males,
Bottom row: 87 weeks’ study high-dose females. Left: 200X magnification; bar indicates 100 µm.
Ductular part of the exocrine pancreas with presence of large interlobular ducts with columnar
epithelium. The duct is surrounded by connective tissue, but still located within the parenchyma.
The duct from high-dose group looks similar to that of the control monkeys. Right:
400X magnification; bar indicates 50 µm. Exocrine acinar cell parenchyma and ductular part of the
exocrine pancreas with presence of medium sized interlobular ducts with cuboidal epithelium. The
duct epithelium is lower and the amount of the surrounding connective tissue is lesser than for the
large ducts. The duct from high-dose group looks similar to that of the control monkeys. The acinar
secretory compartment consists of pyramid-shaped cells where the apical part is filled with eosinophilic zymogen granules and the basophilic basal part contains the nucleus. The secretory compartment from high-dose group looks similar to that of the control monkeys.

Figure 4: 52 weeks’ liraglutide study in nonhuman primates. Male (left) and female (right) from control (upper row) or liraglutide high-dose group (lower row). Double immunohistochemical staining for beta-cells (reddish brown) and non-beta cells (the sum of glucagon, somatostatin, and pancreatic polypeptide, violet/black). Islet structure and distribution of beta-, and non-beta cells from liraglutide dosed animals look similar to what is seen in control animals. 400x magnification; bar indicates 100 µm.

Figure 5: 52 weeks’ liraglutide study in nonhuman primates. Double immunohistochemical staining for glucagon (pink) and Ki-67 (black). Male (left) and female (right) from control (upper row) or liraglutide high-dose group (lower row). Glucagon staining shows high and variable number of alpha-cells in islets, and small number of single cells and small clusters of glucagon positive cells in the acinar exocrine pancreas and associated with ducts. Inserts show Ki-67 labelling in lymph nodes in the same sections. In total 15 of 32 monkeys had lymph nodes in these double stained sections but none of the high-dose treated male monkeys had lymph nodes in such sections. 200x magnification.
Figure 3
216x325mm (600 x 600 DPI)
Figure 4
58x43mm (600 x 600 DPI)
Figure 5
135x102mm (600 x 600 DPI)
Supplementary figure 1: Liraglutide studies in nonhuman primates. Males from 52-week study (left) and females from 87-week study (right) from control (upper row) or liraglutide high-dose group (lower row), ductular part of the exocrine pancreas with presence of large interlobular ducts with columnar epithelium. The duct is surrounded by connective tissue, but still located within the parenchyma. The duct from high-dose group looks similar to that of the control monkeys. HE staining; 400X magnification; bar indicates 50 µm.
Supplementary figure 2: 52-week liraglutide study in nonhuman primates. Male (left) and female (right) from control (upper row) or liraglutide high-dose group (lower row), exocrine acinar cell parenchyma. The acinar secretory compartment consists of pyramid-shaped cells where the apical part is filled with eosinophilic zymogen granules and the basophilic basal part contains the nucleus. The secretory compartment from high-dose group looks similar to that of the control monkeys. HE staining; 400Xmagnification; bar indicates 50 µm.
Supplementary figure 3: 52-week liraglutide study in nonhuman primates. Male (left) and female (right) from control (upper row) or liraglutide high-dose group (lower row). Double immunohistochemical staining for glucagon (pink) and Ki-67 (black). Islets from liraglutide dosed animals look similar to what is seen in control animals with high and variable number of alpha-cells and no staining for Ki-67. Inserts show Ki-67 staining in lymph nodes in the same sections. In total 15 of 32 monkeys had lymph nodes in these double stained sections, but none of the high-dose treated male monkeys had lymph nodes in such sections. Differences in the proliferative activity in lymph nodes from individual animals were observed, but no pattern was detected between males and females or control and liraglutide dosed animals. 400x magnification; bar indicates 100 µm.
Supplementary table 1: Overview of dose levels, duration and group sizes for nonhuman primate studies with liraglutide (daily dosing) and semaglutide (twice weekly dosing)

<table>
<thead>
<tr>
<th>Study group</th>
<th>Dose level (mg/kg) and group size (n/sex)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle control</td>
</tr>
<tr>
<td>4-week liraglutide</td>
<td>0 (3)</td>
</tr>
<tr>
<td>13-week liraglutide</td>
<td>0 (4)</td>
</tr>
<tr>
<td>52-week liraglutide</td>
<td>0 (4)</td>
</tr>
<tr>
<td>87-week liraglutide</td>
<td>0 (5)*</td>
</tr>
<tr>
<td>13-week semaglutide</td>
<td>0 (4)</td>
</tr>
<tr>
<td>52-week semaglutide</td>
<td>0 (4)</td>
</tr>
</tbody>
</table>

* One male was terminated on humane grounds during Week 63. The data from this animal was included in the analysis.

† In the 13-week semaglutide study, the high dose was reduced from 0.98 to 0.47 mg/kg after 6 weeks due to severe dehydration in 2 female animals indicating that 0.98 mg/kg was not a tolerated dose. These two animals were terminated after 36 days and not included in the analysis of pancreas weight, leading to a group size of 2.

NA: Dose level not included in study