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Reanalysis of a recent study on potential adverse pancreatic effects of incretin therapy: methodological deficiencies

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

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

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Inappropriate cohorts for comparisons

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

Additionally, the diagnoses of several of the control “type 2 diabetes” cases are open to question. Some seem more likely to have had type 1 diabetes; in particular, 4 cases were diagnosed with diabetes at a young age and two of these had documented diabetic ketoacidosis (DKA) (nPOD 6110, nPOD 6059) and two others had positive auto-antibodies (nPOD 6149, nPOD 6142); a fifth case nPOD 6109 (age 48.8 y), who was listed as “preclinical” in the database, had insulin auto-antibodies and hemoglobin A_{1c} of 8. Even several of the non-diabetic controls are problematic since nPOD 6015 had had a gastric bypass, nPOD 6158 was positive for both GAD and IAA auto-antibodies and was classified as “prediabetic type 1 diabetes,” and nPOD 6097 was listed as “preclinical type 2 diabetes” with hemoglobin A_{1c} of 7.1.

Certainly there can be difficulty making a distinction between type 1 diabetes and type 2 diabetes, but cases with an ambiguous diagnosis should not have been included in the study. If such ambiguous patients and those under 40 years of age are omitted from the study, only 5 out of 12 type 2 diabetes controls and 8 of 14 non diabetic controls remain. Yet, within the nPOD database, there are 2 subjects with type 2 diabetes and 9 without diabetes (ND) above the age of 40 that were not included in the study with no explanation provided as to why they were excluded.

Methodological problems with quantification

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

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

Questionable conclusions due to the above problems

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

Pancreatic weight was 40% increased in DM-I compared with DM ($P < 0.05$)

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

β -cell mass was decreased [55%] in [control] DM compared with ND but was approximately sixfold increased in DM-I compared with DM

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

Pancreas from type 2 diabetes cases with incretin therapy have “alpha cell hyperplasia and glucagon microadenomas (3/8)...”

The reported increased percentage of alpha cell relative area and mass after incretin therapy are both questionable due to the above mentioned issues of mismatched age comparisons

and methodological issues. However, in many of the included older cases with diabetes (with or without incretin therapy) we observed significant numbers of alpha cells adjacent to or within the ductal epithelium associated with PanINs and focal chronic pancreatitis-like changes (Figure 5); there was no obvious difference between type 2 diabetes +I and control type 2 diabetes pancreas. Additionally the criteria used to distinguish between microadenoma and large hyperplastic islets were not given although this remains an unsettled issue in the field (9). We observed microadenoma/hyperplastic glucagon-rich islets (300 μ m or larger) in about half of the over 40 year-old cases, including the non diabetic cases.

One type 2 diabetes case with incretin therapy had “a neuroendocrine tumor”

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

Type 2 diabetes with incretin therapy reported to have increased PanINs

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

Type 2 diabetes with incretin therapy reported to have “increased exocrine proliferation”

Immunostaining for the antigen Ki67 indicates cells that are in cell cycle, which is equated with cell division (proliferation). We observed Ki67 positive cells to be preferentially located within PanINs in all study groups, with little to no Ki67 positivity in other pancreatic cell types. Since the occurrence of PanINs is age-dependent (8), age-matched comparisons for proliferating cells are critical. Without well-matched controls, no link between incretin therapy and increased Ki67 positivity can be established. Additionally Ki67 positivity has been reported increased in all pancreatic cell types under conditions of prolonged life support (18), but such clinical data are not available for these nPOD cases.

The only finding we were not able to reanalyze was the increased co-localization of insulin and glucagon (16% in incretin treated vs 3% in type 2 diabetes)(1); although the slides are from the same nPOD cases, images of the immunofluorescent staining are not in the nPOD database. Increased incidence of hormone co-localizing cells in type 2 diabetes was recently reported using surgical resections (20) but at a much lower frequency, only 0.82% in newly

diagnosed type 2 diabetes and 0.33% in long standing type 2 diabetes. The finding in itself is thus interesting and should be examined in more detail, provided suitably matched groups are used.

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

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

Figure 2.

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

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

Figure 4.

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

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

Table 1

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

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