

# The DPP4 inhibitor linagliptin delays the onset of diabetes and preserves $\beta$ -cell mass in non-obese diabetic mice

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## Abstract

Recent data indicate that dipeptidyl peptidase 4 (DPP4) inhibitors have anti-inflammatory and  $\beta$ -cell-sparing effects in animal models of type 1 diabetes. To evaluate the effects of the DPP4 inhibitor linagliptin on  $\beta$ -cell mass and insulinitis, we examined the progression of diabetes (blood glucose >11 mmol/l) in non-obese diabetic (NOD) mice with terminal stereological assessment of cellular pancreatic changes. Female NOD mice were fed a normal chow diet or a diet containing linagliptin 0.083 g/kg chow for 60 days. At study end, the incidence of diabetes in linagliptin-treated mice was reduced by almost 50% compared with vehicle (10 of 31 mice

vs 18 of 30 mice,  $P=0.021$ ). The total islet mass and total  $\beta$ -cell mass, identified by insulin immunoreactivity, were greater in non-diabetic linagliptin-treated mice compared with non-diabetic vehicle-treated mice ( $P<0.01$  for both) but were greatly reduced in diabetic mice irrespective of treatment. No changes were seen in the  $\alpha$ ,  $\delta$  and  $\gamma$  endocrine cell pool. Moreover, the total mass of lymphocyte insulinitis was significantly reduced in linagliptin-treated mice compared with vehicle. The data indicate that linagliptin treatment delays the onset of diabetes in NOD mice by protecting  $\beta$ -cell mass.

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## Introduction

The incretin hormone glucagon-like peptide 1 (GLP1) is a gut peptide that is secreted in response to nutrient ingestion. It enhances the glucose-dependent stimulation of insulin secretion and also controls blood glucose (BG) via inhibition of glucagon secretion and gastric emptying (Holst 2007). Long-term treatment with GLP1 receptor agonists reduces food intake and promotes weight loss in both preclinical experiments and clinical studies on patients with or without type 2 diabetes (T2D; Drucker 2006). GLP1 is rapidly degraded in the body by dipeptidyl peptidase 4 (DPP4); thus, inhibition of this enzyme increases GLP1 exposure and thereby its effect on the pancreas (Drucker 2007). For this reason, inhibitors of DPP4 are effective as oral anti-diabetes drugs. Linagliptin, a potent DPP4 inhibitor, is currently approved for the treatment of T2D (Eckhardt *et al.* 2008, Deacon & Holst 2010). In rats, as well as in humans, linagliptin exhibits nonlinear pharmacokinetics and shows concentration-dependent plasma protein binding to DPP4 (Fuchs *et al.* 2009, Heise *et al.* 2009).

GLP1 is believed to exert its incretin actions through specific GLP1 receptors expressed on pancreatic  $\beta$ -cells (Holz 2004). Studies of GLP1 analogues and DPP4 inhibitors on  $\beta$ -cell mass have demonstrated positive effects, either by direct stimulation of  $\beta$ -cell replication or by inhibition of apoptosis

and activation of anti-apoptotic pathways (Xu *et al.* 1999, Stoffers *et al.* 2000, Drucker 2003, Cho *et al.* 2011, Dobrian *et al.* 2011). Thus, besides their use for the treatment of T2D, GLP1 analogues and DPP4 inhibitors may also possess important cytoprotective and regenerative properties.

As the principal mechanism of action of DPP4 inhibitors is based on the amplification of insulin release via the increase of endogenous GLP1 levels, their use for the treatment of T1D is uncertain given the autoimmune T-cell-dependent eradication of the insulin-producing  $\beta$ -cells. However, increased GLP1 levels may modify the natural course of  $\beta$ -cell destruction and/or regeneration, resulting in detectable improvements in insulin secretion and glucose control. Indeed, Kutoh (2011) reported beneficial effects of the DPP4 inhibitor sitagliptin as add-on therapy to insulin in reducing BG levels in a small sample of patients with T1D. In addition, sitagliptin was shown to preserve islet transplants in non-obese diabetic (NOD) mice (Kim *et al.* 2009, 2010). This mouse model spontaneously develops a form of insulin-dependent diabetes similar to the human disease (Delovitch & Singh 1997, Anderson & Bluestone 2005). In this respect, it is worth noting that DPP4 is identical to CD26, a T-cell antigen that plays a role in T-cell-mediated responses (Morimoto & Schlossman 1998).

The purpose of the current study was to evaluate the effects of the DPP4 inhibitor linagliptin on  $\beta$ -cell mass and insulinitis during the progression of diabetes in female NOD mice.

## Materials and Methods

### Animals

A total of 65 female NOD mice (8–9 weeks of age) were obtained from Taconic (Ry, Denmark) and transferred to the Gubra animal stables. On arrival to the animal unit and for the remainder of the study, mice were housed in rodent cages (5–6 per cage) with *ad libitum* access to food and fresh tap water. The animal room environment was controlled within targeted ranges (temperature,  $22 \pm 2$  °C; relative humidity,  $50 \pm 10\%$ ; 12 h light:12 h darkness cycle, lights on from 0600 h).

All animal experiments were conducted in accordance with Gubra Bioethical Guidelines, which are fully compliant with internationally accepted principles for the care and use of laboratory animals. The described experiments were covered by personal licenses for J J (2008/561-1565) issued by the Danish Committee for animal research.

### In vivo procedures

Three weeks after arrival, non-fasting BG levels were measured at 0900 h (Biosen, EKF Diagnostics, Magdeburg, Germany). Animals were then randomised according to BG and body weight into two groups: a vehicle group ( $n=30$ ) with *ad libitum* access to a Sniff Rat-Maintenance diet (Sniff RM, Soest, Germany); or a linagliptin group ( $n=31$ ) with *ad libitum* access to a Sniff RM diet including linagliptin 0.083 g/kg (equivalent to plasma levels of an oral dose of linagliptin of 3–10 mg/kg daily). Four animals were excluded from the study as they had already developed diabetes (BG > 11 mmol/l).

Body weight and mean food intake were measured bi-weekly for the first 3 weeks, then once weekly for the remainder of the study. BG was measured on full blood samples once weekly (10  $\mu$ l obtained in LiHe-coated capillaries) using a glucose oxidase method (Biosen C-line Clinic, EKF Diagnostics). All samples were taken between 2000 and 2100 h. At experimental day 56, all mice were killed using CO<sub>2</sub> anaesthesia followed by decapitation. Terminal blood samples were collected in Na-EDTA tubes containing 1  $\mu$ l DPP4 inhibitor (Millipore (Billerica, MA, USA) catalogue no. DPP4-010, 10  $\mu$ l/ml blood). Plasma was separated and duplicate samples were analysed for active GLP1 using an active GLP1 ELISA kit (Shibayagi, Gunma, Japan) according to the manufacturer's standard procedures.

### Pancreas analyses

The pancreas was removed 'en bloc' and immersion fixed in 4% phosphate-buffered formaldehyde. The dissected pancreata were weighed, dehydrated and infiltrated overnight in paraffin using a VIP-100 histokinette (Sakura) and embedded as whole mounts in blocks of paraffin. Each block was trimmed and three series of 5  $\mu$ m sections were sampled using systematic uniform random sampling principles providing sections at seven to ten levels in total. Sections were mounted

on slides and subsequently subjected to standard haematoxylin–eosin staining for pathological scoring and double immunohistochemical staining procedures for stereological assessment of  $\beta$ -cell mass (insulin immunoreactivity) and non- $\beta$ -cell mass (using an antibody cocktail against pancreatic polypeptide, somatostatin and glucagon). One series was used for double immunohistochemical staining against insulin and the nuclear proliferation marker Ki67, and one series was used to identify and assess CD3 immunoreactivity.

The immunohistochemical staining was performed as follows: after deparaffinisation and microwave oven treatment in citrate buffer (pH 6) for 15 min at 90 °C, sections were stained manually for non- $\beta$ -cells using an antibody cocktail consisting of rabbit anti-glucagon (1:1000, H-028-02, Phoenix Pharmaceuticals, Burlingame, CA, USA), rabbit anti-somatostatin (1:1600, A566) and rabbit anti-pancreatic polypeptide (1:1000, B32-1, Euro-Diagnostica, Malmö, Sweden), followed by a biotinylated F(ab')<sub>2</sub> fragment antibody (1:2000, 711-066-152, Jackson ImmunoResearch Laboratories, West Grove, PA, USA), HRP complex (1:400, P0397) and development with diaminobenzidine and NiSO<sub>4</sub>. This was followed by staining  $\beta$ -cells using a guinea pig anti-insulin (1:100, A0564) combined with HRP-coupled rabbit anti-guinea pig (1:100, P0141) and development with NovaRED (Vector Laboratories, Burlingame, CA, USA). All reagents, including normal sera for blocking, were obtained from DakoCytomation (Copenhagen, Denmark). Double immunohistochemical staining against Ki67 and insulin was performed using a similar approach with the only change being the use of a rat anti-mouse Ki67 antibody (M724, DAKO) diluted 1:400, a secondary non-biotinylated rabbit anti-rat (AI-4001, Vector Laboratories) diluted 1:200 and amplification for 30 min using a MACH 2 goat anti-rabbit HRP-Polymer (RHRP520H, Biocare Medical, Concord, CA, USA). CD3 lymphocytes were detected using an anti-CD3 antibody (CMC363, Cell Marque, Rocklin, CA, USA) diluted 1:500.

### Stereological estimation of $\beta$ -cell mass and lymphocyte insulinitis

The stereological estimation of  $\beta$ -cell mass was performed by an observer blinded to the experimental groups.  $\beta$ -cell mass was estimated by point counting with all points hitting the structure of interest being counted. Sections were scanned in a random systematic way using the newCAST system (Visiopharm, Hørsholm, Denmark) to control the stage and collection of data. A single-point grid per frame was used to estimate pancreas mass and a denser grid (adjusted between animal groups to ensure the accumulation of roughly equal number of 'hits' per sample) was used to estimate  $\beta$ -cell/non- $\beta$ -cell and lymphocyte mass. Similarly, the grid system was used to correct the presence of non-pancreatic elements in the dissected sample. In principle, the point grid is used to estimate the area fraction of counted cell types. The number of points hitting the structure of interest is then converted into mass by taking the grid ratio into consideration

(Bock *et al.* 2003). In general,  $\sim 100$ – $150$  points were counted hitting each structure of interest. The Ki67 proliferation index was calculated using a 2D counting frame superimposed onto the sections. Ki67-labelled  $\beta$ -cells and lymphocytes were counted manually and related to the total area. A mean of 130 Ki67-positive  $\beta$ -cells and 215 proliferating lymphocytes were counted per animal.

### Statistical analysis

Graphical presentations, calculations and statistical analyses were carried out with GraphPad Software (GraphPad Prism version 5.04 for Windows, San Diego, CA, USA; [www.graphpad.com](http://www.graphpad.com)). Statistical analysis of cumulative BG, body weight, food intake and water intake levels was performed using a two-way ANOVA with repeated measures. *Post hoc* analyses were performed in cases with statistical significances ( $P < 0.05$  considered significant; Newman–Keuls *post hoc* analysis). Statistical analysis of individual BG data (i.e. non-diabetic vs diabetic proportions) was performed using Fisher's exact test. All pancreatic measurements were performed using one-way ANOVA followed by Tukey's multiple comparison test.

## Results

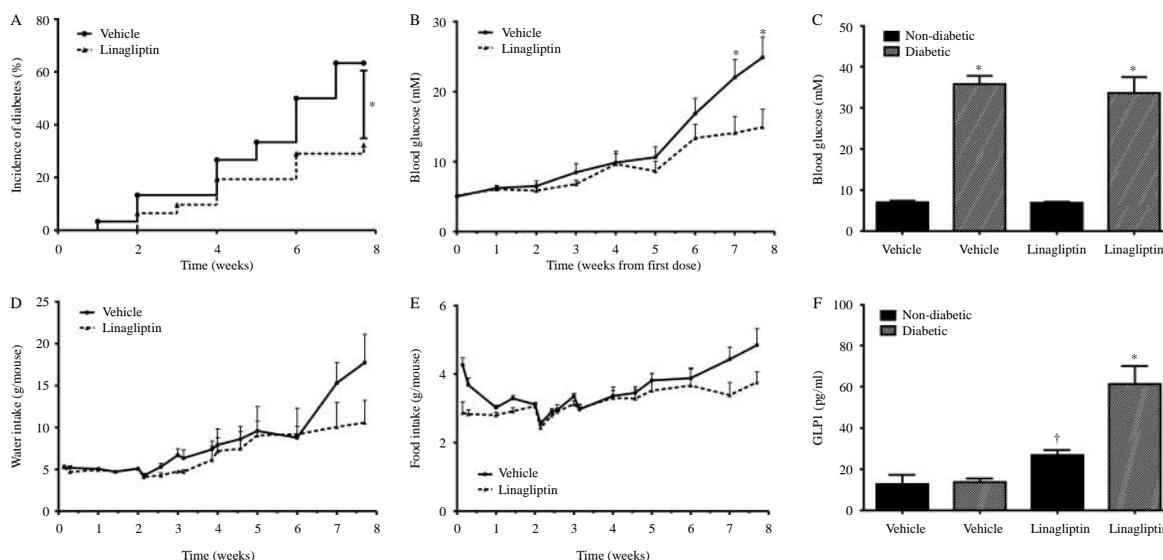
### Development of T1D in the NOD mice

The progression of diabetes over time is shown in Fig. 1A. After 8 weeks of treatment, the incidence of diabetes

(animals with BG  $> 11$  mmol/l) in the linagliptin group was reduced by almost 50% compared with the vehicle group (10 of 31 mice vs 19 of 30 mice developing T1D respectively;  $P = 0.021$ ). Mean levels of BG in mice treated with linagliptin were significantly decreased compared with the vehicle group at the end of the study ( $14.91 \pm 2.54$  vs  $24.88 \pm 2.91$  mM;  $P < 0.05$ ; Fig. 1B) due to the lower number of diabetic animals. No treatment-related differences were observed in BG levels between the diabetic and non-diabetic subgroups (Fig. 1C). Both water and food intake (measured as mean per cage) tended to increase in the vehicle group towards the end of the study, most likely because of the increased number of diabetic animals in this group (Fig. 1D and E). By contrast, no changes in overall body weight were seen between the linagliptin and vehicle groups ( $24.12 \pm 0.41$  vs  $23.17 \pm 0.54$  g). As expected, terminal plasma levels of active GLP1 were significantly increased in the linagliptin group compared with vehicle ( $37.49 \pm 4.34$  vs  $13.38 \pm 1.97$  pg/ml;  $P < 0.01$ ). A subgroup analysis of non-diabetic and diabetic mice revealed that diabetic mice treated with linagliptin had highly elevated GLP1 levels at the study end compared with non-diabetic mice treated with linagliptin ( $61.29 \pm 8.69$  vs  $26.78 \pm 2.54$  pg/ml;  $P < 0.05$ ; Fig. 1F). This finding was most likely a direct consequence of increased linagliptin exposure in the diabetic mice due to the increased food intake.

### Stereological assessment of $\beta$ -cell mass

No changes were seen in total pancreas mass corrected for non-pancreatic tissue elements (fat and lymph tissue parts) between the linagliptin and vehicle groups



**Figure 1** The development of diabetes in NOD mice. The progression of diabetes over time (A;  $*P < 0.05$  vs vehicle), and the effects of linagliptin vs vehicle treatment on mean blood glucose (B;  $*P < 0.05$  vs vehicle), subgroup blood glucose levels (C;  $*P < 0.05$  diabetic vs non-diabetic), water intake (D) and food intake (E) in NOD mice during the 8-week study period. Terminal plasma levels of active GLP1 in NOD mice (F;  $*P < 0.05$  vs non-diabetic linagliptin-treated mice;  $^{\dagger}P < 0.05$  vs non-diabetic vehicle-treated mice).

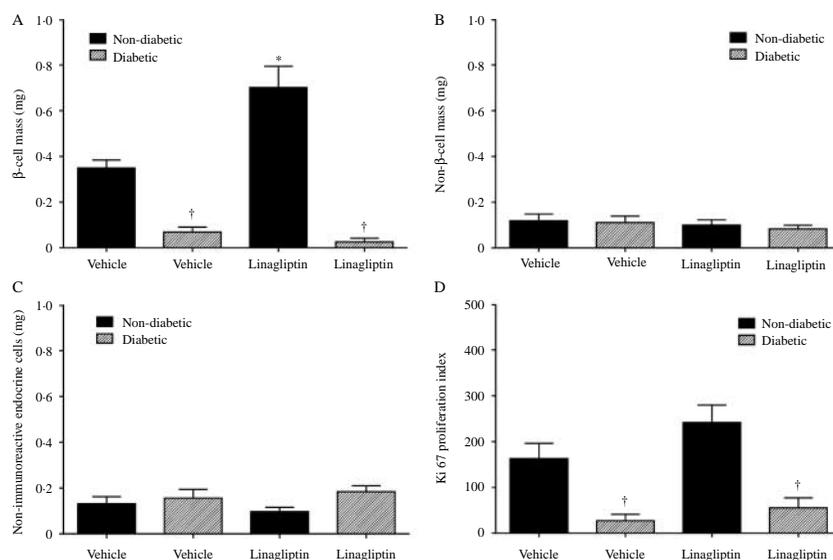
( $277.78 \pm 5.65$  vs  $287.24 \pm 10.0$  mg). The quantitative analyses of immunohistochemically stained islet cell types demonstrated a significantly higher  $\beta$ -cell mass in non-diabetic linagliptin-treated mice compared with non-diabetic vehicle ( $0.70 \pm 0.093$  vs  $0.35 \pm 0.035$  mg), with a highly significant reduction in the diabetic groups irrespective of treatment (Fig. 2A). No significant alterations were observed in non- $\beta$ -cell mass (Fig. 2B) or unstained endocrine cell mass (Fig. 2C). No changes were observed in Ki67 proliferation index between the non-diabetic linagliptin and vehicle groups (number of proliferating  $\beta$ -cell profiles per  $\beta$ -cell area), whereas the index was greatly reduced in diabetic mice (Fig. 2D). The total mass of lymphocyte insulinitis seen in and surrounding the pancreatic islets (Fig. 3) was significantly lower in the non-diabetic linagliptin-treated mice compared with vehicle ( $0.89 \pm 0.16$  vs  $1.60 \pm 0.19$  mg; Fig. 4A), as well as in both diabetic groups (Fig. 4A). A similar pattern was observed for proliferating Ki67 immunoreactive lymphocytes (Fig. 4B). Lymphocytes staining positively for CD3 are shown in Fig. 3F.

## Discussion

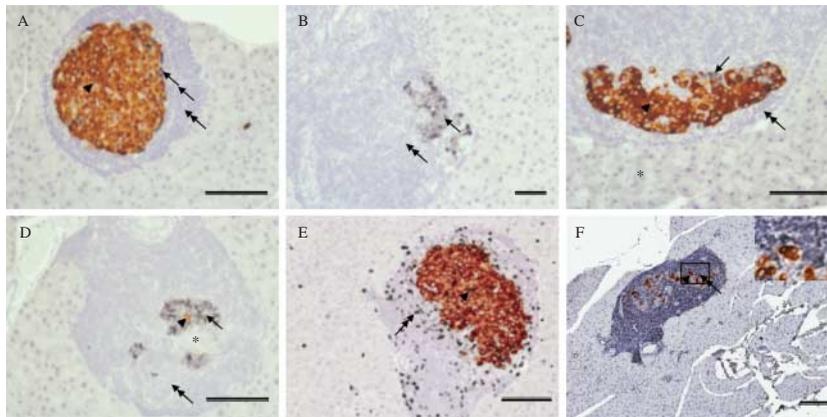
T1D is a multifactorial autoimmune disease characterised by mononuclear cell infiltration in the pancreatic islets (Castano & Eisenbarth 1990, Atkinson & Eisenbarth 2001), leading to an immune response and selective destruction of insulin-producing  $\beta$ -cells. The NOD mouse spontaneously develops autoimmune diabetes and is regarded as a suitable model of human T1D (Delovitch & Singh 1997, Anderson & Bluestone 2005). As in humans, a complex mixture of

mononuclear cells infiltrates the islets of NOD mice from 3 weeks of age, leading to the gradual loss of  $\beta$ -cells (Reddy *et al.* 2000). In this study, we have demonstrated that the DPP4 inhibitor linagliptin reduced the incidence of diabetes and increased  $\beta$ -cell mass in female NOD mice, thereby indicating that linagliptin prevents the autoimmune destruction of pancreatic  $\beta$ -cells.

It still remains unclear whether the effects of DPP4 inhibition on  $\beta$ -cell sparing are mediated through incretins acting directly on  $\beta$ -cells or through incretin-independent effects. GLP1 analogues have received much attention for their potential role in restoring  $\beta$ -cell mass in preclinical models of T2D (Xu *et al.* 1999, Stoffers *et al.* 2000, Rolin *et al.* 2002, Wang & Brubaker 2002). Among the proposed mechanisms underlying this effect are stimulation of cellular proliferation, inhibition of apoptosis and recovery of residual cellular mass (Hadjiyanni *et al.* 2008, Shimoda *et al.* 2011). However, GLP1 is also known to play an immunomodulatory role in rodents, with the GLP1 receptor being expressed in immune cells from the spleen, thymus and lymph nodes of both non-diabetic and diabetic NOD mice (Sherry *et al.* 2007, Zhang *et al.* 2007, Hadjiyanni *et al.* 2008). GLP1 receptor activation has been shown to directly reduce apoptotic cell death after exposure to a diverse number of toxins and cytokines that are likely to contribute to  $\beta$ -cell destruction (Buteau *et al.* 2004). In addition, lymphocytes contain significant amounts of membrane-bound DPP4 (Engel *et al.* 2003), and both splenic DPP4 responsive and lymph node incretin non-responsive T-cell lymphocytes have been shown to infiltrate islets of diabetic NOD mice leading to  $\beta$ -cell destruction (Kim *et al.* 2010). Thus, DPP4 inhibition



**Figure 2** Stereological analyses of NOD mice pancreata. Changes in  $\beta$ -cell mass (A), non- $\beta$ -cell mass (B), non-immunoreactive endocrine cells (C) and Ki67 proliferation index (D) in the linagliptin subgroups compared with vehicle at the end of the 8-week study period. \* $P < 0.05$  vs non-diabetic vehicle-treated mice; † $P < 0.05$  vs non-diabetic mice.

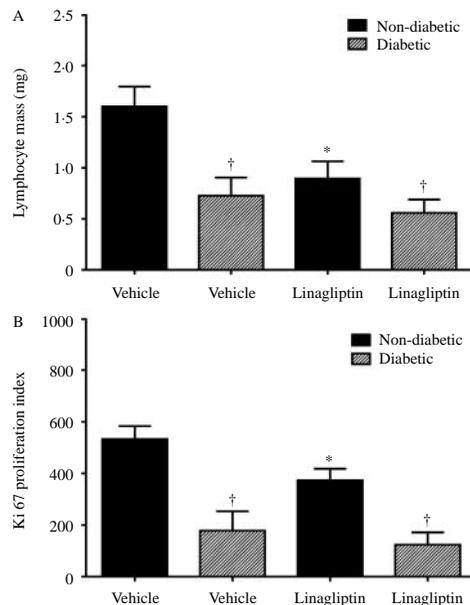


**Figure 3** Islet morphology in NOD mice. Immunohistochemical localisation of  $\beta$ -cells (arrowhead, brown), non- $\beta$ -cells (black, arrow) and infiltrating lymphocytes (double arrow) in non-diabetic vehicle (A), diabetic vehicle (B) and diabetic linagliptin-treated (C) and diabetic linagliptin-treated mice (D). Proliferating Ki67 immunoreactive lymphocytes (double arrow) and  $\beta$ -cells (arrowhead) are shown in E. Anti-CD3 immunoreactive lymphocytes (black, double arrow) surrounding  $\beta$ -cells (brown, arrowhead) are shown in F. Scale bar = 100  $\mu$ m. \*Non-stained endocrine cells.

may exert anti-inflammatory effects in the pancreatic islets in addition to an insulinotropic effect.

A number of studies have previously indicated a beneficial effect of DPP4 inhibition on  $\beta$ -cell mass in different animal models of T1D. Kim *et al.* (2009) demonstrated that sitagliptin was able to reduce the incidence of diabetes in islet-transplanted NOD mice coupled with increased  $\beta$ -cell area and decreased insulinitis (scored as decreased mononuclear cell infiltration). Cho *et al.* (2011) demonstrated that long-term administration of the novel DPP4 inhibitor DA-1229 increased the mass, replication and neogenesis of  $\beta$ -cells in streptozotocin-treated mice. In partial agreement with these data, we demonstrate that non-diabetic linagliptin-treated mice have a markedly higher  $\beta$ -cell mass than their non-diabetic vehicle-treated litter mates. No significant changes were observed in the Ki67 proliferation index of  $\beta$ -cells, indicating that the higher  $\beta$ -cell mass is not an incretin effect on  $\beta$ -cell neogenesis and replication, but rather a  $\beta$ -cell-sparing effect coupled with improvements in glucose homeostasis, or a specific anti-inflammatory effect of DPP4 leading to an increased  $\beta$ -cell survival. This latter interpretation is supported by the decreased mass of infiltrating lymphocytes surrounding the islets in non-diabetic linagliptin-treated mice. Because no changes were observed in non- $\beta$  ( $\alpha$ ,  $\delta$  and  $\gamma$ )-cell mass or in the mass of non-immunoreactive endocrine cells, it appears that the anti-inflammatory effects are  $\beta$ -cell specific. The exact phenotype of the unstained endocrine cells is currently not known. The evaluation of  $\beta$ -cell-specific MafA and PDX1 antibodies was inconclusive, although it is well known that the expression of both markers is highly susceptible to hyperglycaemia in diabetic conditions (Kaneto *et al.* 2009). However, as this pool of cells was not significantly altered across the treatment groups, it does not have an overall importance for the presented findings on  $\beta$ -cell mass. The identification of lymphocytes was based on a histochemical

staining protocol that allows for a clear discrimination of infiltrating cells against the exocrine acinar and duct cells, as well as the immunohistochemically detected endocrine cells, in accordance with previously applied protocols (Hadjiyanni *et al.* 2008, Koulmanda *et al.* 2008, Kim *et al.* 2009). Further immunohistochemical analyses demonstrated



**Figure 4** The total mass of lymphocyte insulinitis in non-diabetic and diabetic linagliptin- and vehicle-treated mice. Insulinitis was significantly lower in non-diabetic linagliptin-treated mice compared with vehicle (A). Moreover, the mass of lymphocytes was reduced in all diabetic groups compared with vehicle (A). A similar pattern was observed in the Ki67 proliferation index (B). \* $P < 0.05$  vs non-diabetic vehicle-treated mice; † $P < 0.05$  vs non-diabetic mice.

that the infiltrating lymphocytes were mainly CD3-positive T-cells that have previously been considered an important contributor to the development of T1D in NOD mice (Fornari *et al.* 2011). The mechanism behind the reduced mass of lymphocytes observed in diabetic mice is presently unknown, but it is likely that the inflammatory process will diminish following the onset of diabetes. A similar pattern was observed for  $\beta$ -cell mass, being markedly reduced in both diabetic groups irrespective of treatment. These data suggest that the onset of diabetes occurs when the mass of  $\beta$ -cells reaches a critical size.

In conclusion, we demonstrate that the DPP4 inhibitor linagliptin is able to reduce the incidence of diabetes and increase the  $\beta$ -cell mass in female NOD mice. Moreover, linagliptin is able to reduce lymphocyte insulinitis. The exact mechanism of action of these effects is currently not known, but the pathogenesis of T1D would appear to suggest a mechanism in which linagliptin inhibits the DPP4-mediated stimulation of autoimmune T-cell activation and islet infiltration. Further studies are required to evaluate this hypothesis.

#### Declaration of interest

J J, N V and S B v W are employees of Gubra ApS, Denmark. M M and T K are full-time employees of Boehringer Ingelheim Pharma GmbH & Co. KG, Germany. The authors were fully responsible for all content and editorial decisions, were involved at all stages of manuscript development and have approved the final version.

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