Chronic administration of Glucagon-like peptide-1 agonists improves trabecular bone mass and architecture in ovariectomised mice

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Abstract

The increased incidence of type 2 Diabetes Mellitus among the aged is associated with an impaired skeletal structure and a higher prevalence of bone fractures. In addition, anti-diabetic therapies can also affect bone mass. Here, we tested the skeletal effects of chronic administration of two Glucagon-like peptide-1 receptor (GLP-1R) agonists and examined the expression and activation of GLP-1R in bone cells. Mice were ovariectomised (OVX) to induce bone loss and four weeks later they were treated with Liraglutide (LIR) 0.3 mg/kg/d, Exenatide (Ex-4) 10 µg/kg/d or saline for four weeks. Mice were injected with calcein and alizarin red prior to euthanasia, to label bone-mineralising surfaces. Tibial micro-architecture was determined by micro-CT and bone formation and resorption parameters measured by histomorphometric analysis. Serum was collected to measure calcitonin and sclerostin levels, inhibitors of bone resorption and formation, respectively. GLP-1R mRNA and protein expression were evaluated in bone, bone marrow and bone cells using RT-PCR and immunohistochemistry. Primary osteoclasts and osteoblasts were cultured to evaluate the effect of GLP-1 agonists on bone resorption and formation *in vitro*. GLP-1 agonists significantly increased trabecular bone mass, connectivity and structure parameters but had no effect on cortical bone. There was no effect of GLP-1 agonists on bone formation *in vivo* but an increase in osteoclast number and osteoclast surfaces was observed with Ex-4. GLP-1R was expressed in bone marrow cells, primary osteoclasts and osteoblasts and in late osteocytic cell line. Both Ex-4 and LIR stimulated osteoclastic differentiation *in vitro* but slightly reduced the area resorbed per osteoclast. They had no effect on bone nodule formation *in vitro.* Serum calcitonin levels were increased and sclerostin levels decreased by Ex-4 but not by LIR. Thus, GLP-1R agonists can have beneficial effects on bone and the expression of GLP-1R in bone cells may imply that these effects are exerted directly on the tissue.

Introduction

Osteoporosis, osteoarthritis, joint deformities and fractures affect a large proportion of the elderly population and represent important causes of morbidity. There is now a growing body of evidence that the incidence of these conditions is significantly increased in the presence of type 1 and 2 diabetes mellitus (DM) (1-5). Clinical data indicate that bone of diabetic patients is fragile and of poor quality, despite a bone mineral density (BMD) that is often normal. Circulating levels of sclerostin, a negative regulator of bone formation produced by osteocytes, are elevated in type 2 diabetic patients (6). Although the mechanisms leading to the poor bone strength and quality in DM patients are not entirely known, accumulation of advanced glycation end products, changes in collagen cross-linking and suppression of bone turnover are significant contributors (7-9). In addition to the effect of DM itself on bone, adverse impacts on bone health of some anti-diabetic drugs, such as thiazolidinediones (TZDs), have been reported. Several studies have indeed shown that TZDs increase fracture risk (10,11). In contrast, metformin, another widely prescribed anti-diabetic drug (12), is osteogenic *in vitro* (13,14) and reduces the risk of fracture in DM patients (15), as well as inhibiting the bone loss induced by ovariectomy (OVX) in rats (16,17). However, our recent studies showed no beneficial effect of metformin on bone mass and fracture healing in rodents (18).

Incretin hormones such as Glucagon-like-peptide 1 (GLP-1), GLP-2 and Glucose-dependent insulinotropic peptide (GIP) are peptides secreted in the gastrointestinal tract in response to ingestion of nutrients (19) with insulin-independent anti-diabetic properties (20). Following its secretion from the intestinal L-cells, GLP-1 binds to its receptor (GLP-1R) on pancreatic β-cells to stimulate insulin secretion (19). As GLP-1 is quickly degraded in the circulation by the ubiquitous protease dipeptidyl peptidase-IV (DPP-4), GLP-1 agonists with an extended half-life by virtue of their resistance to degradation by DPP-4 have been developed for clinical use (21), including Exendin-4 (Ex-4) and Liraglutide (LIR) (22,23). GLP-1 agonists, administered either as a monotherapy or in combination with other existing oral anti-diabetic drugs (24), are now increasingly used for the treatment of DM, as they provide additional extra-glycaemic effects, such as weight loss (25).

Several studies have demonstrated that GLP-1 agonists affect bone turnover (26,27). GLP- 1 has been shown to indirectly inhibit bone resorption via stimulation of calcitonin production induced by its binding to the GLP-1 receptor (GLP-1R) in thyroid C cells (28). Accordingly, mice with deletion of pancreatic GLP-1R develop cortical osteopenia and show increased bone resorption through a calcitonin-dependent pathway (29). Another study showed a similar positive effect of GLP-1R activation on bone strength and quality, as mice lacking GLP-1R showed significantly impaired mechanical properties, a decrease in cortical thickness and bone outer diameter and a reduction in the maturity of the collagen matrix (30). Similarly, double incretin receptor knock-out (DIRKO) mice exhibit dramatic and profound alterations of bone microarchitecture and strength, confirming the importance of incretin hormones in the regulation of bone quality (31). 3 days' infusion or daily injections of GLP-1 agonist for 3 days was

shown to be anabolic in bone of normal, insulin-resistant (IR) and T2DM rodent models (32-36). In addition, it was recently demonstrated that long-term treatment with the GLP-1 agonist Ex-4 prevents osteopenia in aged ovariectomised rats, a model of bone loss that mimics osteoporosis (37). It is, however, unclear whether the mechanism of action of GLP-1 agonists in bone is direct through a functional GLP-1R expressed by bone cells or indirect via an increase in calcitonin production. Furthermore, there are inconsistencies in the literature regarding the expression of GLP-1R in bone and thus the basis for direct skeletal effects of GLP-1. While previous *in vivo* studies indicate indirect effects of GLP-1 on the skeleton via a calcitonin-dependent pathway (29), it has recently been shown that mouse osteoblast-like MC3T3-E1 cells express a functional receptor for GLP-1, different from the cAMP-linked GLP-1R expressed in the pancreas, suggesting a possible direct skeletal action of GLP-1 (38,39). In contrast, expression of the pancreatic-type GLP-1R mRNA was identified in human osteoblastic cell lines, although its expression varied between them (40). The presence of pancreatic GLP-1R has also been reported in osteocytic MLO-Y4 cells and osteocytes in rat femurs (35), as well as in mesenchymal stem cells (41). GLP-1R expression is increased during osteogenic differentiation of adipose derived stem cells (ADSCs), suggesting that GLP-1R activation may contribute to osteogenesis (42).

In this study, we investigated the effects of chronic administration of two different GLP-1 agonists on bone mass, architecture, cellular activities *in situ* , calcitonin and sclerostin productions in osteopenic ovariectomised mice. We also aimed to determine if GLP-1 agonists can directly affect bone cell function *in vitro* through a receptor expressed in bone cells.

Material and methods

Animals and study design

Thirty female C57Bl/6NCrl mice twelve-weeks-old were obtained from Charles River laboratories (Inc., Margate, UK). Mice were all ovariectomised (43,44) and four weeks later, divided randomly into three treatment groups: one group (n=10) was treated with 10 µg/kg/d Exenatide (Bachem) dissolved in saline, the second was treated with 0.3 mg/kg/d Liraglutide (Bachem) dissolved in saline and the last group received saline (control). All treatments were administered by daily subcutaneous injections for 4 weeks. At days 6 and 3 prior to euthanasia, mice were intraperitoneally injected with calcein (20 mg/kg) and alizarin red complexone (30 mg/kg) (Sigma-Aldrich), respectively, to label bone-mineralising surfaces in trabecular bone. At the end of the experiment, mice were sacrified, the serum collected for sclerostin and calcitonin measurements, right tibiae dissected for micro-CT analysis and left tibiae for bone histomorphometry. Since we did not have a SHAM group, the success of ovariectomy was

confirmed by observation of uterine atrophy during dissection. All animal experimentation procedures were performed in compliance with local ethical committee and Home Office Project Licence under the auspices of the UK Animals (Scientific Procedures) Act 1986.

Micro-CT analysis of tibiae

Right tibiae were fixed in 10% neutral-buffered formalin for 24-72h and stored in 70% ethanol at 4°C. They were then scanned using high-resolution (5µm pixel size) micro-computed tomography (micro-CT) (skyScan-1172/F BRUKER, Belgium), as previously described (45). After scanning, the data was reconstructed with NRecon version 1.6.4.1 (NRecon®). Trabecular and Cortical bone areas were analysed with CT-Analyser (CTAn) version 1.11.10.0. For analysis of trabecular bone in proximal metaphyses, the cortical shell was excluded by operator-drawn regions of interest and 3D algorithms were used to determine the relevant parameters, which included: bone volume percentage (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), structure model index (SMI), trabecular pattern factor (Tb.Pf), trabecular separation (Tb.Sp) and degree of anisotropy (DA). Analysis of cortical bone in midshaft diaphyses was performed using a 0.49mm long segment (or 100 tomograms) at 37% and 50% percent of tibial length from its proximal end. Cortical bone parameters consisted of: tissue area (Tt.Ar), tissue perimeter (Tt.Pm), bone area (Ct.Ar), eccentricity (Ecc), moment of inertia (MMI polar) and cross-sectional thickness (Ct.Th).

Bone Histomorphometry

Left tibiae were fixed in 4.5 % formaldehyde for 2 days at 4°C, dehydrated in acetone for 24h and embedded in methyl methacrylate (MMA) at low temperature to preserve enzymatic activity (46). Unstained 8-µm-thick longitudinal sections were used for fluorescence microscopy to assess mineral apposition rate (MAR, µm/day). Area of mineralising surfaces was expressed as alizarin red-labelled surfaces per bone surfaces (MS/BS, %) and the bone formation rate was calculated as MS/BS×MAR (BFR/BS, µm3/µm2/day) (47). Alternatively, sections were stained for tartrate-resistant acid phosphatase (TRAP) (Leucognost® SP; Merck, Germany) and counterstained with Mayer's haematoxylin solution. Histomorphometric parameters were measured on the trabecular bone of the metaphysis, on a region of interest consisting of 2 mm width below the growth plate. Measurements were performed using image analysis software (Tablet'measure; Explora Nova, La Rochelle, France). Histomorphometric parameters were reported in accordance with the ASBMR Committee nomenclature (48).

Immunohistochemistry for GLP-1 R

Adult wild-type C57BL/6 mouse femurs were fixed in 10% neutral buffered formalin, cast in paraffin and sectioned at 6μm. Endogenous peroxidase activity was blocked using 3% H_2O_2 in methanol (10min), tissue sections were permeabilised in 1% Sodium Dodecyl Sulphate (SDS) in Tris Buffered Saline (TBS) for 5min and blocked with 3%BSA in 20% goat serum. Samples were incubated overnight at 4°C with rabbit polyclonal antibody anti-GLP-1R (1μg/ml) (ab39072, Abcam, Cambridge) and rabbit IgG for control sections (Vector labs). Sections were washed with TBS-Tween (TBST) and incubated in biotinlylated secondary antibody (goat anti-rabbit; 1:300) for 1hour at room temperature in TBST containing 1% BSA. Sections were washed with TBST, incubated with Avidin Biotin Complex (ABC) to amplify the target antigen signal before a second wash with TBST and and a final incubation with DAB (3, 3'-diaminobenzidine). Sections were counterstained briefly with haematoxylin and imaged using a light microscopy.

Cell culture

Osteocytic cell line MLO-A5

Murine pre-osteocytic cells (MLO-A5) were cultured in minimal essential medium (MEM), supplemented with 10% foetal calf serum (FCS), 2mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2.

Osteocytic cell line IDG-SW3

The mouse osteoblastic-late-osteocytic cell line IDG-SW3 was kindly provided by Prof Linda Bonewald and cultured as previously described (49) in MEM containing L-glutamine supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. 50 U/ml of recombinant Mouse Interferon-gamma (INF-γ) (Invitrogen) was added to induce expression of the SV40 large tumor antigen and maintain proliferation of this cell line. The IDG-SW3 cells were expanded on rat tail collagen type 1-coated plates (Becton Dickson Bioscience) at 40 000 cells/cm^2 at 33 ºC. Osteogenesis was induced by replacing medium at confluence with fresh growth medium supplemented with 50 mg/L ascorbic acid and 4mM β -glycerophosphate without IFNγ at 37 ºC. Cells were maintained for 30 days in osteogenic medium, which was changed 3 times weekly.

Primary osteoblast culture

Primary mouse osteoblastic cells were obtained by sequential enzyme digestion of excised calvarial bones from 2 day old C57BL/6 mice using a 3-step process (1% trypsin in PBS for 10 min; 0.2%

collagenase type II in Hanks balanced salt solution (HBSS) for 30 min; 0.2% collagenase type II in HBSS for 60 min) (50). The first two digests were discarded and the cells resuspended in MEM supplemented with 10% FCS, 2 mM L-glutamine, 1% gentamicin, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin. Cells were cultured for 2-4 days at 37 °C in 5% CO_2 until they reached confluence. They were then cultured in 6-well trays in MEM supplemented with 2 mM β-glycerophosphate and 50 µg/ml ascorbic acid, with half medium changes every 3 days. Exendin-4 (0, 10, 25, 50 and 100 nM) or Liraglutide (0, 10, 100, 500, and 1000 nM) were added to the culture (1 plate/treatment). All experiments were carefully pH-controlled because bone mineralisation is extremely sensitive to inhibition by acidosis (51).

Bone nodule formation by osteoblasts was measured after 28 days of culture. Experiments were terminated by fixing cell layers in 4% paraformaldehyde for 10 min; mineralised bone nodules were visualised and quantified unstained. Plates were then stained for alkaline phosphatase (ALP) activity with 10mg/ml naphtol AS-MX-phosphate (Sigma) in 10% dimethyl formamide mixed with Fast Violet salt in 0.1M Tris–HCl at 37 °C for 30 min. Cell layers were imaged at 800 dpi using a high-resolution flat-bed scanner. Binary images of each individual well were then subjected to automated analysis (Image J), using constant “threshold” and “minimum particle” levels, to determine the number and surface area of mineralised bone nodules and the surface of ALP staining as previously described (52).

Primary osteoclast culture

Osteoclast formation and activity were studied in an *in vitro* model in which osteoclasts are derived from the bone marrow of juvenile mice (53,54). Bone marrow from two 6 week old female C57BL/6 mice was flushed from their long bones. Marrow cells were centrifuged at 300g and resuspended in MEM supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml amphotericin B, containing 10^{-7} M prostaglandin E_2 and 50 ng/ml M-CSF (referred as MEM_1) (R&D Systems Europe Limited, Abingdon, UK). The cell suspension was incubated for 24 h at 37 °C in 5% CO_2 to allow attachment of stromal cells. Non-adherent cells were collected and resuspended at 5×10^6 cells/ml in supplemented MEM (referred as MEM_2) complemented with 10^{-7} M prostaglandin E_2, 150 ng/ml M-CSF and 3 ng/ml RANKL (Receptor activator of nuclear factor kappa-B ligand) (R&D Systems Europe Limited, Abingdon, UK). 106 cells were plated into a 96 well plate containing dentine discs in each well and incubated for 24 h to allow attachment of osteoclast precursors. Dentine discs were then transferred to 6 well trays containing MEM_2. Exendin-4 (Sigma) (0, 10, 25, 50 and 100 nM) or Liraglutide (Bachem) (0, 10, 100, 500, and 1000 nM) were added to the culture in two wells/group (10 disks). Cultures were grown for 8 days with half medium changes every 2-3 days. Culture medium was maintained at pH 7.4 for the first 6 days, then reduced to pH 7 by addition of HCl in order to activate osteoclast resorption activity.

Experiments were terminated by washing discs in PBS, followed by fixation in 2.5% glutaraldehyde. Discs were stained for TRAP using a leukocyte acid phosphatase kit (Sigma) according to the manufacturer's instructions. The total number of osteoclasts on each disc was assessed ‘‘blind’’ by transmitted light microscopy and the plan surface area of resorption pits was measured ‘‘blind’’ using reflective light microscopy and dot-counting morphometry (Image J) (53).

RT-PCR

Long bones of wild-type C57BL/6 mice were flushed with PBS to remove the bone marrow, then individually powdered with a mortar and pestle under liquid nitrogen. MLO-A5 and IDG-SW3 cells were trypsinised with 0.05% trypsin-EDTA (Life Technologies). Osteoclast RNA was extracted from control conditions as described above at day 2 (OC precursors), day 5 (early OC), day 7 (mature OC) and day 9 (resorbing OC) by scraping the dentine disks. Osteoblast RNA was extracted from control cells as described above at day 7 (proliferating OB), day 14 (differentiating OB) and day 21 (mature-mineralising OB) of culture after addition of 0.05% trypsin-EDTA to the wells.

Total RNA from bones, bone marrow, MLO-A5 cells, IDG-SW3 cells, primary osteoclasts and osteoblasts was extracted using TRIzol® reagent or RNeasy Mini Kit (Qiagen) according to the manufacturers' protocols. Total RNA concentration and purity were estimated by absorbance at 260 and 280 nm, respectively and integrity by visualisation of ribosomal bands after agarose gel electrophoresis.

GLP-1 receptor mRNA was amplified by RT-PCR in 50μL volumes and the amplification parameters consisted of initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 30 s and extension at 72 °C for 1 min. The resulting products were visualised by electrophoresis on a 2% agarose gel. The mouse primers used for amplification were 5’-TCCTTCGTGAATGTCAGCTG-3’ and 5’-TGGTGCAGTGCAAGTGTCTG-3’ (designed using Blast).

Serum quantifications of calcitonin and sclerostin

Quantification of calcitonin in mouse serum was performed using a competitive inhibition enzyme immunoassay, Murine ELISA kit for calcitonin (Uscn Life Science Inc.). Serum levels of sclerostin were detected using a Solid Phase Sandwich ELISA, ®ELISA Mouse/Rat SOST kit (R&D Systems Europe, Ltd., Abingdon, UK) according to the manufacturer's recommendations.

Statistics

Data are presented as mean ± SD. Multiple comparisons were performed using one-way analysis of variance, using the Dunnett's Post-hoc test where appropriate. P values less than 0.05 were considered to be statistically significant. *In vitro* results are representative of experiments each performed 3 times.

Results

Chronic GLP-1 agonist treatment improves trabecular bone mass and architecture in ovariectomised mice

We examined the effects of chronic LIR and Ex-4 treatment on bone mass and architecture of OVX mice using micro-CT. There were no significant differences in tibial lengths between the three groups (Table 1). However, LIR and Ex-4 improved bone mass, as both treatments significantly increased the BV/TV and Tb.N of ovariectomised mice compared to saline-treated controls by 49% and 35% respectively for BV/TV and 33% and 45% respectively for Tb.N (Table 1). LIR and Ex-4 also improved bone structure and connectivity, as shown by the decreases in Tb.Pf by 13% and 11% respectively and SMI by 9% and 7% respectively (only significant with LIR). There was no effect of either LIR or Ex-4 on Tb.Th, Tb.Sp and DA. GLP-1 agonists had no effect on cortical architecture (Table 1).

Chronic GLP-1 agonist treatment does not affect bone formation *in vivo* but stimulates bone resorption

Because of the beneficial effects of GLP-1 agonists on trabecular bone architecture with micro-CT, we wanted to confirm them and examine bone cellular activities using histomorphometry. Bone histomorphometry similarly showed an improvement in trabecular bone mass and architecture as a result of GLP-1 agonist treatment (Table 2). Both LIR and Ex-4 increased BV/TV and Tb.N in the trabecular bone of ovariectomised mice. Tb.Th was not affected, but Tb.Sp was decreased as a result of treatment with both LIR and Ex-4, when quantified by histomorphometry (Table 2). However, this effect was not observed using Micro-CT (Table 1).

Ex-4 and LIR significantly increased the percentage of TRAP-positive surfaces (osteoclast surfaces) but only EX-4 enhanced the number of osteoclasts compared to control mice (Table 2). Analysis of bone formation activity showed that mineralising surfaces and MAR were not affected by either Ex-4 or LIR treatment (Table 2). Bone formation rate was also not changed by GLP-1 agonist treatment (Table 2). In addition, Ex-4 and LIR had no effect on adipocyte number in trabecular bone (Table 2).

Chronic Exenatide but not Liraglutide treatment increases serum calcitonin levels and decreases sclerostin levels

Serum calcitonin concentration was significantly higher by 177% in the Ex-4-treated mice than in control mice. Calcitonin levels in Liraglutide- treated mice increased by 53% compared to control mice but the effect was not significant (Figure 1A).

Serum sclerostin concentration was significantly lower by 23% in the Ex-4-treated mice than in control mice. However, no difference in sclerostin levels was observed between Liraglutide- treated mice and control mice (Figure 1B).

GLP-1 receptor is expressed in bone tissue and bone cells

To determine whether the observed effects of GLP-1 agonist treatment might be mediated through a direct skeletal mechanism, we next determined whether the pancreatic-type GLP-1R is expressed in mouse bone and bone marrow, primary osteoblasts and osteoclasts and osteocyte-like MLO-A5 and IDG-SW3 cells. As shown in Figure 2A, GLP-1R mRNA expression was detected in mouse bone and bone marrow. Although no expression of the GLP-1R mRNA was detected in the MLO-A5 osteocytic cell line, it was detected in the IDG-SW3 osteocytic cell line. Figure 2 (B, C) illustrates the presence of GLP-1R mRNA in primary osteoblasts and osteoclasts at different stages of differentiation. In order to further validate these results, we examined the distribution of GLP-1R in mouse bone using immunohistochemistry using muscle as a control of the specificity of GLP-1R antibody (Figure 3). GLP-1R was expressed in the periosteum (Figure 3A), bone marrow cells (Figure 3B) and in some osteocytes, but not all (Figure 3C). Immunohistochemistry and TRAP staining of consecutive trabecular bone sections confirmed that GLP-1R was also expressed in osteoclasts (Figure 3D).

GLP-1 agonists increase osteoclast number but decrease osteoclast activity *in vitro*

As GLP-1R is expressed in osteoclasts, we next determined whether GLP-1 agonists could influence bone resorption *in vitro*. To determine whether GLP-1 agonists can directly affect osteoclastic formation and activity, osteoclast progenitors from mouse bone marrow were cultured with either Ex-4 (10 nM to 100 nM) or LIR (25 nM to 1000nM) (Figure 4). Osteoclast number was increased in a dose dependent manner for Ex-4 and was enhanced at 1000 nM for LIR. However, addition of Ex-4 (≥ 50 nM) to mature osteoclasts decreased bone resorption per osteoclast by up to 20%, while LIR (1000 nM) decreased bone resorption per osteoclast by up to 13%, showing that despite an increase in the number of osteoclasts, the overall resorption is decreased.

GLP-1 agonists have no effect on bone formation *in vitro*

To determine whether GLP-1 agonists can directly affect *in vitro* bone formation, mouse osteoblast progenitors were cultured in osteogenic medium with either Ex-4 (10 nM to 100 nM) or LIR (25 nM to 1000 nM) (Figure 5). As previously published, abundant mineralised bone nodules with characteristic trabecular features formed after 4 weeks of culture (50). However, neither treatment had an effect on the area of bone nodule formation compared to control.

Discussion

This is the first extensive study that examined the long-term effects of two different GLP-1 agonists on bone mass, architecture and bone cellular activities *in situ* and assessed the skeletal expression of GLP-1R as well as the action of these two GLP-1 agonists on bone resorption and formation *in vitro*. We demonstrate that long-term treatment with two GLP-1 agonists improves trabecular bone mass and architecture in ovariectomised mice, despite no effect of Ex-4 and LIR on bone formation *in vivo* and an increase in osteoclast number and surfaces with Ex-4. We also show that GLP-1R is expressed in bone cells implying that GLP-1 agonists could exert direct effects on bone.

The demonstration that GLP-1 agonists improve trabecular bone mass in ovariectomised mice, confirms previous studies that have reported osteogenic effects of GLP-1 agonist treatment in normal, IR, T2DM and ovariectomised rodent models (32-37). The novelty of our study is that we have investigated the longer term skeletal effect of LIR and Ex-4 in OVX mice, having administered these drugs daily for 4 weeks, and that we examined bone architecture together with bone cellular activity *in situ*. Both drugs increased tibial bone volume due to the formation of new trabecular bone, mostly manifested in an increase of the number of trabeculae, rather than modifications in their thickness. Silva and Gibson (55) have shown that it is more important to maintain trabecular number than thickness to preserve bone mass, indicating that drugs, such as GLP-1 agonists, which restore the number of trabeculae in bone, could be advantageous in individuals predisposed towards osteoporosis. Our results also indicate an improvement in trabecular architecture with GLP-1 agonist treatment and a normalising effect upon the OVX-impaired connectivity and anisotropy factors (Tb.Pf and SMI), an effect that has also been observed in short term studies with GLP-1 agonists in T2DM and IR (33,37). These positive effects of GLP-1 analogues on bone mass and architecture were confirmed in histology sections using bone histomorphometry. In contrast, GLP-1 agonists had no effect on cortical bone, also in agreement with most previous studies (36,37), although one recent paper did report a beneficial effect of LIR on cortical bone of T2DM rats that was associated with increases in cortical thickness and cortical area (36). This may have been the result of the higher dose of LIR used in this study and also the rats received the drug at week 2 of age, a time of high bone growth. Ma et al (37) found a greater

effect of Ex-4 on trabecular bone than ours at a dose of 3 μg/kg/d, but the rats received the treatment for 16 weeks.

No report has previously compared the skeletal effects of LIR and Ex-4. Our data suggests that LIR is more efficacious in bone than Ex-4, although this might be due to the doses of drugs chosen and/or to a different mechanism of action on bone of LIR and Ex-4. The half-life of Ex-4 is 2.4 hours while LIR has a half-life of 13 hours, therefore Ex-4 would need to be given in regular doses to build up and maintain a high enough concentration in the blood to be therapeutically effective (56). As a result, LIR treatment is formulated as once daily injection in contrast to Ex-4 which is formulated as twice daily injections because of its short half-life (57). However, in our study we administrated both treatments once daily and thus this may have contributed to the less pronounced effect of Ex-4 on bone, although the doses for both drugs were chosen because they had osteogenic effects in previous studies (32-35,37). Clinical studies have shown that LIR is more efficacious than Ex-4 in patients with T2DM, as it induces a significant greater reduction in HbA1c (56). Although our *in vitro* results do not indicate any major differences in the effects of each drug on bone formation and resorption, we observed a marked difference in the effects of these drugs on calcitonin and sclerostin levels in mouse serum, as Ex-4 stimulates calcitonin and inhibits sclerostin production but not LIR. The effect of Ex-4 on sclerostin production matches other findings (35) but the effect of LIR was not investigated. It is unclear yet why there is this major difference and further studies on the mechanisms of actions of these drugs are required.

Since GLP-1 agonists improved trabecular bone architecture, we examined bone resorption and formation activities in trabecular bone using bone histomorphometry. Our results showed no effect of GLP-1 agonists on *in vivo* bone formation, but both drugs significantly increased the number of osteoclast surfaces implying an increase in osteoclastic differentiation. In contrast, Ma et al (37) showed enhanced bone formation and decreased numbers of osteoclasts per millimetre of trabecular bone surface after 16 weeks' administration of Ex-4 to OVX rats. The reason for this discrepancy is unclear, but it may be possible that the bone loss induced by OVX in our experiment was too severe to measure any significant changes in bone formation, as the trabecular bone volume percent was very low (1-2%). Both mice and rats experience rapid bone loss following ovariectomy, but we have previously observed using this strain of mice that the trabecular bone volume in control mice is lower than in rats. This increase in bone resorption surfaces as a result of GLP-1 agonist treatment is surprising, as it has been suggested that incretins inhibit bone resorption after the ingestion of fat or protein (26,27). Among these incretins, GIP has been shown to both stimulate osteoblastic differentiation (58) and inhibit osteoclastic activity (59), and has a role in age-induced bone loss (60). Also, recent studies have shown that daily administration of GLP-2 decreased bone resorption markers (61). However, with histomorphometry, we only measure osteoclast surfaces and we can't assess if osteoclastic resorptive activity is impaired.

Much less is known about GLP-1's putative role as a modulator of bone turnover. Mice with homozygous deletion of the pancreatic GLP-1 receptor develop cortical osteopenia and bone fragility as well as increased osteoclastic bone resorption that might be due to a reduction in thyroid calcitonin secretion (29). Despite a few studies showing a beneficial effect of GLP-1 agonists on bone architecture, the mechanisms by which GLP-1 regulates bone turnover are unknown. It has been reported that GLP-1 might directly stimulate *in vitro* osteoblastic cells via a specific GLP-1 receptor, different or similar to the one expressed in the pancreas. While murine MC3T3 osteoblastic cells were shown to express a GLP-1 receptor different from the cloned GLP-1 receptor in the pancreas (38), other human osteoblastic cell lines in contrast express the GLP-1R mRNA (40). This receptor is also expressed in the osteocytic cell line MLO-Y4 and in osteocytes *in vitro* and *in situ* (35) but was not detected in primary osteoblasts and osteoclasts cultured on plastic using qPCR (30). Here we used two techniques to establish whether and where GLP-1R is expressed in bone and examined the direct effects of GLP-1 agonists on bone cell functions *in vitro*. Our results demonstrate that GLP-1R mRNA is expressed in mouse primary osteoblasts and osteoclasts in addition to whole bone and bone marrow. In their study, Mabilleau et al (30) cultured osteoblastic cells isolated from bone marrow for 3 days, whereas we cultured osteoblasts isolated from calvaria for 28 days. At 3 days, only proliferating osteoblasts would have been obtained, while a longer culture period is necessary to obtain differentiating osteoblasts and osteoblasts producing collagen matrix. In our experiment, osteoblasts formed after 28 days and express all markers of differentiated cells including osteocalcin and the formation of bone nodules *in vitro*, their ultimate purpose (50). Similarly, our *in vitro* bone resorption model uses osteoclasts cultured on dentin disks to stimulate their differentiation and activity, which is more comparable to the *in vivo* situation (53). Furthermore, in the earlier study (30), osteoclasts were cultured on plastic, which does not promote bone resorption. Our results show no expression of GLP1R in the early osteocytic cell line MLO-A5, while it was expressed in the late osteocytic cell line IDG-SW3. These results were confirmed *in situ* using immunohistochemistry and we found that GLP-1 R was specifically expressed in the periosteum, bone marrow, osteoclasts and in some osteocytes but not all, suggesting that expression may be influenced by osteoblastic differentiation status.

Only one previous study assayed GLP-1R expression in bone *in situ*, which similarly identified the receptor in some osteocytes (35). The presence of GLP-1R in bone cells *in vitro* and *in situ* implies that GLP-1 agonists could have a direct effect on bone cells. However, our data show no major effect of GLP-1 agonists on osteoblasts *in vitro,*. In contrast, GLP-1 agonists did affect osteoclast differentiation and activity *in vitro*, as the area resorbed per osteoclast was decreased with Ex-4 and LIR, despite an increase in osteoclast number, suggesting that GLP-1 agonists may inhibit osteoclastic resorptive activity. Overall, our *in vivo* and *in vitro* results indicate that GLP-1 agonists stimulate osteoclastic differentiation but impair their resorptive activity. This may partly explain the increase in trabecular bone volume that we observed, although this is probably not the main mechanism of action of GLP-1 agonists in bone as the reduction in bone resorption is mild. Differentiation of bone marrow mesenchymal stem

cells (BMMSCs) into osteoblasts or adipocytes is crucial for bone remodelling and our immunohistochemistry data show that GLP-1R is expressed both in bone marrow and in the periosteum, which contains many osteoprogenitor cells. We can't exclude that BMMSCs are also a key target of GLP-1 agonists, although we didn't observe any effect of these drugs on the number of adipocytes in bone marrow *in situ*. Previous *in vitro* studies have shown a reduction in adipocyte differentiation from BMMSCs when cultured with Ex-4 (37).

Since no major changes in bone cellular activities were observed *in vitro* and *in vivo,* despite a mild reduction in bone resorption activity *in vitro*, it is difficult at the present time to explain the increased bone mass observed with GLP-1 agonists. One possible explanation could be that the duration of bone formation is increased with GLP-1 agonists despite a similar rate of bone formation by osteoblasts. This can be determined by the average volume of bone matrix made by each team of osteoblasts and is revealed by the measurement of the wall thickness of trabecular bone packets (62). It is however difficult to measure in mouse bone. Another reason could be that GLP-1 agonists do not act directly on bone cells. It has been shown that GLP1-R is also expressed in C cells of the thyroid gland and exerts, when activated by GLP-1 or its stable analogues, a stimulating effect on calcitonin secretion in rodents (63,64), a potent inhibitor of bone resorption. As proposed by Yamada et al (29), GLP-1 agonist effects on bone might be indirect rather than direct, acting mainly by targeting calcitonin secretion from the thyroid to modulate bone turnover. Our data indicate that Ex-4 stimulates calcitonin secretion and thus can induce an inhibition of resorption. However we found no effect of LIR on calcitonin secretion while studies have reported that both Ex-4 and LIR stimulate calcitonin secretion (63,64). Our data are not completely consistent with this mechanism since we observed an increase in resorption surfaces with GLP-1 agonists whereas other studies have reported that calcitonin decreased resorption surfaces (65). Calcitonin function is mainly to suppress osteoclast activity which is not always reflected by osteoclasts surfaces.

Sclerostin, the protein product of the SOST gene, can bind to bone morphogenetic proteins (BMP) and inhibit canonical Wnt/β-catenin signalling, which is essential for bone formation (66). Interestingly, sclerostin levels are increased in diabetes and can be regulated by GLP-1 agonists (6,35). We show here that Ex-4 treatment reduces serum sclerostin in mice. These findings are in agreement with Kim et al. (35) and suggest an alternative mechanism that could contribute to the increased trabecular bone mass in mice treated with Ex-4. LIR, nevertheless, had no effect on sclerostin production.

Another recently proposed mechanism of action of GLP-1 agonists is the activation of 5' adenosine monophosphate-activated protein kinase (AMPK), a master regulator of cellular energy homeostasis. We demonstrated several years ago that AMPK activation regulates osteoblastic differentiation and bone mass (67). The energy sensor AMPK has been suggested to be an attractive target for the pharmacological treatment of T2DM (68) and is involved in the mechanism of action of several anti-diabetic drugs (66,67). Although our results demonstrate that bone formation is not directly enhanced

by GLP-1 agonists, we have not investigated whether GLP-1 agonists affect AMPK activation in bone which could impact on bone cellular energy homeostasis.

Taken together, our results suggest that GLP-1 agonists improve trabecular bone mass and architecture in ovariectomised mice, although their mechanism of action is still unclear. Our findings may guide selection of therapeutic strategies to prevent and improve the low bone mass and deterioration of bone tissue associated with aged postmenopausal osteoporosis. Considering the impaired bone structure and elevated risk of fractures in diabetic patients, the use of GLP-1 agonists as an anti-diabetic therapy may have additional beneficial effects on the skeleton avoiding the occurrence of additional diabetic complications. More studies are therefore required to examine the long-term potential beneficial effects of GLP-1 therapy in diabetic patients that are at risk of concurrent osteoporosis.

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Figure legends:

Figure 1: Effect of GLP-1 agonists on serum calcitonin and sclerostin levels in 5-month-old ovariectomised mice. (A) Calcitonin levels were assessed by competitive ELISA and (B) Sclerostin levels were assessed by sandwich ELISA in serum from 5-months-old ovariectomised mice treated with saline, Liraglutide (0.3 mg/kg/d) or Exenatide (10 µg/kg/d). Bars represent mean ± SD of n=5 mice/group *, P < 0.05 versus control

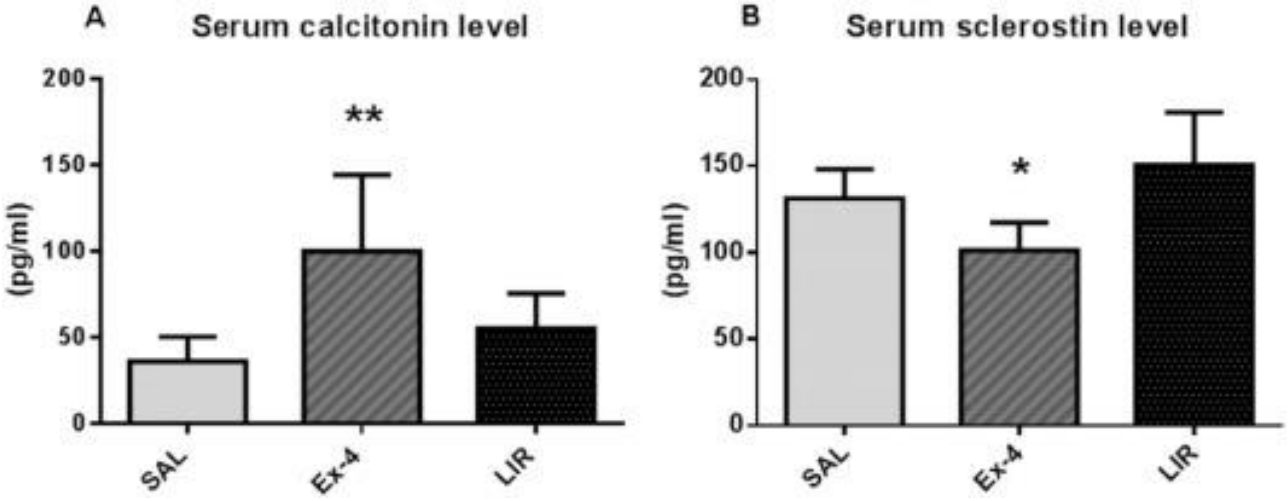

Figure 2: Expression of GLP-1 receptor mRNA in mouse tissues and bone cells using RT-PCR. (A) The GLP-1 receptor mRNA is expressed in bone marrow, bone, in the late osteocytic cell line IDG-SW3 but not in the pre-osteocytic cell line MLO-A5. Liver and pancreas were used as positive controls (B) Expression of the GLP-1 receptor mRNA in primary osteoblasts (OB) at different stages of maturation (proliferating OB, differentiating OB and mature mineralising OB). (C) Expression of the GLP-1 receptor mRNA in primary osteoclasts (OC) derived from mouse bone marrow at various stages of differentiation (OC precursors, early OC, mature OC and resorbing OC).

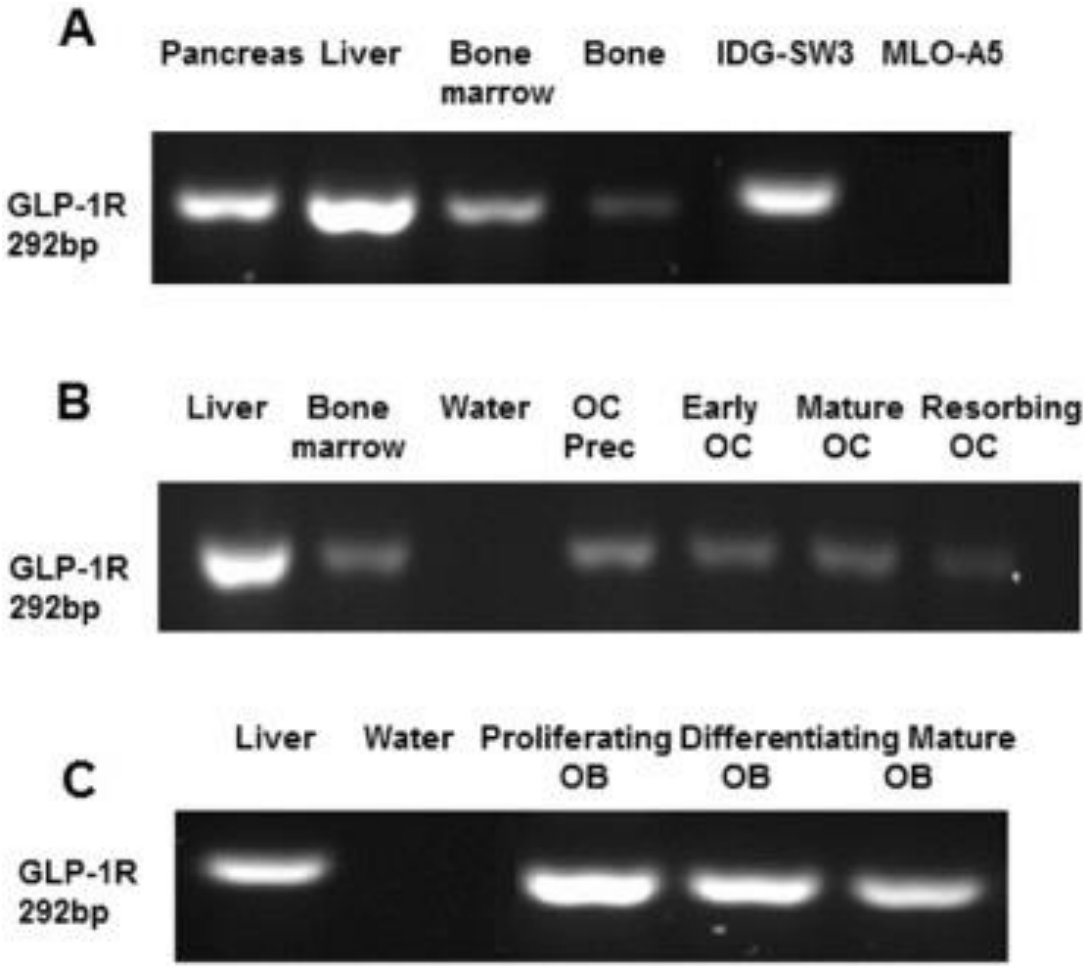

Figure 3: Expression of the GLP-1 receptor in mouse femur by immunohistochemistry. Immunostaining of mouse femur with GLP-1R antibody and counterstain with hematoxylin (A) Cortical bone staining x 10, (B) Bone marrow x40, (C) Osteocytes in cortical bone x40, (D) Trabecular bone x40; consecutive sections immunostained with GLP-1R antibody and TRAP-stained respectively to visualise osteoclasts.

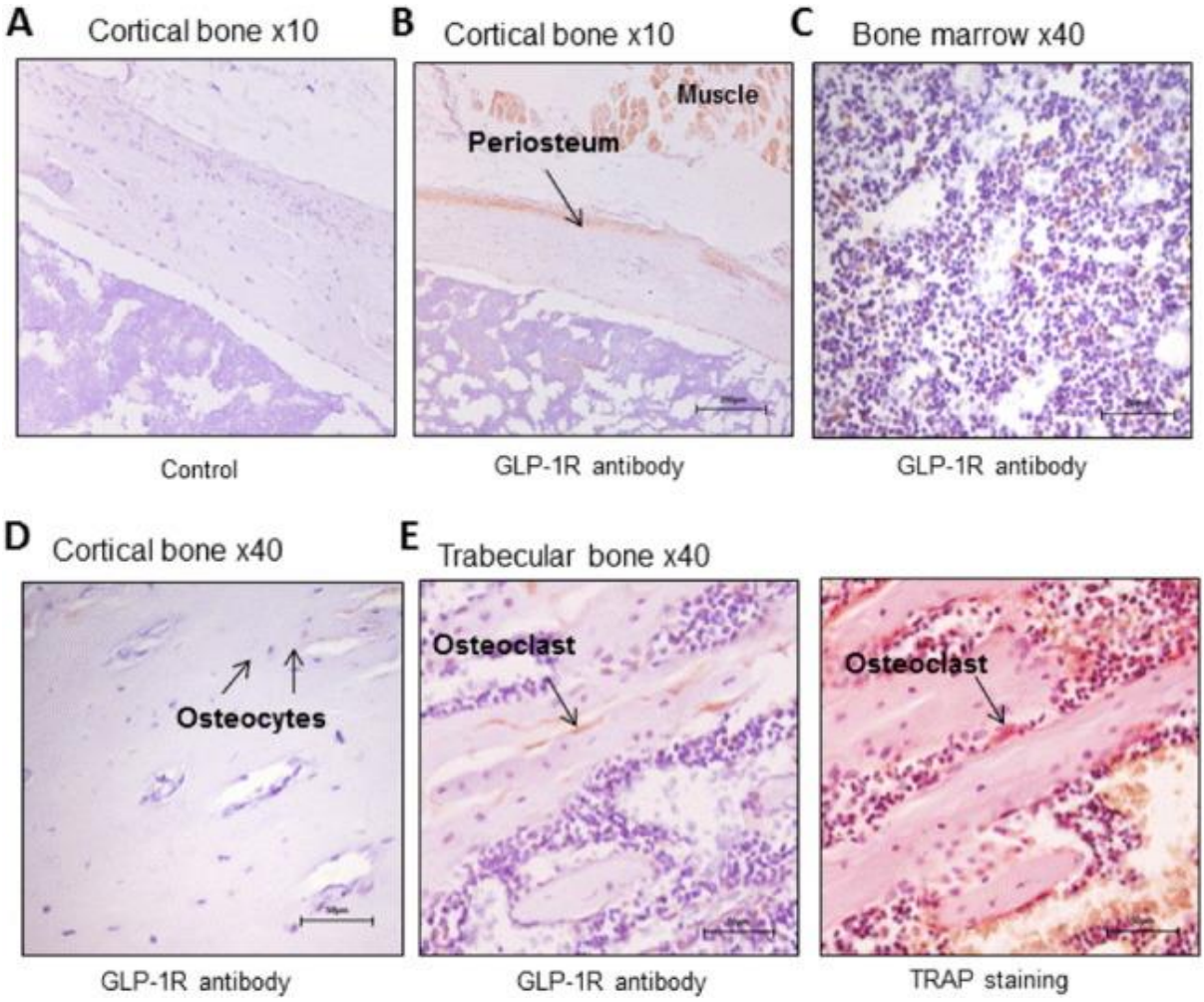

Figure 4: Effects of GLP-1 agonists on osteoclastic differentiation and bone resorption *in vitro*. Primary osteoclasts were isolated from mouse bone marrow and cultured on dentine disks. Either Exenatide or Liraglutide was added to the medium at various concentrations. Osteoclasts were stained for TRAP activity and viewed by transmitted light. (A) Number of osteoclasts per dentine disk in cultures treated with Exenatide, (B) Resorption area per dentine disk in cultures treated with Exenatide , (C) Number of osteoclasts per dentine disk in cultures treated with Liraglutide, (D) Resorption area in cultures treated with Liraglutide. Mean ± SD of 10 disks/group. **, P < 0.01; *, P < 0.05 versus control

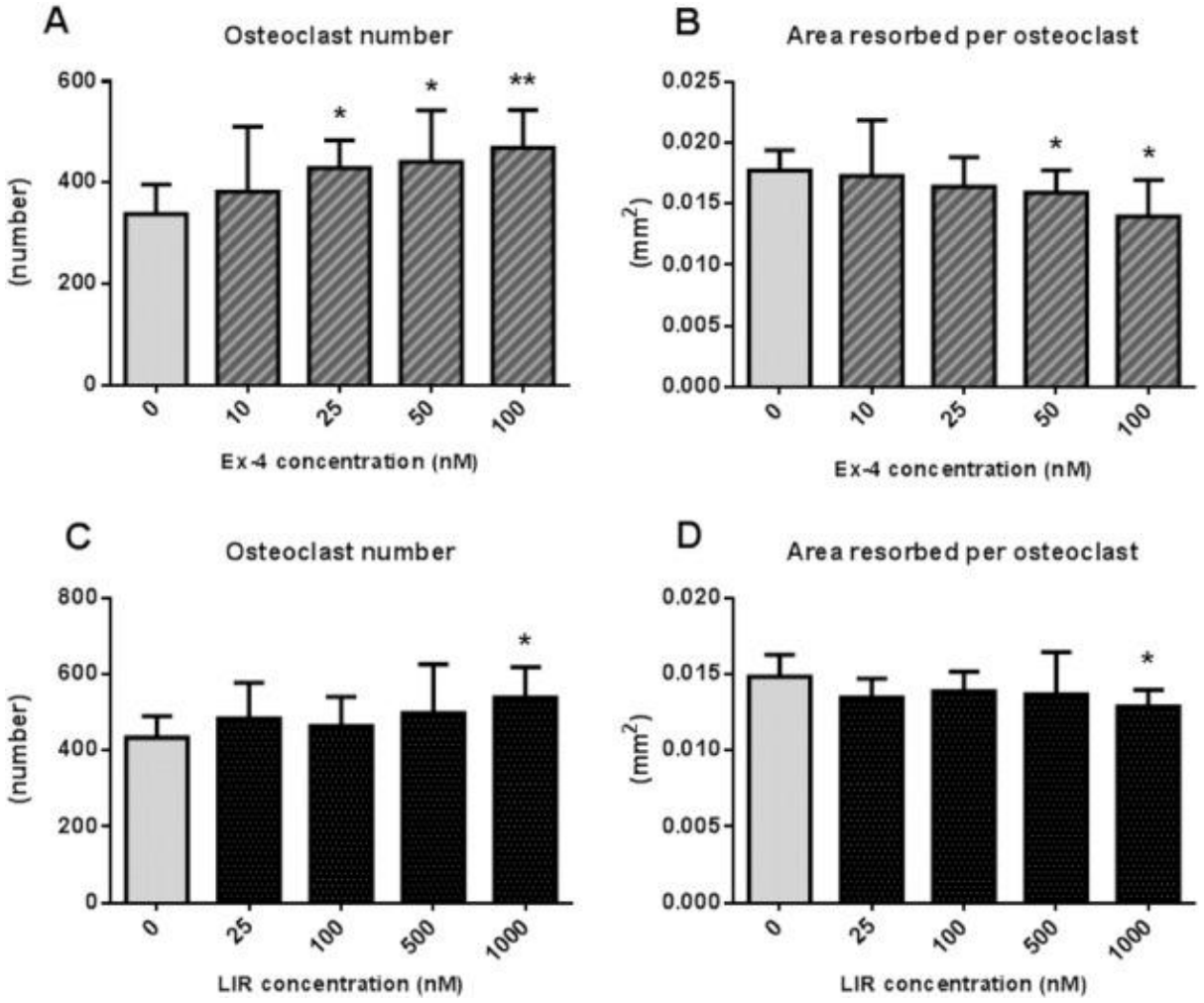

Figure 5: Effects of GLP-1 agonists on bone nodule formation *in vitro*. Either Exenatide or Liraglutide was added to the 28-day primary culture of mouse osteoblasts isolated from calvarial bone and cultured in osteogenic medium at increasing concentrations. (A) Area of bone formation representing the mineralised bone nodule observed with reflected light scans of unstained cell wells by osteoblasts cultured with Exenatide, (B) Area of bone formation by osteoblasts cultured with Liraglutide. Mean ± SD of 6 wells/group *, $P < 0.05$ versus control.

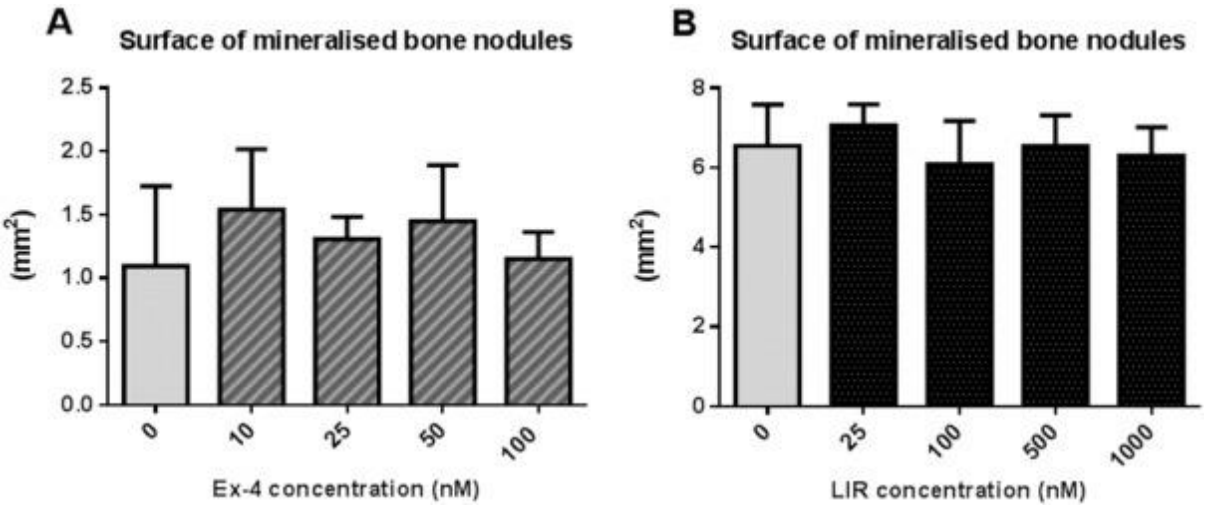

Table 1: Effect of Liraglutide and Exenatide on trabecular bone parameters in tibia of 5-months-old ovariectomised mice. Measurements were assessed by micro-CT in the proximal tibial metaphysis of mice treated with either saline, Liraglutide or Exenatide. Mean ± SD of n=10 mice/group. *, P < 0.05; **, P < 0.01; versus saline.

Parameters	Saline	Exenatide(10 µg/kg/day)	Liraglutide(0.3 mg/kg/day)
Length of tibia (mm)	17.8 ± 0.5	17.8 ± 0.3	17.8 ± 0.4
Trabecular architecture			
BB/TV (%)	1.38 ± 0.42	1.89% ± 0.48 *	2.06% ± 0.52 **
Tb.N (number/mm)	0.280 ± 0.97	0.373 ± 0.098 *	0.407 ± 0.085 **
Tb.PF (factor/mm)	33.31 ± 3.62	29.57 ± 2.42 *	29.04 ± 2.66 **
Tb.Th (mm)	0.0501 ± 0.0048	0.0487 ± 0.0047	0.0502 ± 0.0046
Tb/Sp (mm)	0.676 ± 0.081	0.621 ± 0.076	0.631 ± 0.080
SMI	2.51 ± 0.23	2.33 ± 0.19	2.28 ± 0.080
DA	2.66 ± 0.43	2.86 ± 0.35	3.05 ± 0.29
Cortical architecture			
Tt.Ar (mm2)	1.238 ± 0.063	1.270 ± 0.079	1.204 ± 0.097
Tt.Pm (mm)	5.34 ± 0.28	5.54 ± 0.35	5.34 ± 0.36
Ct.Ar (mm2)	0.739 ± 0.054	0.758 ± 0.049	0.732 ± 0.065
Ecc	0.789 ± 0.035	0.806 ± 0.035	0.778 ± 0.042
MMI polar (mm4)	0.278 ± 0.040	0.301 ± 0.042	0.269 ± 0.050
Ct.Th (mm)	0.201 ± 0.011	0.204 ± 0.011	0.202 ± 0.011

Table 2: Histomorphometric measurements of bone architecture and cellular activities in 5-month old ovariectomised mice treated with GLP-1 agonists. Cellular parameters were measured by bone histomorphometry on sections of the trabecular region of mouse tibia from mice treated with saline, Liraglutide (0.3 mg/kg/day) or Exenatide (10 µg/kg/day). BV/TV: bone volume percent, Tb.N: trabecular number, Tb.Th: trabecular thickness, Tb.Sp: trabecular separation, TRAP Oc·S/BS: osteoclastic surfaces per millimetre of trabecular bone surface, TRAP Oc·N/BS: number of osteoclast per millimetre of trabecular bone surface, MS/BS: alizarin red-labelled surfaces per bone surfaces, MAR: mineral apposition rate, BFR: bone formation rate. Mean ± SD of n = 10 mice/group, *P < 0.05 **, P < 0.01 versus saline.

Parameters	Saline	Exenatide(10 µg/kg/day)	Liraglutide(0.3 mg/kg/day)
BV/TV (%)	3.38 ± 1.69	5.58 ± 2.76 *	6.22 ± 1.90 **
Tb.N (number/mm)	0.98 ± 0.34	1.46 ± 0.50 *	1.47 ± 0.39
Tb.Th (mm)	0.0335 ± 0.0076	0.03376 ± 0.0095	0.0422 ± 0.0061
Tb.Sp (mm)	1.086 ± 0.365	0.710 ± 0.212 *	0.686 ± 0.225 *
TRAP Oc S/BS (µm) % control	100 ± 50	150.6 ± 22.8 *	142.6 ± 31*
TRAP Oc N/BS (1/mm) % control	100 ± 29.7	162 ± 65.6 *	138 ± 30.9
MS/BS (%)	41.3 ± 7.8	43.8 ± 6.6	44.5 ± 5.7
MAR (µm/day)	2.15 ± 0.43	1.96 ± 0.31	1.96 ± 0.32
BFR/BS (µm3/µm2/day)	0.890 ± 0.231	0.846 ± 0.216	0.875 ± 0.194
Adipocyte number (number/mm2)	93 ± 32	92 ± 44	103 ± 43

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