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Effects of GIP, GLP-1 and GLP-1RAs on bone cell metabolism

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Abstract

The relationship between gut and skeleton is increasingly recognised as part of the integrated physiology of the whole organism. The incretin hormones gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are secreted from the intestine in response to nutrient intake and exhibit several physiological functions including regulation of islet hormone secretion and glucose levels. A number of GLP-1 receptor agonists (GLP-1RAs) are currently used in treatment of type 2 diabetes and obesity. However, GIP and GLP-1 cognate receptors are widely expressed suggesting that incretin hormones mediate effects beyond control of glucose homeostasis, and reports on associations between incretin hormones and bone metabolism have emerged. The aim of this review was to provide an overview of current knowledge regarding the in vivo and in vitro effects of GIP and GLP-1 on bone metabolism.

We identified a total of 30 preclinical and clinical investigations of the effects of GIP, GLP-1 and GLP-1RAs on bone turnover markers, bone mineral density (BMD), bone microarchitecture and fracture risk. Studies conducted in cell cultures and rodents demonstrated that GIP and GLP-1 play a role in regulating skeletal homeostasis, with preclinical data suggesting that GIP inhibits bone resorption whereas GLP-1 may promote bone formation and enhance bone material properties. These effects are not corroborated by clinical studies. While there is evidence of effects of GIP and GLP-1 on bone metabolism in preclinical investigations, clinical trials are needed to clarify if similar effects are present and clinically relevant in humans.

Introduction and Background

Bone remodelling is the regenerative mechanism that maintains the skeletal health and biomechanical competence through resorption of bone by osteoclasts and formation of new bone by osteoblasts. These processes are highly coordinated and under the influence of several hormonal factors [1].

Incretin hormones encompass a group of hormones characterised by their ability to enhance insulin secretion in response to intake of nutrients, such as glucose and fat. The most important incretin hormones are gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) [2]. Both of these hormones interact with cognate receptors, which are widely expressed in human tissues: the GIP receptor (GIPR) is expressed in pancreas, central nervous system, thyroid as well as adipose tissue [3], and the GLP-1 receptor is expressed in the kidney, stomach, duodenum, central nervous system and pancreas [4]. Both the GIP and the GLP-1 receptors are 7-transmemembrane proteins, and activation of these receptors by binding of either GIP or GLP-1 increases intracellular levels of cyclic adenosine monophosphate (cAMP) and Ca^{2+} [5].

GIP and GLP-1 are secreted from enteroendocrine cells of the small intestine. Once secreted, GLP-1 binds to the GLP-1 receptors expressed on pancreatic β-cells, leading to a glucose-dependent insulin secretion but the effect is short-lived as GLP-1 is quickly degraded by the dipeptidyl peptidase-4 (DPP-4) enzyme, which is expressed on the surface of several cell types [2]. Observations of lower postprandial levels of GLP-1 in patients with type 2 diabetes has led to the development of DPP-4 inhibitors and GLP-1 receptor agonists (GLP-1RAs) resistant to enzymatic degradation by DPP-4, and these drugs are currently widely used for the treatment of type 2 diabetes due to their glucose-lowering properties [2]. GLP-1RAs vary in several ways including their degree of homology with native human GLP-1 and pharmacokinetics. For example, compared to Exendin-4, a naturally occurring peptide resistant to degradation by DPP-4, liraglutide, which is a GLP-1RA marketed for the treatment of type 2 diabetes, has a substantially higher degree of homology to native human GLP-1 (97% vs. 53%) and a longer half-life (12h vs. 2.4h) [6, 7].

Unlike GLP-1, there is limited information regarding the physiological functions of GIP. GIP stabilizes blood glucose levels by enhancing glucose-stimulated insulin secretion and promoting glucagon secretion at low glucose levels. Plasma levels of GIP are normal or increased in type 2 diabetes, and the secretion of GIP

in response to an oral glucose tolerance test (OGTT) or a meal test in patients with type 2 diabetes is not different from healthy controls [3].

The role of the skeleton in regulating overall energy metabolism is increasingly recognised and thought to be mediated by interaction between insulin and bone secreted "metabolic hormones" e.g. osteocalcin [8]. Investigations of the effects of incretin hormones on bone metabolism have emerged, providing support of an integration of nutrient intake and bone metabolism [9]. Thus, the aim of the present review is to investigate our current knowledge regarding the effects of GIP, GLP-1 and GLP-1RAs on bone turnover and bone cell metabolism in animals and humans. In order to focus on the explicit effects of incretin hormones, DPP4-inhibitors were not included in this review.

Materials and Methods

On October 15th 2016 we searched the databases PubMed, DARE and MedLine and used PubMatrix to crosscheck our search results using the following control words: "GLP-1", "GIP", "Glucagon Like Peptide-1", "Exenatide", "Lixisenatide", "Albiglutide", "Liraglutide", "Dulaglutide", and "Semaglutide" together with the one of the next search words: "Bone", "Fracture", "Osteoblast" and "Osteoclast". Eighty-nine articles were identified and screened independently by two authors. Articles in English language that reported the expression of incretin hormone receptors in bone cells or effects of GIP, GLP-1 or GLP-1RAs on bone metabolism were included whereas reviews were excluded. A total of twenty-one preclinical studies (see Table 1) and nine clinical studies (see Table 2) were included.

Results

Incretin hormones and bone cells

GIP

GIPRs have been reported to be present in human and rodent osteoblastic cell lines that mirror different stages of osteoblast differentiation, including MG63, Saos-2 and ROS 17/2.8 osteosarcoma cell lines [10, 11], TE-85 cells [11], murine MC3T3-E1 [12] as well as primary murine osteoblasts and human osteoblasts [10]. The expression of GIPR has been reported to increase with osteoblastic cell maturity [11]. Furthermore,

it has been shown that GIPR expression is glucose-dependent in MC3T3-E1 osteoblastic murine cells [12]. However, similar effects have not been reported in human bone cell lines or primary cells.

GIPR signalling in osteoblastic cells is associated with increased intracellular cAMP concentration and increased cell viability, collagen type 1 expression and alkaline phosphatase activity [10, 11]. Furthermore, GIP has been reported to inhibit osteoblastic cell apoptosis [13, 14], bone marrow stem cell apoptosis [14], and osteoclastic cell activity in a dose-dependent manner in vitro, and GIP decreases both PTH and receptor activator of nuclear factor κβ ligand (RANKL)-induced osteoclastic bone resorption, suggesting that GIP has a direct and inhibitory effect on osteoclasts [15], indicating that GIP may promote bone formation and impede bone resorption.

GLP-1 - animals

Several investigators have assessed the effect of GLP-1 on animal bone cells, but the results are inconsistent. Expressions of GLP-1Rs have been reported in murine osteoblasts, osteocytes and osteoclasts [12, 16, 17], and studies have shown glucose-dependent expression of GLP-1R in osteoblastic cells during bone morphogenetic protein (BMP)-2-induced differentiation [12]. Furthermore, binding of GLP-1 to murine osteoblastic MC3T3-EI cells has been reported [18]. By contrast, GLP-1R expression was not detected in a number of other investigations of bone cells including murine MC3T3-E1 osteoblastic cells [18], primary osteoblasts [19, 20] and osteoclasts [19]. Although the GLP-1R was not identified, treatment of osteoblastic MC3T3-E1 cells with GLP-1 induced hydrolysis of glycosylphosphatidylinositols (GPI) and increased phosphatidylinositol-3 kinase and mitogen activated protein kinase (MAPK) activity, suggesting that GLP-1 influences osteoblast differentiation and activity through an unknown GPI-coupled receptor [18]. Furthermore, high doses of GLP-1 reduced the expression of genes involved in the development of osteoblasts such as the runt-related transcription factor 2 and major genes of the Wnt pathway, had no effect on mRNA levels of the RANKL decoy receptor osteoprotegerin but increased the expression of osteocalcin, which is a marker of late osteoblast maturation, suggesting that GLP-1 may promote the activity of mature osteoblasts rather than osteoblast differentiation. Additionally, it has also been reported that GLP-1 has no

effect on intracellular cAMP levels [18, 20] or osteoblastic apoptosis (Saos-2 cell lines) or osteoclast differentiation and activity in vitro [20].

GLP-1 - humans

GLP-1R expression has been reported in human osteoblastic cell lines but may depend on level of osteoblast development as the GLP-1R was expressed in all except the most developed human cell lines (MG63 and TE-85 but not Saos-2) [11]. GLP-1Rs were expressed in human bone marrow stem cells and adipose-derived stem cells retrieved from subcutaneous fat tissue [21, 22]. Additionally, GLP-1R expression was augmented during in vitro osteoblastic differentiation of adipose-derived stem cells [22]. Correspondingly, treatment of osteoblastic cell lines (MG63 and TE-85) with GLP-1 (7-36 amide) increased the viability but reduced the secretion of bone formation marker procollagen type 1 amino-terminal propeptides (P1NP), but not of alkaline phosphatase, which also reflects bone formation [11]. GLP-1 treatment of human bone marrow stem cells caused an increase in cell proliferation, decreased apoptosis and inhibited adipocyte differentiation by decreasing of the expression of the peroxisome proliferator-activated receptor γ (PPARγ) [21]. Furthermore, GLP-1 treatment of adipose-derived stem cells stimulated osteoblast differentiation and decreased adipogenic differentiation in a dose-dependent manner [23].

In vivo *and* ex vivo *animal studies - GIP*

Total and femoral BMD assessed by dual energy X-ray absorptiometry (DXA) were lower in four weeks old $GIPR^{-/-}$ mice, and total BMD remained lower in $GIPR^{-/-}$ mice at five months of age, supporting that inactivation of the GIP and its receptor influence bone metabolism [24]. Furthermore, micro-computed tomography (micro-CT) scans showed smaller bones with impaired microarchitecture and abnormal trabecular structure in five months old $GIPR^{-/-}$ mice [24]. The GIPR-knockout was obtained by targeted disruptions of exon 4 and 5 in the GIPR gene and only female mice were used. Interestingly, when using another GIPR-knockout method targeting exons 6–10, increased trabecular number and volume as assessed by micro-CT were reported in another study, which comprised of 16-week old $GIPR^{-/-}$ and wild type (WT) mice [25].

Measurements of bone turnover markers revealed higher urinary deoxypyridinoline, a marker for bone resorption, as well as lower levels of bone formation markers (osteocalcin and alkaline phosphatase) in eight weeks old GIPR$^{-/-}$ mice than in matched WT mice [13]. In contrast, no change in serum deoxypyridinoline was observed in five months old GIPR$^{-/-}$ mice [24]. Consistent with the increased urinary elimination of deoxypyridinoline, GIPR$^{-/-}$ mice (eight weeks old) had increased number of osteoclasts assessed by histochemical analyses and lower bone formation rate assessed by bone histomorphometry [13]. By contrast, fewer osteoclasts, unaffected number of osteoblasts and increased bone formation rate as assessed by histomorphometry and increased runt-related transcription factor 2, osteocalcin and collagen-I gene expressions and decreased RANKL gene expression were observed in GIPR$^{-/-}$ cultured osteoblastic cells [25]. In the same study, biomechanical tests revealed weaker bone in GIPR$^{-/-}$ mice suggesting that bone matrix deteriorates in the absence of GIP signalling [25]. Impairment of bone strength was also observed in five months old GIPR$^{-/-}$ mice, presumably due to increased bone resorption and impaired bone formation [24].

On the other hand, transgenic mice overexpressing GIP exhibited increased bone formation and decreased bone resorption markers (osteocalcin and deoxypyridinoline, respectively) as well as fewer osteoclasts and increased bone mass [26]. Consistent with the proposed anti-resorptive effect of GIP, six weeks of daily injection of GIP attenuated bone-loss in ovariectomized eight-week old mice [27]. Furthermore, while treatment of eight-week old streptozotocin-induced diabetic mice with daily GIP-injection for a total of 21 days failed to improve the deleterious effects of diabetes on cortical bone mineral density, thickness and strength, nanoindentation tests showed that GIP increased bone mechanical properties and collagen integrity in mice [28].

In vivo and ex vivo animal studies - GLP-1

The number of osteoclasts in the tibia and bone resorption but not bone formation was higher in six-weeks old GLP-1R$^{-/-}$ mice than in WT mice [20]. Additionally, cortical but not trabecular bone mass of the tibia and spine as well as bone strength were lower in 10-weeks old GLP-1R$^{-/-}$ mice compared to WT mice [20]. In agreement with these results, bone mineral content, bone diameter, cortical thickness and bone material properties such as work to fracture and yield strength were decreased in 16-weeks old GLP-1R$^{-/-}$ mice [19].

Three days of continuous infusion of human GLP-1 in 10 weeks old healthy, D-fructose-induced insulin resistant and streptozotocin-induced diabetic Wistar rats revealed beneficial effects of GLP-1 treatment on bone architecture measured by trabecular separation and trabecular bone pattern factor but not BMD or bone volume when compared to normal rats [29]. Micro-CT scans showed higher femoral and vertebral bone mass in 11-week old hyperlipidemic rats than in WT rats during treatment with GLP-1 [30], which was associated with increased mRNA levels of osteoprotegerin and osteocalcin, indicating promotion of bone formation. Because disruption of the GIP receptor leads to a compensatory increase in GLP-1 secretion and vice versa [31, 32], $GIP^{-/-}$ and $GLP\text{-}1R^{-/-}$ double knockout (DIRKO) mice have been generated. Trabecular bone mass was higher whereas bone diameter and cortical bone mass were lower in 26-week-old DIRKO female mice than in matched WT mice. In addition, several measures of bone mechanical properties including maximal load, hardness and dissipated energy determined by nanoindentation were also impaired in DIRKO mice. Importantly, hyperglycemia impairs bone formation whereas insulin appears to have bone anabolic properties [9]. Therefore, the increased levels of glucose and decreased levels of insulin in DIRKO mice as observed after an intra-peritoneal glucose tolerance test may have contributed to the development of the bone phenotype [33]. The investigation did not include dynamic histomorphometry, therefore, it remains unanswered if bone formation was different in DIRKO mice.

Additionally, GLP-1 may promote collagen maturation as less mature collagen was observed in $GLP\text{-}1R^{-/-}$ mice [19], and combined $GLP\text{-}1R^{-/-}$ and $GIP^{-/-}$ mice [33]. Furthermore, GLP-1R is expressed in the calcitonin-secreting C-cells in the murine thyroid gland, and GLP-1 has been shown to promote secretion of calcitonin in rodents [34]. Treatment with calcitonin reduced bone resorption in $GLP\text{-}1R^{-/-}$ mice, suggesting that GLP-1 acts on bone through secretion of calcitonin in rodents [20].

Effects of GLP-1RAs on bone parameters in animals

Exendin-4

Recently, exendin-4 was shown to increase mouse osteoblastic MC3T3-E1 cell proliferation and the expression of the bone formation markers alkaline phosphatase and osteocalcin together with increased expression of runt-related transcription factor 2, possibly due to increased activity of the MAPK pathway

[17]. Feeding six-week old Wistar rats with a high fat diet for 35 days decreased the osteoprotegerin to RANKL ratio, increased bone resorption and reduced bone mass [30]. Treating a similar group of high fat diet rats for three days with exendin-4 was associated with a higher osteoprotegerin to RANKL ratio, as well as a less pronounced bone loss [30]. Compared to high fat diet rats, histomorphometry showed that osteoclast number and area of eroded surface and osteoid were lower and bone volume and trabecular thickness substantially greater in high fat diet rats treated with exendin-4 [30], suggesting that the GLP-1RA exendin-4 may prevent the deleterious effects of insulin resistance.

Treatment of osteoporotic 12-month old ovariectomized rats for 16 weeks with exendin-4 increased femoral and vertebral BMD dose-dependently [35]. A subgroup of these rats were treated with estradiol and used as positive controls. While estradiol and exendin-4 both reduced the level of bone resorption markers, formation markers osteocalcin and P1NP were higher in rats treated with exendin-4 compared to rats treated with estradiol. Histomorphometric investigations showed that estradiol and exendin-4 increased bone formation rate and mineral apposition rate. Also, the number of osteoblasts was higher in bone tissue from rats treated with exendin-4 compared to estradiol-treated animals. In a comparable study of 12-week old osteoporotic ovariectomized mice, four weeks of treatment with exendin-4 led to increased bone mass as well as trabecular number as assessed by micro-CT and bone histomorphometry [16]. In that study, changes in mineral apposition and bone formation rates were not reported but a decrease in bone resorption was observed [16].

Effects on osteoclastic bone resorption may be explained by changes in the level of calcitonin, which increased during exendin-4 treatment [16]. Single injections of exendin-4 have been shown to increase calcitonin gene expression [20], and long-term treatment with exanatide (exendin-4) at high doses increased calcitonin secretion in WT mice but not in GLP-$1^{-/-}$ mice [34], further supporting that exenatide may act on bone through promotion of calcitonin secretion in rodents. The ratio of Wnt-pathway activator low-density lipoprotein receptor-related protein 5 to sclerostin increased following three days treatment with exendin-4 [29], and long-term treatment with exendin-4 reduced sclerostin levels in mice [16], indicating that exendin-4 could increase bone formation.

Liraglutide

In vitro, liraglutide enhanced osteoblast and reduced adipocyte differentiation of rat and human bone marrow stem cells by down-regulating the adipocyte-specific transcription factor PPARγ and increasing the expression of runt-related transcription factor 2. Furthermore, expression of collagen-I and alkaline phosphatase were increased, jointly indicating that liraglutide promotes bone formation [36].

Similar to what has been observed with exendin-4 treatment, four weeks of treatment with liraglutide prevented trabecular bone loss in 12-week old ovariectomized mice [16]. Bone histomorphometric analyses revealed no changes in bone formation rate suggesting that liraglutide decreases bone resorption and does not affect bone formation. Changes in calcitonin or sclerostin levels were not reported [16].

In another study, treatment with liraglutide for two months prevented bone loss in five-month old ovariectomized rats and increased trabecular bone volume, trabecular thickness and number as well as cortical bone thickness and density despite inducing significant weight loss [36]. Fasting glucose levels were similar in vehicle and liraglutide treated as well as sham-operated rats, demonstrating that glucose-lowering effects of liraglutide cannot account for changes in bone mass. Glucose tolerance, however, was increased by liraglutide treatment, which may have positive effect on bone formation [9].

The effects of liraglutide on bone metabolism have also been investigated in hyperglycaemic conditions. Treatment of Goto-Kakizaki rats, which develops type 2 diabetes early in life in part due to hypercorticonsteronemia, for four weeks with liraglutide increased both trabecular and cortical bone volume, thickness and density, which was associated with increased expression of the runt-related transcription factor 2, alkaline phosphatase, osteocalcin and collagen type 1 suggesting an anabolic effect on bone [37]. These effects were not observed in study of eight-week old rats with streptozotocin-induced (insulinopenic) diabetes, where deterioration in cortical bone microarchitecture, lower bone turnover and impaired bone strength were not prevented by treatment with liraglutide for 21 days [28]. Interestingly, microindentation showed that liraglutide improved several tissue material properties, and while collagen maturity and glycation indices were unchanged, liraglutide increased collagen integrity index, suggesting that the drug improved bone strength at tissue level irrespective of coexisting hyperglycaemia [28].

While GLP-1 appears to promote secretion of calcitonin in rodents, possibly causing the observed changes in bone resorption, long-term treatment of monkeys with very large doses of liraglutide caused neither c-cell

hyperplasia nor secretion of calcitonin [34]. These observations highlight that the effects of GLP-1 on bone metabolism may vary substantially between mammals.

Clinical studies

Clinical studies on incretin hormones and bone metabolism

Few human clinical studies have examined the effects of incretin hormones on bone metabolism. Associations between a GIPR polymorphism known to reduce GIPR activity and lower BMD as well as higher fracture risk was observed in a prospective cohort study comprising perimenopausal women followed for up to 16 years [38]. An intravenous bolus of glucose along with either a single subcutaneous of GLP-1 or an intravenous infusion of GIP in healthy, obese individuals with a mean age of 55 years resulted in non-significant decrease in bone resorption marker collagen type 1 cross-linked C-telopeptide (CTX) with no changes in bone formation marker osteocalcin despite incretin hormone levels exceeding normal postprandial levels by 10 times [39]. By contrast, infusion of GIP at a normal physiological level for one hour reduced bone resorption marker CTX in healthy individuals, and the reduction was higher during hyperglycaemia [40]. Furthermore, during an euglycaemic intravenous glucose infusion neither GIP, GLP-1 nor GLP-2 infusions reduced bone resorption marker CTX as much as an oral glucose tolerance test. However, the combination of GIP, GLP-1 and GLP-2 at physiological doses reduced bone resorption similar to that observed during the oral glucose tolerance test, suggesting that postprandial impairment of bone resorption is explained by combined rather than single effects of incretin hormones on bone remodeling [41].

Clinical studies and GLP-1RAs

In a 24-week study of patients with type 2 diabetes, no effects of treatment with exenatide (synthetic exendin-4) were observed on either bone turnover markers or BMD [42]. In an open-label randomized controlled trial comparing effects of 1.2 mg liraglutide once daily to no treatment in 37 perimenopausal non-diabetic women on diet restriction, significantly lower total body and peripheral bone mineral content (BMC) were observed in the untreated group. Liraglutide increased bone formation markers (P1NP) but not bone resorption markers (CTX) [43]. However, although total hip and lumbar spine BMD were not reported, total and peripheral BMD did not differ between groups. In another 52-week, randomized, double-blind controlled clinical trial comparing BMD in individuals with type 2 diabetes treated with liraglutide 1.2 mg

once daily (n=20), liraglutide 1.8 mg once daily (n=23) or glimepiride 8 mg once daily (n=18), no difference in BMD between groups from baseline to end of the study (104 weeks) was observed [44].

GLP-1RA had no effect on fracture risk as reported in a meta-analysis, which included a total of 19 fractures identified among six randomized, controlled trials including 4255 individuals treated for a mean duration of 67.4 weeks [45] (Table 2). While the I^2 was unreported, the investigators used a random-effects model, possibly indicating heterogeneity between trials. In a more recent meta-analysis based on randomized controlled clinical trials on liraglutide or exenatide with comparators that included fracture data, lower fracture risk was observed among those treated with liraglutide whereas fracture risk was higher in the exenatide-treated individuals. The analysis was based on 11206 individuals but only 48 fracture cases [46] (Table 2). Importantly, the investigators did not observe any heterogeneity (I^2= 0%). In support of these results, a Danish nation-wide register-based case-control study comprising more than 200.000 fracture cases and a similar number of controls including 255 users of GLP-1RAs among fracture cases and 220 GLP-1 users in the control group showed that current treatment with a GLP-1RA was not associated with changes in fracture risk. A number of potential confounders such as body mass index and glucose control were not adjusted for in this study, and the mean duration of treatment was only 36 weeks [47] (Table 2).

Discussion

Currently available data suggest that GIP and GLP-1 influence bone metabolism, but the mechanisms have not been established and may comprise both direct and indirect effects as presented in Figures 1 and 2. However, the vast majority of the current knowledge of the relationship between GIP, GLP-1 or GLP-1RAs and bone metabolism is based on preclinical studies, which have not been confirmed in clinical investigations.

Preclinical investigations of the effects of GIP on bone metabolism based on activation of the GIPR suggest that GIP inhibits bone resorption. Furthermore, while there is some evidence to suggest that GIP may promote bone formation, the effects of GIP on the quality of the bone matrix remain uncertain. The investigations of effects of GIP on bone metabolism that have been based on GIPR-knockout mice have provided contradictory results, showing biochemical and microarchitectural signs of both increased and

decreased bone resorption, which may be explained by differences in the methods used to generate and investigate GIPR-knockout mice. Thus, the discrepancy between the reports regarding the bone phenotype in $GIPR^{-/-}$ mice could be explained by several factors including variation in age, strain of the mice and nutrition, which are critical for interpretation of the results. Furthermore, deletions of different regions of the extracellular domain of the GIPR [13, 24, 25] may have resulted in dissimilarities of the structure as well as the function of the receptor. It should be acknowledged that the preclinical investigations may not reflect effects GIP on human bone metabolism as interspecies differences between rodents and human GIP receptor ligand and receptor signalling have been reported [48]. Currently available clinical data support that GIP decreases bone resorption in humans as in rodents, but further clinical investigations including treatment of a longer duration and assessment of bone histomorphometry are needed to corroborate these observations. Contrary to GIP, it remains to be established if GLP-1 or any of the GLP-1 receptor analogues cause changes in rodent and in particular human bone metabolism. Preclinical studies based on assessments of GLP-1 receptor inactivation or treatment indicate that GLP-1 promotes bone formation, impairs bone resorption and may promote both bone microstructure and strength, but the mechanisms by which GLP-1 changes these aspects of bone are not clear. Beneficial effects of liraglutide on bone microarchitecture in rats with type 2 diabetes associated with hypercorticosterone but not in an insulinopenic rat model of diabetes could indicate that the effect may depend on insulin levels and insulin sensitivity. While GLP-1 and GLP-1RAs may inhibit bone resorption in rodents by promoting the secretion of calcitonin, studies have consistently shown that GLP-1 and GLP-RAs have no effect on calcitonin secretion in monkeys and humans [34]. This indicates that an indirect pathway between calcitonin stimulation by GLP-1 or GLP-1RAs and beneficial effects on bone remodeling is determined by interspecies differences rather than differences in pharmacokinetics between GLP-1RAs. Therefore, it is unlikely that different GLP-1RAs should have diverging effects on calcitonin in humans, and a stimulating effect on bone remodeling through a GLP-1 and calcitonin-coupled pathway is not to be expected in humans.

GLP-1 may influence proliferation and differentiation of human bone marrow stromal stem cells [21, 23]. Moreover, GLP-1-induced down regulation of PPARγ activity might suggest a commitment of human bone marrow stem cells to osteogenic rather than adipogenic differentiation, ultimately leading to increased bone

formation [49]. Along these lines, preclinical investigations based on cultured human osteoblasts and osteoclasts including both early stages and fully mature cells indicate that GLP-1 primarily acts on early stages of cell development and may not have any substantial impact on fully developed bone cells. It is currently unknown if GLP-1 and different GLP-1RAs have similar effects on differentiating and mature bone cells. Based on available preclinical data, exendin-4 seems to prevent bone loss, deterioration of bone microstructure and bone strength as observed in conditions known to influence bone remodelling such as post ovariectomy, type 2 diabetes and hyperlipidaemia. Preclinical studies have also demonstrated that exendin-4 enhances Wnt-signalling and possibly bone formation by increasing osteoblast activity and bone formation, possibly by decreasing the levels of sclerostin, which inhibits Wnt-signalling. Similarly, liraglutide appears to have anabolic effects on bone remodelling, possibly due to promotion of bone marrow stem cell differentiation towards the osteogenic lineage. Jointly, preclinical studies indicate that exendin-4 and liraglutide have similar effects on bone metabolism, implying that other GLP-1RAs are likely to have analogous effects on bone, possibly promoting bone formation and impairing bone resorption.

Clinical investigations of the effects of GLP-1 or different GLP-RAs on the differentiation of bone marrow stem cells as well as osteoblasts, osteoclasts and osteocytes are largely missing, and it also remains to be investigated if GLP-1 and its analogues influence Wnt-signalling or indeed any of the other pathways that are highly involved in the regulation of bone metabolism such as the NF-kB pathway.

Clinical investigations of the effects of GLP1-RAs are limited in number and have not been designed to address the effects of these drugs on bone turnover specifically. Liraglutide was reported to increase the level of a bone formation marker (P1NP) in one intervention study, but the study participants were on a weight-reducing diet, which may have influenced the results. Importantly, the potential promotion of bone formation by liraglutide was not corroborated by increases in BMD at the spine and hip, which would have been anticipated if liraglutide increased bone formation substantially. These observations are in agreement with subgroup investigations of the effect of liraglutide in the LEAD trial and assessments of effects of exanatide on bone turnover and bone mineral density. It should be noted that the available clinical investigations are of short duration, appear not to have been designed to assess effects on bone metabolism as a primary endpoint

and do not provide in-depth assessments of bone remodelling, *i.e.* dynamic histomorphometry of bone biopsies, which limit the possibilities to interpret the results.

Currently available clinical data on effects of GLP-1RAs on human bone metabolism are limited to investigations based on exenatide and liraglutide. The search strategy in the present review included assessment of publications on presently approved GLP-1RAs and aspects of bone metabolism but not GLP-1RAs that used to be or currently is under investigation, such as taspoglutide and semiglutide. It is not beyond the realms of possibility that these investigational drugs may influence bone metabolism, however, in the interest of space, this review was focused on marketed GLP-1RAs.

From a clinical perspective, increased fracture risk is the most important outcome of any pharmacologically induced change in bone mass and bone remodeling. Fractures occurring during GLP-1RA treatment have been reported in some but not all intervention studies, and these results have been explored in a number of meta-analyses. Based on available data, GLP-1RAs seem to neither increase nor decrease fracture risk. While further investigations including longer exposure times and adjustment for confounders known to influence fracture risk are desirable, currently available data lend credence to GLP-1RAs as being safe with regard to fracture risk.

Conclusions

Preclinical investigations suggest that incretin hormones may have profound effects on bone remodeling and ultimately bone structure and strength. In vitro and in vivo studies in animals suggest that mainly GIP inhibits bone resorption (Figure 1) whereas GLP-1 may promote bone formation, inhibit bone resorption and enhance bone material properties (Figure 2). In humans, GIP might inhibit bone resorption during an intravenous infusion, and the anti-resorptive effect appears to be even more pronounced during hyperglycaemia. Exendin-4 and liraglutide appear to be beneficial to bone remodelling, with studies showing mainly anti-resorptive effects in animals. By contrast, the effects of incretin hormones and GLP-1RAs in humans remain elusive. In vitro studies suggest that GLP-1 may increase bone formation in humans due to effects on osteoprogenitor cell differentiation, but similar studies on effects of GLP-1 as well as GLP-1RAs on mature osteoblastic cells are lacking. Clinical trials designed to test the effect of GLP-1 or GLP-1RAs on

bone remodeling have not been conducted. Based on existing albeit sparse data GIP but not GLP-1 appears to confer alterations to bone remodeling in humans.

Perspectives

The physiological effects of GIP, GLP-1 and GLP-1RAs on bone metabolism have not been established, and research on the impact of incretin hormones on human osteoblast differentiation and osteoclast activity including their potential interaction with Wnt- and NF-kB-signalling is needed. Importantly, due to dissimilarities between the expression of GLP-1Rs in animals and humans, the potential effects of GLP-1RAs on bone cells in vitro and in vivo may differ significantly between preclinical and clinical investigations. Several GLP-RAs have emerged as antidiabetic drugs, and liraglutide has recently been introduced for the treatment of obesity. Clinical studies assessing bone remodeling during treatment with GLP-1RAs in diabetic due to insulin resistance or insulinopenia, obese and non-diabetic individuals are needed.

Table 1. Preclinical investigations reporting effects of GIP, GLP-1 or GLP-1-RAs on bone cells or bone metabolism in animals.

	Cell or animal type(s)	Intervention	Outcome
GIP and bone cells			
Bollag[10]	Rat osteocytes		GIPR transcripts expressed in osteocytes
Pacheco-Pantoja[11]	Human osteoblast-like cells (Saos-2, TE-85 and MG-63)	GIP (0.01 nM and 1 nM)	GIPR expressed in all cell lines with highest levels in Saos-2 and lowest levels in MG-63 GIP increased alkaline phosphatase and P1NP production in Saos-2 and decreased P1NP secretion by TE-85 cells GIP had no effect on osteocalcin secretion
Aoyama[12]	Murine osteoblast-like cell (MC3T3-E1)		GIPR expressed in murine MC3T3-E1
Tsukiyama[13]	Murine osteoclasts	GIP (100 μM)	No effect of GIP on osteoclast activity
Berlier[14]	Human osteoblast-like cells (Saos-2) and bone marrow stem cells	GIP (0.1 and 1 μM)	GIP reduced cell apoptosis
Zhong[15]	Rat osteoclasts	GIP (10 and 50 nM)	GIPR expressed in rat osteoclasts GIP dose-dependently impaired the effects of PTH-induced bone resorption GIP decreased expression of markers of osteoclast differentiation
GLP-1 + bone cells			
Pacheo-Pantoja[11]	Human osteoblast-like cells (Saos-2, TE-85 and MG-63)	GLP-1 (0.1nM and 1 nM)	GLP-1R expressed in all but the most mature osteoblastic cell line (Saos-2) GLP-1 decreased P1NP secretion dose-dependently in MG-63 cells and decreased P1NP secretion in TE-85 cells GLP-1 had no effects on alkaline phosphatase or osteocalcin

			secretion
Aoyama[12]	Murine osteoblast-like cells (MC3T3-E1)		GLP-1R expressed in MC3T3-E1 cells undergoing differentiation induced by BMP-2
Nuche-Berenguer[18]	Murine osteoblast-like cells (MC3T3-E1)	GLP-1 (10 nM)	GLP-1 binding to MC3T3-1 cells GLP-1 decreased expression of genes coding the Wnt pathway and runt-related transcription factor 2 (early marker of osteoblast differentiation) GLP-1 increased osteocalcin expression and down-regulated
Yamada[20]	Murine primary osteoblasts and human osteoblast-like cells (Saos-2)	GLP-1 (10 μM for differentiation and 100 μM for osteoclast activity studies)	GLP-1R was not expressed in murine osteoblasts GLP-1 had no effect on apoptosis of osteoblast-like cells. GLP-1 without effect on osteoclast resorption activity
Pereira[16]	Murine IDG-SW3 osteocystic and MLO-A5 osteoblast-late-osteocystic cells Murine primary osteoblast and osteoclast cell cultures	Exenatide 1 (0, 10, 25, 50 and 100 nM) and Liraglutide (0, 10, 100, 500, and 1000 nM)	GLP-1R expressed in IDG-SW3 but not ML-A5 osteocytic cells GLP-1R expressed in primary murine osteoblasts and osteoclasts at different stages of differentiation GLP-1RAs increased osteoclast numbers but decreased area resorbed per osteoclast ex vivo resulting in decreased bone resorption overall
Feng[17]	Murine MC3T3-cells	Exenatide (0.1nM - 10 μM) Exenatide (100 nM)	GLP-1R expressed in murine osteoblast-like cells Exenatide increased osteoblast-like cell viability Increased expression of markers of bone formation (runt-related transcription factor 2, alkaline phosphatase, collagen type 1 and osteocalcin) Exendin-4 induced phosphorylation of MAPK and the effect was blocked by MAPK-inhibitors
Mabilleau[19]	Murine primary		GLP-1R not expressed in primary

	osteoblast and osteoclast cultures		osteoblasts or osteoclasts
Incretins + human cells			
Tsukiyama[13]	Human osteoblast-like cells (Saos-2)	GIP (1μM for expression and 1-100 nM in apoptosis studies)	GIPR indicated but not confirmed GIP protected osteoblast-like cells from apoptosis in a dose-dependent manner
Sanz[21]	Human mesenchymal stem cells	GLP-1 (10 nM)	GLP-1R expressed in Human mesenchymal stem cells GLP-1 promoted differentiation and decreased apoptosis of Human mesenchymal stem cells GLP-1 inhibited adipocyte differentiation, partly through decreasing PPARγ
Jeon[22]	Human adipose-derived stem cells		GLP-1R expressed in human adipose-derived stem cells
Lee[23]	Human adipose-derived stem cells	GLP-1 (10 and 100 nM)	GLP-1 treatment increased alkaline phosphatase expression (at 100 nM GLP-1). GLP-1 decreased adipogenic differentiation of human adipose-derived stem cells (at 100 nM). GLP-1 stimulated osteogeneic differentiation of human adipose-derived stem cells (at 100 nM).
GIP + animal studies			
Gaudin-Audrai[25]	**Mice** Sixteen-weeks old $GIPR^{-/-}$ mice and WT		$GIPR^{-/-}$ mice had: Lower bone resorption (CTX) Fewer osteoclasts and augmented number of osteoblasts Higher osteoblast activity Higher trabecular bone mass
Mansur[28]	**Mice** Streptozotocin-induced diabetic (insulinopenic) 8 weeks old mice	GIP (enzymatic-resistant, 25nmol/kg) or saline for 21 days	GIP prevented reduction in mineral apposition rate (formation of bone) GIP did not prevent loss of whole bone strength or cortical microarchitecture

			GIP prevented deterioration of bone quality
GLP-1 + animal studies			
Yamada[20]	**Mice** Ten weeks old GLP-1R$^{-/-}$ and WT mice		GLP-1R$^{-/-}$ mice had: Increased number of osteoclasts and resorption activity Decreased calcitonin expression in the thyroid Normal bone formation Decreased cortical BMD (tibia and spine) and total BMD (tibia)
Mabilleau[19]	**Mice** Sixteen weeks old GLP-1R$^{-/-}$ and WT mice		GLP-1R$^{-/-}$ mice had: Lower bone diameter and cortical thickness Lower bone strength Decreased collagen maturation
Nuche-Berenguer[29]	**Rats** Streptozotocin-induced diabetic (insulinopenic) 8-9 weeks old rats, rats on high fat diet for 35 days (insulin resistant) and WT rats	GLP-1 (0.86 nmol/kg/hr) for 3 days or saline	In both types of diabetic rats, GLP-1 Increased expression of osteocalcin and ratio between osteoprotegerin and RANKL, indicating higher bone formation Decreased anitrophy of bone tissue (assessed using µCT)
Nuche-Berenguer[30]	**Rats** Six weeks old rats fed normal or hyperlipidaemic chow for 35 days	GLP-1 (0.86 nmol/kg/h) or saline for 3 days.	GLP-1 Increased osteocalcin and osteoprotegerin expression Increased osteoprotegerin to RANKL ratio Increased BMD in the spine and femoral bone Normalized bone volume per total tissue volume, eroded bone surface, proportion of bone tissue covered by osteoblasts and osteoclasts as well as mineral apposition rate

Exendin-4 + animal studies			
Nuche-Berenguer[30]	**Rats** Six weeks old rats fed normal or hyperlipidaemic chow for 35 days	Exendin-4 (0.1 nmol/kg/h) or saline for 3 days.	Exendin-4 - Increased osteocalcin and osteoprotegerin expression Increased osteoprotegerin to RANKL ratio (more than GLP-1) Increased BMD in the spine and femoral bone Normalized bone volume per total tissue volume, eroded bone surface, proportion of bone tissue covered by osteoblasts and osteoclasts as well as mineral apposition rate
Bjerre Knudsen[34]	**Mice** 5-10 weeks mice	Exenatide (0.25-1.0 mg/kg/d as single injections or for 12 weeks).	Exenatide did not increase calcitonin levels in mice
Pereira[16]	**Mice** Ovariectomized or sham operated 12 weeks old mice (intervention 1 month after surgery)	Exenatide (10 µg/kg/d) or saline for 4 weeks	Exenatide - Increased BV/TV and trabecular numbers and connectivity Increased the number of osteoclasts but reduced the amount of bone resorbed per osteoclast Had no effect on mineral apposition rate Increased levels of sclerostin.
Yamada[20]	**Mice** Ten weeks old mice	Exendin-4 (24nM/kg)	Exendin-4 increased thyroid calcitonin mRNA levels in WT mice.
Liraglutide + animal studies			
Pereira[16]	**Mice** Ovariectomized or sham operated 12 weeks old mice (intervention 1 month after surgery)	Liraglutide (0.3 mg/kg/d) or saline for 4 weeks	Liraglutide - Increased BV/TV and trabecular numbers and connectivity Had no effects on sclerostin levels Had no effect on mineral apposition rate
Lu[36]	**Rats**	Liraglutide (0.6 mg/d)	Liraglutide -

	Ovariectomized or sham operated 6 weeks old rats (intervention 3 months after surgery)	and ovariectomized, saline and ovariectomized or saline + sham for 2 months	Increased collagen type 1, alkaline phosphatase and runt-related transcription factor 2 and reduced PPARγ expression Increased trabecular thickness, number and BMD (levels in sham operated not reached)
Bjerre Knudsen[34]	**Mice, Rats and Monkeys** 5-10 weeks mice, 6-9 weeks GLP1-R$^{-/-}$ mice, 6-7 weeks old rats, 1-2 years old cynomolgus monkeys.	Liraglutide (Mice: 0.03-3.0 mg/kg/d in as single injections, for 9 and 104 weeks. Rats: 0.075-0.75 mg/kg/d as single injections, for 4 weeks and 16 months. Monkeys: 5 mg/kg/d as single injections and up to 87 weeks)	Liraglutide - Increased levels of p-calcitonin in mice and rats (short and long-term treatment) Did not increase calcitonin levels in GLP-1R$^{-/-}$ mice Did not increase calcitonin levels in monkeys (short and long-term treatment)
Mansur[28]	**Mice** Streptozotocin-induced diabetic (insulinopenic) 8 weeks old mice	Liraglutide (25 nmol/kg) or saline for 21 days	Liraglutide - Did not prevent reduction in mineral apposition rate (formation of bone) or loss of whole bone strength or cortical microarchitecture Prevented deterioration of bone quality

Table 2. Clinical investigations reporting effects of GLP-1-Ra's on bone mineral content or density and bone turnover.

	Investigational drug	Study design	Number of participants	Outcome
Clinical trials				
Iepsen[43]	Liraglutide (no active control)	Randomized, controlled trial. 52 weeks.	37	P1NP increased 16% (7 ± 3 µg/L). CTX did not change. Stabile BMC (total, pelvic, arm/leg) but loss in controls
Gilbert[44]	Liraglutide vs. glimepiride	Randomized, double-blind, active-control trial. 52 weeks.	61 (subgroup among a 776 individuals enrolled in the LEAD-3 trial)	No difference from baseline in BMD in those receiving 1.2 or 1.8 mg Liraglutide
Henriksen[39]	None – GIP, GLP-1 and GLP-2	Three different studies using GLP-1,GIP and GLP-2 respectively.	GLP-1: 7 GIP: 8 GLP-2: 60 postmenopausal women	CTX did not change in GLP-1 or GIP-studies. Dose-dependent and significantly reduction in CTX after GLP-2 injection. Osteocalcin -levels were not affected.
Nissen[40]	None - GIP		10	During euglycemia and hyperglycemia injection with GIP significantly decreased CTX.
Lund[41]	None – GIP, GLP-1 and GLP-2	Three different case-control study	Study 1: 20 Study 2: 20 Study 3: 10	Combined infusion of GIP, GLP-1 and GLP-2 in patients with type 2 diabetes significantly reduced CTX to levels seen in OGTT.
Bjerre Knudsen [34]	Liraglutide, active comparator and placebo	Eight phase-III clinical trials (LEAD I-VI, a Japanese (type 2 diabetes studies) and an obesity study)	5673	No increase in calcitonin levels
Li[42]	Exenatide vs. Insulin Lispro vs. Pioglitazone	Randomized, parallel-group trial. 24 weeks.	62	No difference from baseline in bone turnover markers or BMD
Meta-analyses and case-control studies				
Su[46]	Exenatide or Liraglutide vs. comparators	Meta-analysis of randomized, controlled trials (n=16)	5,040 vs. 3,410 comparators	No increase in fracture risk (MHOR = 1.05, 95 % CI 0.59–1.87). Liraglutide reduced fracture risk (OR 0.38 (0.17-0.87)). Exenatide increased fracture risk (OR 2.09 (1.03-4.21))
Mabilleau[45]	Exenatide or Liraglutide vs. comparators	Meta-analysis of randomized, controlled trials (n=7)	2,918 vs. 1,227 comparators	No increase in fracture risk (OR 0.75 (0.28–2.02))

Driessen[47]	GLP-1 (Liraglutide and Exenatide)	Register-based case-control study	229,145 cases (incl. 255 on GLP-1-Ra) and 229,145 controls (incl. 220 on GLP-1-Ra)	GLP-RAs not associated with fracture (OR 1.16; 95 % CI 0.83–1.63])

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407 **Figures**
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409 **Figure 1**
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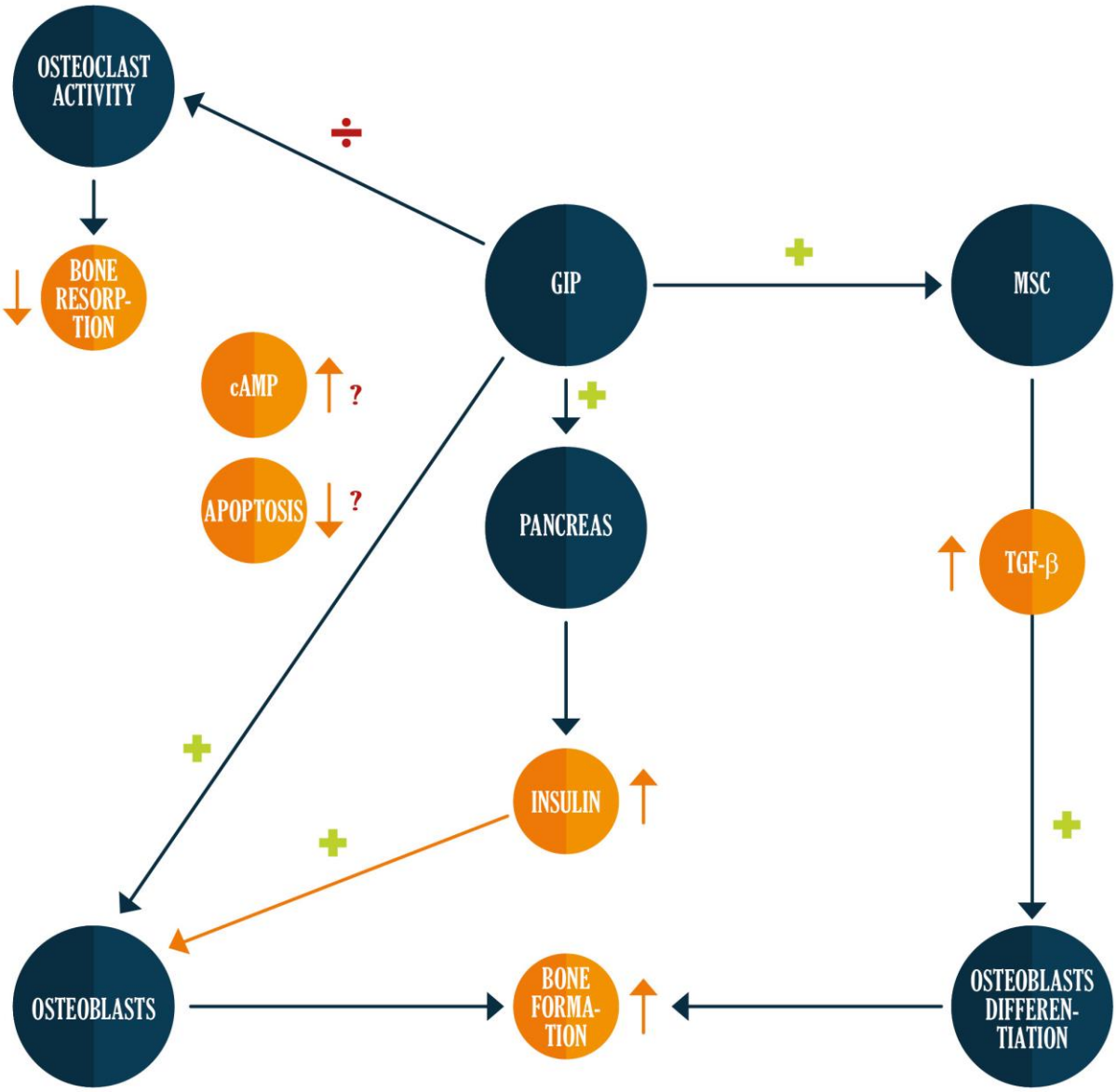

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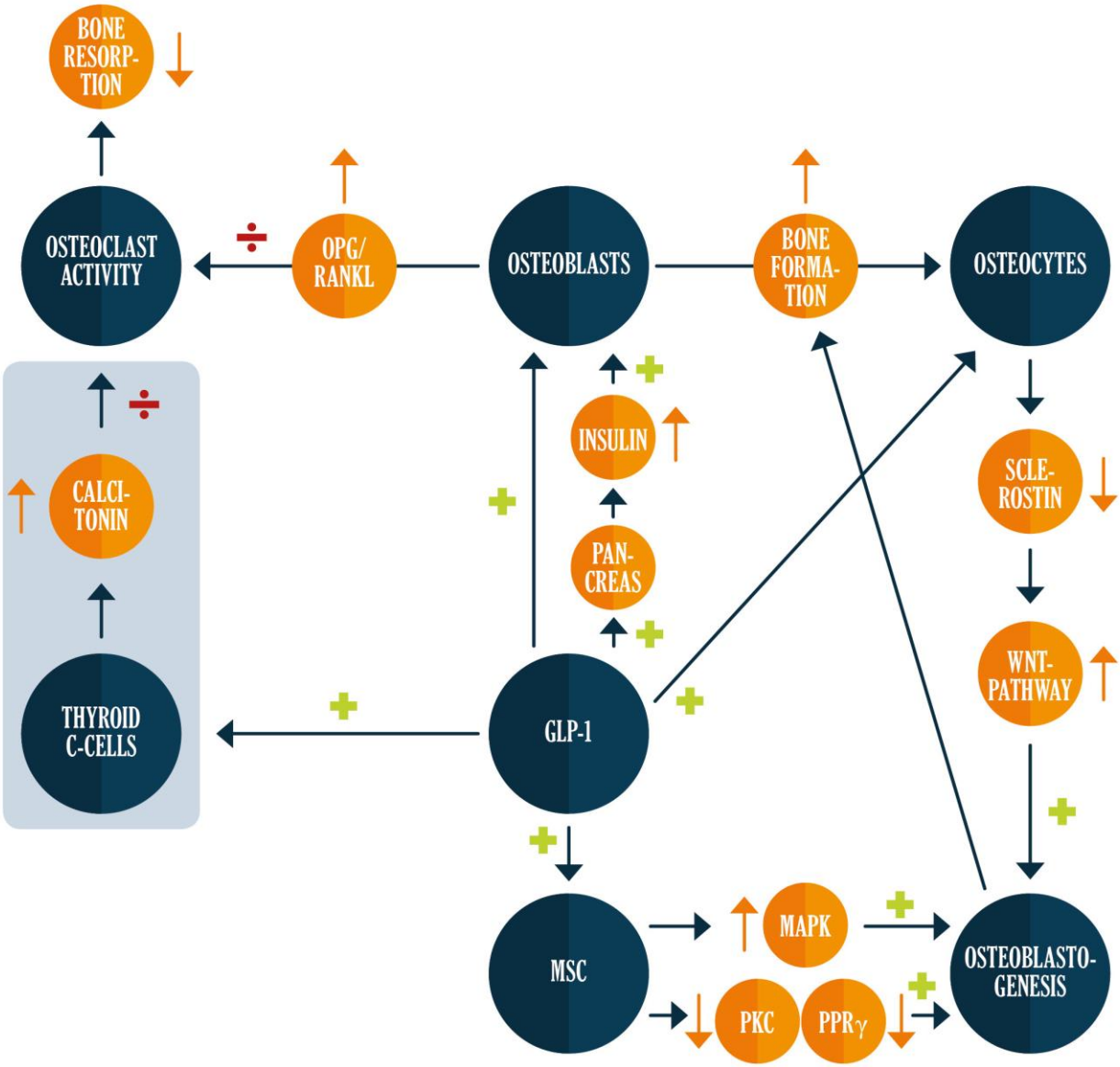

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Figures legends

Figure 1

Potential mechanisms of how GIP acts on bone metabolism.

Stimulation is illustrated as "plus" and inhibition as "minus". Upward arrows illustrate up-regulation (downward arrows the opposite). GIP appears to act directly on osteoblasts and increase osteoblastic activity resulting in increased bone formation, possibly through cAMP-mediated pathways and through inhibition of osteoblastic apoptosis. GIP may act directly on early stages of mesenchymal stem cells (MSC). GIP may increase secretion of insulin and amylin from pancreatic islets, possibly promoting bone formation.

Figure 2

Potential mechanisms of how GLP-1 acts on bone metabolism.

Stimulation is illustrated as "plus" and inhibition as "minus". Upward arrows illustrate up-regulation (downward arrows the opposite). GLP-1 may act on osteoblasts by a) increasing osteoprotegerin (OPG)/RANKL ratio thereby inhibiting osteoclast activity resulting in decreased bone resorption and b) stimulation of GLP-1 on osteoblasts, causing increased levels of the bone formation markers osteocalcin, alkaline phosphatase and collagen α1. GLP-1 acts directly on pancreas, increasing levels of insulin, which acts on osteoblasts to stimulate bone formation.

GLP-1 also acts on osteocytes, inhibiting levels of sclerostin, which increases the activity of Wnt-pathway resulting in increased osteoblastogenesis and eventually increases bone formation. GLP-1 acts on MSCs and osteoblastogenesis through a) increasing activity of MAPK-pathway, which stimulates osteoblastogenesis resulting in increased bone formation and through b) decreasing activity of the Protein Kinase C (PKC)-pathway resulting in decreased levels of PPARγ, which results in increased activity of osteoblastogenesis and decreased activity of adipogenesis resulting in increased bone formation.

In rodents only (grey area), GLP-1 acts on thyroid C-cells, and stimulates secretion of calcitonin, which inhibits osteoclast activity and decrease bone resorption.

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