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Guillaume Mabilleau, Marie Pereira, Chantal Chenu

Accepted Preprint first posted on 30 August 2017 as Manuscript JOE-17-0278

Novel skeletal effects of Glucagon-like peptide-1 (GLP-1) receptor agonists

Guillaume Mabilleau[1], Marie Pereira[2], Chantal Chenu[3]

[1]: GEROM Groupe Etudes Remodelage Osseux et biomatériaux, IRIS-IBS Institut de Biologie en Santé, CHU d'Angers, Université d'Angers, 49933, ANGERS Cedex, France.

[2]: Centre for Complement and Inflammation Research (CCIR), Department of Medicine, Imperial College London, London W12 0NN, UK

[3]: Department of Comparative Biomedical Sciences, Royal Veterinary College, London NW1 OTU, UK

Corresponding author:

Chantal Chenu, PhD

Department of Comparative Biomedical sciences

Royal Veterinary College, London NW1 OTU, UK

cchenu@rvc.ac.uk

Short title: GLP-1 receptor agonists and bone

Abstract

Type 2 Diabetes Mellitus (T2DM) leads to bone fragility and predisposes to increased risk of fracture, poor bone healing and other skeletal complications. In addition, some anti-diabetic therapies for T2DM can have notable detrimental skeletal effects. Thus an appropriate therapeutic strategy for T2DM should not only be effective in re-establishing good glycaemic control but also in minimising skeletal complications. There is increasing evidence that Glucagon-like peptide-1 receptor agonists (GLP-1RAs), now greatly prescribed for the treatment of T2DM, have beneficial skeletal effects although the underlying mechanisms are not completely understood. This review provides an overview of the direct and indirect effects of GLP-1RAs on bone physiology, focusing on bone quality and novel mechanisms of action on the vasculature and hormonal regulation. The overall experimental studies indicate significant positive skeletal effects of GLP-1RAs on bone quality and strength although their mechanisms of actions may differ according to various GLP-1RAs and clinical studies supporting their bone protective effects are still lacking. The possibility that GLP-1RAs could improve blood supply to bone, which is essential for skeletal health, is of major interest and suggests that GLP-1 anti-diabetic therapy could benefit the rising number of elderly T2DM patients with osteoporosis and high fracture risk.

Lay Abstract

Bone weakening is an important complication in individuals with type 2 diabetes (T2DM). This review summarises the effects on skeletal health of drugs that are similar to the hormone Glucagon-like peptide-1 (GLP-1), which are now used increasingly for the treatment of T2DM and could lead to a stronger skeleton.

1 Introduction

Diabetes mellitus (DM) is a chronic disease that progresses worldwide at alarming rates. For instance, in 2013, it has been estimated that DM affected 382 million individuals (Federation, 2013). Projections for 2035 indicate a global burden of 55% to reach up to 592 million individuals (Federation, 2013). Associated complications are commonly cardio-vascular events, nephropathy, retinopathy, neuropathy and bone fragility that dampen the quality of life of affected individuals.

Type 2 diabetes mellitus (T2DM) is by far the most common form of DM and is characterised by chronic hyperglycaemia and hyperinsulinaemia mostly caused by insulin resistance (IR) in peripheral tissues such as the liver and muscle. The aetiology of bone fragility in T2DM is unclear. Indeed, bone mineral density is normal or slightly elevated in T2DM despite an increase risk of femoral neck fracture, suggesting alterations of bone "quality" rather than bone mass (Vestergaard et al., 2005, Schwartz et al., 2011, Napoli et al., 2016). Bone quality is an umbrella term that regroups factors such as bone microarchitectures, tissue material properties and bone toughness (Chappard et al., 2011). Another important contributor for bone fracture is represented by an increased risk in falls in this population (Schwartz et al., 2002, Schwartz et al., 2008). At the cellular and molecular levels, T2DM is characterised by a reduction in bone turnover suggesting modifications of bone cell behaviours (Vestergaard, 2007). Furthermore, low testosterone and vitamin D levels, and high plasma sclerostin, are common features observed in T2DM patients (Sellmeyer et al., 2016).

Current treatment options of T2DM rely on lifestyle intervention and oral or injectable drugs, when needed, to reach an HbA_{1C} level of 7% or less. Among the most prescribed drugs, the glucagon-like peptide-1 receptor agonists (GLP-1RAs) have recently attracted attention as *Glp-1r* knockout animals and GLP-1 supplemented animals exhibited modifications of bone strength and quality as described below.

Endogenously, GLP-1 is produced by post-translational processing of the glucagon gene in enteroendocrine cells, mainly L-cells (Habib et al., 2012). Two forms of GLP-1 are produced in the intestine, $GLP\text{-}1_{7\text{-}36NH2}$ and $GLP\text{-}1_{7\text{-}37}$ although the major circulating form is $GLP\text{-}1_{7\text{-}36NH2}$ (Orskov et al., 1994). L-cells are an open type endocrine cells highly polarised with secretory granules at their basolateral pole ready to be released in the capillary network running

through the *lamina propria*. This secretion is regulated by intraluminal contents, neural stimuli and hormones (Baggio and Drucker, 2007). Beyond its endocrine mode of action, GLP-1 has also been suspected to act via the autonomous nervous system and hypothalamic and brainstem nuclei (Holst and Deacon, 2005).

To act, GLP-1 engages its receptor; the GLP-1r that is coded by the human *GLP1R* gene comprising 13 exons that span approximately 13.8kb (Yamada et al., 1995) and localised on chromosome 6p21 (Gremlich et al., 1995). The GLP-1r is expressed in the endocrine pancreas, gastro-intestinal tract, lung, heart, kidney and several regions of the brain (Baggio and Drucker, 2007). Recent evidences also suggest that GLP-1 can bind in specific circumstances to the glucagon receptor (Weston et al., 2015). The principal physiological role of GLP-1 is to potentiate glucose-dependent insulin secretion (McIntosh et al., 2010). Extrapancreatic actions of GLP-1 results in reduction of food intake through the CNS, inhibition of gastric emptying, positive actions on the cardiovascular system and a role in energy expenditure (McIntosh et al., 2010).

GLP-1RAs are GLP-1 with extended half-life to be more resistant to degradation by the dipeptidyl peptidase-4 (DPP-4) enzyme. Several molecules listed in Table 1 have been developed by the pharmaceutical industry and now been approved for the treatment of T2DM. The aim of the present review is to provide the reader with a comprehensive analysis of the effects of GLP-1RAs on bone physiology with special focuses on the mode of action including effects on bone quality, blood flow to bone, and on the hormonal regulation of bone metabolism.

2 Pathogenesis of bone fragility in diabetes

As mentioned in the introduction section, the aetiology of diabetes seems linked to bone quality rather than bone quantity. As such it is important to understand what alterations of bone tissue are observed in T2DM individuals.

2.1 Alterations in bone microarchitecture and bone material properties

Often, the assessment of bone microarchitecture and material properties require the use of bone biopsy as a source of bone tissue for experimental investigation. However, such biopsies are not available and microarchitecture and material properties have been investigated in humans by high resolution peripheral quantitative computed tomography (HR-pQCT) and bone microindentation. In term of bone microarchitecture, most studies tend to indicate a preserved trabecular bone microarchitecture but an increase in cortical bone porosity in diabetic individuals with or without fracture (Burghardt et al., 2010, Farr et al., 2014, Patsch et al., 2013). A limitation of HR-pQCT is that it can only be performed at peripheral skeletal sites and may not reflect the full bone phenotype.

In terms of bone material properties, the use of the OsteoProbe bone microindentation device showed that postmenopausal women with T2DM had significantly lower bone material strength index (BMSi) as compared to age- and sex-matched postmenopausal women without diabetes, suggesting altered bone material properties (Farr et al., 2014).

2.2 Advanced glycation endproducts

Prolonged hyperglycaemia leads to the formation of advanced glycation endproducts (AGEs) in the bone matrix that can impair its mechanical properties and the behaviour of bone cells. The most studied AGEs in humans is pentosidine because of its easiness to be measured in clinical samples such as blood or urine. As such, serum and urine pentosidine levels have been correlated with clinical fractures in T2DM patients (Schwartz et al., 2009, Yamamoto et al., 2008). However, further work is required to determine the extent to which circulating levels of AGEs reflect those in human bone tissue.

2.3 Bone turnover markers and circulating sclerostin levels

Multiple studies in humans have found that serum markers of bone formation and resorption are reduced in diabetic individuals vs. non-diabetic controls (Dobnig et al., 2006, Gerdhem et al., 2005, Krakauer et al., 1995, Shu et al., 2012). In contrast, circulating levels of sclerostin have reported to be higher in diabetic individuals (Garcia-Martin et al., 2012,

Gaudio et al., 2012, Gennari et al., 2012) and with regards to sclerostin's potent inhibitory action on bone formation, this may exacerbate the low bone formation phenotype in those patients. As such, long-standing low bone turnover observed in diabetes may result in a defective microdamage repair and increased bone microcrack accumulation that can further contribute to the observed fracture risk. Enzymatic cross-linking of type I collagen by lysyl oxidase is reduced in diabetes (Khosravi et al., 2014, Saito et al., 2006). As circulating markers of bone resorption are based on cross-linked fragments of type I collagen, it is possible that bone resorption is underestimated in diabetes.

3 Skeletal effects of GLP-1RAs: direct and/or indirect mechanisms of action

3.1 Clinical studies

Clinical data on the skeletal effects of GLP-1RAs are scarce. Bone turnover markers and bone mineral density have been assessed in T2DM patients treated with exenatide and liraglutide. However, all these studies reported no effects of GLP-1RA treatment on circulating bone markers or bone mineral density (Li et al., 2015, Bunck et al., 2011, Gilbert et al., 2016). Interestingly, the effects of liraglutide administration on bone turnover markers have been reported not in diabetic but in the obese population for the weight-loss action of liraglutide. In that study, bone formation was improved as indicated by higher values for N-terminal propeptide of type 1 procollagen reported in the liraglutide arm, but no effects on bone resorption were observed (Iepsen et al., 2015).

Two meta-analyses have also been performed on the use of GLP-1RAs and the possible effects of these medications on fracture risk. They showed divergent effects on bone fractures and differences among GLP-1RAs. It was demonstrated that liraglutide significantly reduced the risk of bone fractures whereas exenatide treatment was associated with an elevated risk of incident bone fractures (Su et al., 2015). The other meta-analysis however found neutral effect of both liraglutide and exenatide as compared with other anti-diabetic medications (Mabilleau et al., 2014). Interestingly, Driessen and colleagues (Driessen et al., 2015b, Driessen et al., 2015a) investigated in the British and Danish populations the incidence of bone fracture in GLP-1RA takers as compared with non-takers. No significant

difference was observed and they suggested that the effect of both GLP-1RA-type was neutral in the human diabetic population.

However, interpretation of the above clinical studies and meta-analyses/observational studies should be done carefully as they have some limitations:

- Bone fractures were not the principal end-points and as such are often disclosed as a serious adverse event, although this represents only a fraction of all fractures
- There is a lack of information on bone status (bone mineral density, microarchitecture, bone quality) and calcium and phosphorus metabolism at baseline and at the end of studies that could highlight the possible action of GLP-1RAs on bone strength
- The duration of studies may not be long enough to allow for improvement in bone quality independently of bone turnover markers
- The incidence of GLP-1RAs on falls, and hence a possible mechanism of action to reduce fracture, is very scarce.

Furthermore, as discussed below, a reduction in bone fracture has been evidenced with DPP-4 inhibitors (Monami et al., 2011). However, several differences exist between DPP-4 inhibitors and GLP-1RAs. First, GLP-1RAs induce a modest weight loss whilst DPP-4 inhibitors are neutral on that aspect (Amori et al., 2007, Inzucchi et al., 2012). After the age of 50, weight loss is associated with an increased risk of fracture in overweight and obese individuals (Jensen et al., 1994, Langlois et al., 2001). Secondly, the most common treatment-emergent adverse events with GLP-1RAs are nausea, vomiting and diarrhoea and it is plausible that they result in malabsorption of mineral and nutrients, negatively affecting bone physiology.

In clinical trials, GLP-1RAs have been effective in reducing HbA1c level and hence chronic hyperglycaemia. Data on AGEs and pentosidine in response to GLP-1RAs on the other hand are limited. Tanaka et al, (Tanaka et al., 2015a) demonstrated that despite evident action of liraglutide in reducing circulating glucose in Japanese overweight/obese patients with T2DM, the effects of such molecule on circulating pentosidine were null.

3.2 Effect of DPP-4 inhibitors on the skeleton

The other class of pharmacotherapeutic agents that uses the incretin system are DPP-4 inhibitors which inhibit the principal enzyme responsible for the degradation of endogenous GLP-1. By decreasing clearance of GLP-1, concentrations of active GLP-1 are increased by 2- to 3-fold, resulting in a lowering of fasting and postprandial glucose concentrations.

Data regarding the effects of DPP-4 inhibitors on human skeletal health are quite scarce. A meta-analysis carried out on 28 trials suggests a reduced fracture risk with DPP-4 inhibitors, dependent on the treatment duration (Monami et al., 2011). However, not all studies are showing a positive effect of DPP-4 inhibitors on fracture risk, BMD and bone turnover (Monami et al., 2011, Driessen et al., 2014). Recent preclinical studies showed protective effect of DPP-4 inhibitors on the skeleton of diabetic rats (Glorie et al., 2014, Eom et al., 2016) while others have shown no effect (Gallagher et al., 2014). *In vitro* studies have also indicated neutral effects of DPP-4 inhibitors on bone formation (Gallagher et al., 2014). Therefore, DPP4 inhibitors could have a possible protective effect mediated by an increase of the circulating concentrations of GLP-1 or no adverse effect. Overall, although the interest in this new anti-diabetic treatment effect on bone is high, unfortunately to date data on DPP-4 inhibitors do not allow the stating of recommendations.

3.3 Experimental studies

The first understanding of GLP-1 actions in skeletal physiology arises from *Glp1-r* KO mouse. At 10 weeks of age, these mice exhibited a small reduction in bone mass associated with an increased number of osteoclasts and eroded surfaces (Yamada et al., 2008). On the other hand, the mineral apposition and bone formation rates appeared unaffected by GLP-1r inactivation (Yamada et al., 2008). Similarly, observations in the same KO model at 16 weeks of age and in the double incretin receptor knockout model at 26 weeks of age corroborated these findings (Mabilleau, 2017, Mieczkowska et al., 2015). Taken together these results suggested a control of bone resorption (osteoclast differentiation and/or action) by the GLP-1r. According to the literature, this effect on resorption seems to be indirect through a

reduction in calcitonin gene expression in GLP-1r-deficient animals (Yamada et al., 2008) but further evidences are warranted.

While it is well established that GLP-1RAs increase bone mass in rodents (see paragraph 4), previous investigations of their effects on bone turnover are conflicting. It has been reported that 3 μg/kg/day and 4.2 μg/kg/day exenatide induced bone formation by osteoblast activation in old ovariectomised (OVX) rats (Ma et al., 2013) and in hindlimb-unloading rats (Meng et al., 2016) by promoting the osteogenic differentiation and inhibiting BMSC adipogenic differentiation. A decrease of osteoclastic surfaces was also observed (Ma et al., 2013). In contrast, we found no effect of both 10 μg/kg/day exenatide and 0.3 mg/kg/day liraglutide on bone formation and mineralisation rates in OVX mice and a slight increase of osteoclastic surfaces with the drug using bone histomorphometry (Pereira et al., 2015). The reasons for those discrepancies are unclear and may involve differences in bone turnover in mice and rats and/or in the duration of GLP-1RA treatment. Interestingly, our recent unpublished data demonstrate that GLP-1RAs increase bone formation in a T2DM mouse model but not in lean control mice, suggesting that glucose levels and/or low bone turnover may also influence the skeletal effects of GLP-1RAs. It is possible that the efficacy of GLP-1RAs on the skeleton may be improved in situations where there is a disproportionate reduction in bone formation as compared with resorption such as in T2DM.

3.4 *In vitro* studies

While several studies have reported that GLP-1RAs could have beneficial effects on the skeleton, the downstream molecular mechanisms underlying the osteogenic effect have not been identified (Bjarnason et al., 2002, Clowes et al., 2002). It is indeed unclear whether the mechanism of action of GLP-1RAs in bone is direct, through a functional GLP-1r expressed by bone cells, or indirect, via an increase in calcitonin production by the thyroid C-cell which inhibits bone resorption (Yamada et al., 2008). Furthermore, the presence and the identity of the GLP-1r in bone were controversial until recently and thus the basis for direct skeletal effects of GLP-1 has not been established.

We recently demonstrated that GLP-1 might directly affect bone cells via a GLP-1r identified in primary mouse osteoblasts isolated from calvaria and bone marrow-derived osteoclasts (Pereira et al., 2015) and this was confirmed *in situ* using a GLP-1r antibody (abcam). Similarly, other studies showed that mouse osteoblast-like MC3T3-E1 cells express a functional receptor for GLP-1 (Aoyama et al., 2014). In contrast, expression of the pancreatic-type GLP-1r mRNA was identified in human osteoblastic cell lines deriving from osteosarcomas, but its expression was dependent on the stage of osteoblastic development (Pacheco-Pantoja et al., 2011). However, other study failed to demonstrate the presence of GLP-1r at the mRNA level in primary murine osteoblasts or osteoclasts (Mabilleau et al., 2013). Similarly, the presence of the pancreatic GLP-1r in osteocytic cells was controversial as it has been reported in some cell lines, but not all (Pereira et al., 2015, Kim et al., 2013), as well as in osteocytes in rat femurs (Kim et al., 2013).

The presence of GLP-1r in bone cells *in vitro* and *in situ* implies that GLP-1RAs could have direct effects on bone cells. A study has indeed identified potential skeletal beneficial effects of 10 nM of exenatide by promoting osteoblastogenesis and restraining adipogenesis through a β-catenin pathway, during BMMSC differentiation (Meng et al., 2016). Despite increased osteoblastogenesis, no direct effect of GLP-1RAs on bone nodule mineralisation *in vitro* was shown with up to 100□M of exenatide and 1000nM of liraglutide (Ma et al., 2013, Pereira et al., 2015). It is well established that exposure of primary osteoblast cells to high glucose levels inhibits *in vitro* bone nodule formation (Pereira et al., 2016, Balint et al., 2001). Interestingly, despite no effect of exenatide on bone formation in normal glucose conditions, unpublished results from our group demonstrate that it can reduce the deleterious effect of glucose on bone formation *in vitro*, in a dose-dependent manner. This could be due to upregulated GLP-1r expression in high glucose conditions, which could in turn magnify the effect of GLP-1RAs (Aoyama et al., 2014). Regarding the effects of GLP-1RAs on osteoclastogenesis *in vitro*, we showed that both liraglutide and exenatide increased osteoclastogenesis, while decreasing the area resorbed per osteoclast, suggesting that GLP-1RAs stimulate osteoclastic differentiation but impair their resorptive activity (Pereira et al., 2015).

3.5 GLP-1RA effects on the balance between adipogenesis and osteogenesis and adipocytes

Bone marrow mesenchymal stem cells (BMMSCs) have the ability to differentiate into various cell types, including osteoblasts and adipocytes and can be targeted by anti-diabetic drugs (e.g. thiazolidinedione). Considering the reciprocal relationship between osteogenic and adipogenic differentiation, GLP1-RAs may also indirectly affect bone formation by modulating adipogenesis. Several previous *in vitro* studies have indeed shown that GLP-1 stimulates adipose-derived stem cells (Lee et al., 2015, Cantini et al., 2015) and BMMSC (Lu et al., 2015) towards osteoblast differentiation whereas it inhibits adipocytic differentiation. Furthermore, the GLP-1R is expressed by adipocytes and GLP-1RAs down-regulates adipogenic/lipogenic genes on adipose tissue explants and cultured adipocytes while increasing lipolytic markers and expression of adiponectin (Cantini et al., 2015, El Bekay et al., 2016, Wang et al., 2017). While skeletal effects of adiponectin are multi-faceted and not always concordant, it was suggested that it may be a negative regulator of bone metabolism (Naot et al., 2017), adding to the complexity of the indirect effects of GLP-1RAs on the skeleton. Adipocyte accumulation in the bone marrow during ageing and obesity was recently shown to inhibit bone healing in mice and this was reversed by DPP-4 inhibitors, suggesting that targeting adipocytes with GLP-1RAs may also have beneficial effects on skeletal health (Ambrosi et al., 2017).

3.6 Potential signalling mechanisms of GLP-1 in bone

GLP-1RA binding to the classical pancreatic GLP-1r activates the main (cAMP-PKA) and alternative phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) downstream signalling pathways. While it is still unclear whether GLP-1RAs' skeletal effects are direct via a GLP-1r expressed in bone or indirect, Cantini et al, (71) suggested that in tissues other than pancreas, GLP-1 and GLP-1RAs may not exert their actions through the classical GLP-1r but via unknown alternative pathways. This was observed in cardiomyocytes, liver and muscles (Cantini et al., 2016).

Similarly, GLP-1 action in bone could be mediated by a receptor different from the classical pancreatic cAMP-linked GLP-1r. In fact, Nuche-Berenguer et al, (72) identified in a mouse

osteoblastic line a receptor different from the pancreatic cAMP-linked GLP-1r. Moreover, they showed that GLP-1 binding to this different receptor induced an immediate hydrolysis of glycosylphosphatidylinositols, generating inositolphosphate glycan and activating PI3K and MAPK, without affecting cAMP/PKA classical signalling (Nuche-Berenguer et al., 2010b). Thus, the hypothesis of an indirect action of GLP-1RAs cannot be excluded.

4 GLP-1RAs and bone quality

Unfortunately, as neither peripheral quantitative computed tomography (pQCT) nor iliac crest bone biopsy are part of the usual care in diabetic clinical trials, human data on the effects of GLP-1RAs on all aspects of bone quality are presently missing. As such, the following summary of action of GLP-1RA is based on pre-clinical data obtained in animal models. Several animal models of either osteoporosis or T2DM have been used to assess the effects of two GLP-1RAs, exenatide and liraglutide, on bone quality and strength. However, data concerning potential bone effects of other GLP-1RAs, and especially those administered once weekly, are currently missing. Mice presenting a deletion of GLP-1r have also been generated and represented a suitable model to investigate the role of the GLP-1/GLP-r pathway in bone.

4.1 Effects of GLP-1RAs on bone strength

Our knowledge of the effects of the GLP-1/GLP-1r pathway on bone strength has been markedly improved by the use of *Glp-1r* KO mice. Indeed, although these animals are not diabetic, they exhibited a significant reduction in bone strength represented by lower ultimate load and stiffness (Mabilleau et al., 2013). Bone strength in response to the GLP-1RA exenatide has also been investigated in osteoporotic animal models generated either by ovariectomy or disuse. In ovariectomy-induced osteoporosis, the use of exenatide at a concentration as low as 1 µg/kg/day for 16 weeks, led to improvement in maximum load and stiffness as well as Young's modulus and ultimate stress, suggesting amelioration in bone microarchitecture and/or tissue material properties (Ma et al., 2013). In the rat tail suspension model, the administration of exenatide (4.2 µg/kg/day) for 4 weeks resulted in

higher value for maximum loading, stiffness, stress and Young's modulus, suggesting here again ameliorations in bone microarchitecture and/or tissue material properties (Meng et al., 2016). However, bone strength has not been measured after treatment with liraglutide.

4.2 Effects of GLP-1RAs on bone microarchitecture

In *Glp-1r* KO animals, unpublished observations from our group, revealed that these animals presented with a reduction in cancellous bone volume associated with a lower trabeculae numbers and higher trabecular spacing. These data have been confirmed by the elegant study of Yamada et al, (Yamada et al., 2008) who reported a significant reduction in cancellous bone mineral density in the same transgenic animal model. Alterations of cortical bone in this mouse model were also evidenced with lower outer bone diameter and cortical thickness (Mabilleau et al., 2013). Exenatide and liraglutide have been used as a treatment option in pre-clinical animal models of osteoporosis. They demonstrated positive effects on trabecular bone microarchitecture in the axial and appendicular skeleton evidenced by amelioration of structural parameters in lumbar vertebra and long bones as early as 4 weeks treatment. Indeed, both liraglutide and exenatide treatments resulted in higher bone volume/total volume (BV/TV) values (24% to 148%, depending on dose and treatment duration) and higher values for trabecular number (Tb.N), thickness (Tb.Th) and reduction in separation (Tb.Sp) (Ma et al., 2013, Meng et al., 2016, Pereira et al., 2015, Sun et al., 2016, Lu et al., 2015). When comparing the effects of both GLP-1RAs, liraglutide (0.3mg/kg/day) was more potent than exenatide (10µg/kg/day) (Pereira et al., 2015). The effects of GLP-1RAs on cortical microarchitecture were only observed after a minimum of 8 weeks treatment with exenatide or liraglutide, but highlighted significant increases in cortical thickness with 20µg/kg/day of exenatide or 0.6mg/kg/day of liraglutide (Lu et al., 2015, Sun et al., 2016).

In opposition to what is commonly observed in humans, animal models of T2DM exhibit significant alteration of trabecular and cortical microarchitectures. The effects of GLP-1RAs in diabetic animal models have also been reported. The use of exenatide at a regimen of 10µg/kg/day for 3 days in T2DM animals resulted in improvement in trabecular microarchitecture at the femur and lumbar spine (Nuche-Berenguer et al., 2011, Nuche-

Berenguer et al., 2010a). The use of liraglutide was also investigated in the Goto-Kakizaki T2DM rat model at a dose of 0.4mg/kg/day for 4 weeks. This regimen led to significant improvement in trabecular and cortical bone microarchitectures in the femur and lumbar vertebra (Sun et al., 2015).

The effects of liraglutide on bone microarchitecture have also been investigated in a T1DM mouse model. In this study, the administration of 0.093mg/kg/day liraglutide for 3 weeks did not demonstrate ameliorations of neither trabecular nor cortical microarchitectures (Mansur et al., 2015).

4.3 Effects of GLP-1RAs on tissue material properties

With respect to the improvement in bone strength and intrinsic properties (Young's modulus, stress), that are independent of the bone architecture, one could suspect action of GLP-1RA on tissue material properties. However, very little information has been reported. Tissue material properties represent a set of parameters that describe the modification of biochemical composition or organisation of the bone matrix at the molecular and nanoscale levels (Chappard et al., 2011). This encompasses for a thorough assessment of the mineral and collagen compartment. Most of our knowledge on the action of GLP-1 on tissue material properties is based on Glp1r KO mice. Indeed, in these animals, a significant reduction in enzymatic collagen cross-linking has been evidenced and associated with alteration of bone strength at the tissue level (Mabilleau et al., 2013). However, in opposition to what has been seen with the sister incretin hormone GIP, Glp-1r deletion did not alter the mineral compartment (Mieczkowska et al., 2013). Data regarding the potential effects of GLP-1RAs on tissue material properties in osteoporotic animals are lacking. However, an elegant study conducted by Mansur et al, (Mansur et al., 2015) investigated the effects of 0.093 mg/kg/day liraglutide over a period of 3 weeks in a T1DM mouse model. These authors reported no amelioration of enzymatic collagen cross-linking or collagen glycation but an unexpected reduction in collagen destruction (Mansur et al., 2015).

5 GLP-1RAs and blood flow to bone

Diabetes leads to poor circulation and vascular diseases are the principal causes of death and disability in people with diabetes. Consequently, wound and fracture healing are delayed in diabetic patients, one of the main reasons being the impairment in vascularisation (Falanga, 2005, Loder, 1988). Particularly, diabetes was shown to induce a decrease in endothelial progenitor cells (EPC) that are important for angiogenesis and vascular repair (Fadini et al., 2005, Rigato et al., 2015). It is now well established that blood flow is crucial to bone vascular function and osteogenesis (Ramasamy et al., 2016) and that disrupted blood supply to bone is associated with reduced bone mass, osteonecrosis and impaired bone regeneration (Loder, 1988, Vogt et al., 1997, Atsumi and Kuroki, 1992). Very little work has however examined whether the bone vasculature and bone blood flow are reduced in diabetic bone and if it is possible to restore them with the use of anti-diabetic drugs. Fajardo (Fajardo, 2017) recently reviewed the literature regarding the microvascular complications in diabetic bone but evidences are still lacking to support the link between skeletal fragility in diabetes and those vascular complications.

Incretin-based therapy seems very promising for the prevention of diabetic vascular complications (Mima, 2016). The potential for GLP-1RAs to enhance vascular function has been demonstrated in several studies (Nystrom et al., 2004, Zhou et al., 2015, Sufiun et al., 2015, Smits et al., 2015). The improvement of vascular endothelial function restores impaired glucose tolerance by ameliorating insulin resistance in skeletal muscle (Kubota et al., 2011). Interestingly, two weeks administration of 0.5µg/kg/d exenatide was shown to accelerate diabetic wound healing by increasing angiogenesis in the wound and the number of circulating EPCs (Roan et al., 2017). Our recent, not yet published, work also demonstrates that 10µg/kg exenatide can have beneficial effects on bone vascularisation in diabetic bone by acutely increasing blood flow to bone in db/db mice. This suggests that the increased bone formation induced by exenatide treatment in diabetic mice could be attributed in part to this increased skeletal perfusion. No study has yet examined the effect of liraglutide on bone blood flow. More work is therefore needed to examine whether skeletal perfusion is linked to bone formation in diabetic bone and if GLP-1RAs could be used as treatment to increase vascularisation in diabetic patients with poor fracture healing.

6 GLP-1RAs and hormones that regulate bone metabolism

A major breakthrough in the bone research field has been the finding that bone is an endocrine organ that can affect other organs via the release of hormones such as osteocalcin and sclerostin. There are increasing reports showing that GLP-1RAs can affect the release of these hormones by bone cells *in vitro* and in animal models but the clinical evidence is however still very scarce.

6.1 Sclerostin

The discovery of the importance of the Wnt/ β catenin pathway for bone formation has led to extensive work examining the function of sclerostin in bone. Sclerostin is a product of the *SOST* gene expressed mainly by osteocytes which is secreted and acts as a potent antagonist of Wnt signalling (Bellido, 2014). Its deficiency or its pharmacological neutralisation increases bone formation, making it a potential target for treatment of bone diseases associated with bone loss, such as osteoporosis (Hamann et al., 2013, Ominsky et al., 2010). Most studies have shown that serum sclerostin levels are elevated in diabetic patients, suggesting that sclerostin could contribute to the decreased bone formation observed in diabetic patients (Garcia-Martin et al., 2012, Gaudio et al., 2012, Gennari et al., 2012). However the association between sclerostin levels and increased fracture risk in T2DM patients is not always conclusive and further studies are required to confirm the link (Yu et al., 2017). Some differences in serum sclerostin levels measurements could be explained by the fact that sclerostin could be derived from other non-skeletal sources so that serum levels may not always reflect the production in bone (Roforth et al., 2014) and also because the ELISA kits for sclerostin measurements were found to lack accuracy (Piec et al., 2016, Costa et al., 2017).

To address this issue, experimental studies were conducted examining if sclerostin production by osteocytes is modified in bone of diabetic rodents or *in vitro* when osteocytes are cultured in high glucose levels. Although an *in vitro* study reported an increased production of sclerostin by osteocyte-like cell line when cultured in hyperglycemia (Pereira

et al., 2016, Tanaka et al., 2015b), our recent study shows that the impaired bone microarchitecture and cellular turnover associated with T2DM-like conditions in diabetic ZDF rats are not correlated with changes in serum sclerostin levels, bone sclerostin expression or osteocyte viability (Pereira et al., 2016). On the other hand, high fat diet in mice resulted in increased serum sclerostin and dramatic alterations of osteocyte network organisation (Mabilleau et al., 2016).

Few studies have investigated if GLP-1RAs could affect sclerostin production by osteocytes. Although GLP-1r is mainly expressed by immature osteoblasts, it can be present in osteocytes where it co-localise with sclerostin (Kim et al., 2013), suggesting that GLP-1RAs may affect sclerostin production. Kim et al, (Kim et al., 2013) have indeed shown that sclerostin levels are increased in diabetic rats compared to controls and can be down-regulated by exenatide treatment. More recently, they demonstrate that the DPP-4 inhibitor vildagliptin lowers the increased levels of sclerostin induced by thiazolidinedione (Eom et al., 2016). Our own results showed that exenatide but not liraglutide decreased sclerostin levels in OVX mice (Pereira et al., 2015).

Overall, despite some controversy, the majority of clinical and experimental studies suggest that sclerostin may play a role in the decreased bone turnover in patients with T2DM and be a potential target for GLP-1 therapy. The origin of sclerostin in serum is however still unclear and more studies are required to examine whether bone production of sclerostin is affected in T2DM patients.

6.2 Osteocalcin

Osteocalcin (OC) is a small protein produced in bone by osteoblasts during bone formation which has traditionally been used as a serum marker for bone formation (Ducy et al., 1996). This protein has however regained a different interest in recent years due to the demonstration that when it is in its uncarboxylated form (GluOC) which does not bind to bone, it can circulate, act as a hormone and regulate glucose metabolism (Lee et al., 2007). GluOC can stimulate the release of GLP-1 from the small intestine and therefore indirectly promote insulin secretion by the pancreatic □□cell (Mizokami et al., 2013).

It was suggested that incretins could contribute to whole body energy metabolism by modulating osteocalcin synthesis in osteoblasts. The effects of GLP-1RAs on osteocalcin production by osteoblasts were examined and once again the results are inconsistent. While Kim et al, (Kim et al., 2013), Nuche-Berenguer et al, (Nuche-Berenguer et al., 2010a) demonstrate an increase in serum osteocalcin levels with exenatide in T2DM and IR rats, this was not the case with liraglutide treatment (Iepsen et al., 2015, Conte et al., 2015). A recent study demonstrates that incretins inhibit thyroid hormone-stimulated osteocalcin synthesis in osteoblasts *in vitro*, suggesting that incretins could stimulate bone formation by reducing the osteocalcin levels (Kainuma et al., 2016), although this is not confirmed *in vivo*. Osteocalcin concentration significantly increases during calcification and arterial calcification is an important complication of diabetes due to the differentiation of vascular smooth muscle cells into osteoblast-like cells. Although some work demonstrates an inhibitory effect of GLP-1RAs on vascular calcifications, this is not always the case (Zhan et al., 2014, Davenport et al., 2015).

6.3 Calcitonin

Calcitonin is a peptide hormone produced by the thyroid parafollicular cells, commonly named "C-cells," that regulate calcium homeostasis (Warshawsky et al., 1980). Increases in serum calcium activate the release of calcitonin from the C-cells, which consecutively inhibits bone resorption by the osteoclast and calcium absorption by the intestine. It was therefore one of the first agents to be used as a treatment for osteoporosis. As mentioned previously, several animal studies suggest that GLP-1RAs can affect bone metabolism indirectly via the release of calcitonin by thyroid C cells which express the GLP-1r (Yamada et al., 2008, Lamari et al., 1996). The expression of the GLP-1r in thyroid glands has indeed been documented in rodents (Bjerre Knudsen et al., 2010), but there is an uncertainty regarding its expression in humans (Gier et al., 2012, Hegedus et al., 2011). Furthermore, basal and stimulated calcitonin did not change during 1 year of liraglutide treatment (Lunati et al., 2016). Our own work demonstrates that serum levels of calcitonin were indeed increased by exenatide treatment in ovariectomised mice (Pereira et al., 2015), although this was not shown when mice were treated with liraglutide, suggesting once again that these two GLP-1 agonists may have a different mechanism of action.

7 Differences in mechanisms of action between liraglutide and exenatide

Some divergent skeletal effects of liraglutide and exenatide observed in clinical and experimental studies suggest possible different mechanisms of action. Although overall similar in action, liraglutide and exenatide treatments differ in several aspects mainly due to their differences in molecular structures. Indeed, liraglutide is an analog of human naïve GLP-1 with 97 % homology, whereas exenatide only share 50% homology with human naïve GLP-1. This molecular divergence determines the differences in pharmacokinetic profiles between liraglutide and exenatide (Jespersen et al., 2013). The half-life of liraglutide is five time longer than exenatide, therefore exenatide treatment requires twice daily injections in patients. Moreover, while exenatide is mailnly eliminated in the kidney, liraglutide is fully degraded within the body and no specific organ or enzyme is responsible for its elimination (Giorda et al., 2014). Exenatide administration also results in higher frequency of antibody formation than that of liraglutide (Buse et al., 2011). Thus, the favourable role of liraglutide on bone fractures risk and its more potent effect *in vivo* could be explained in part by its similar pharmacokinetic profile with human naïve GLP-1. On the other hand, exenatide possesses distinct absorption, elimination and antibody formation properties. Whether those different properties of exenatide could interact with some bone metabolism and turnover pathways needs to be clearly elucidated but this may explain the distinctive effects of these two GLP-1RAs on bone hormones production.

8 Conclusion

Based on several rodent studies, GLP-1 therapy emerges as one of the most promising anti-diabetic therapy for treating the skeletal fragility associated with diabetes. It was shown to increase bone mass, improve trabecular and cortical architectures, enhance bone strength and tissue material properties, affecting the collagen compartment rather than the mineral one. The possible mechanisms of action of GLP-1RAs on the skeleton are illustrated in Figure 1. They are however still not very clear and different GLP-1RAs may have different means of action. Among the potential ones, the stimulation of bone blood flow by GLP-1RAs seems very interesting and extremely promising in situations of osteoporotic and diabetic fractures. Clinical data are however still lacking and those establishing the relationship

between the GLP-1RA use and decrease fracture risk have been so far negative. There is therefore a need for long-term clinical studies comparing the skeletal effects of different GLP-1RAs.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research presented

Funding

Some of this work was funded by the Society for Endocrinology (Early career grant for Marie Pereira).

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Figure 1: Simplified scheme of the possible skeletal effects of GLP-1RAs

GLP-1RAs exert multiple beneficial effects on the skeleton. They increase bone mass, improve trabecular and cortical architectures, enhance bone strength and collagen content. They however do not affect bone mineral density (BMD). Several potential mechanisms of action have been described to explain these positive effects of GLP-1RAs on bone. They include indirect effects of GLP-1RAs on bone turnover mediated via hormonal changes. GLP-1RAs were indeed shown to upregulate calcitonin production by C-cells in the thyroid leading to a decrease in bone resorption; alternatively they can down-regulate sclerostin production by osteocytes and increase bone formation. Their beneficial effects on bone blood flow could also contribute to a stimulation of bone formation. GLP-1RAs can also have direct effects on bone cells mediated by the GLP-1R expressed in primary osteoblasts, osteoclasts and in some osteocytes. *In vitro* studies suggest that GLP-1RAs may stimulate bone formation in condition of hyperglycemia and impair osteoclast bone resorptive activity. However, some divergent skeletal effects of liraglutide and exenatide were observed in clinical and experimental studies, suggesting that different GLP-1RAs may use various mechanisms of action.

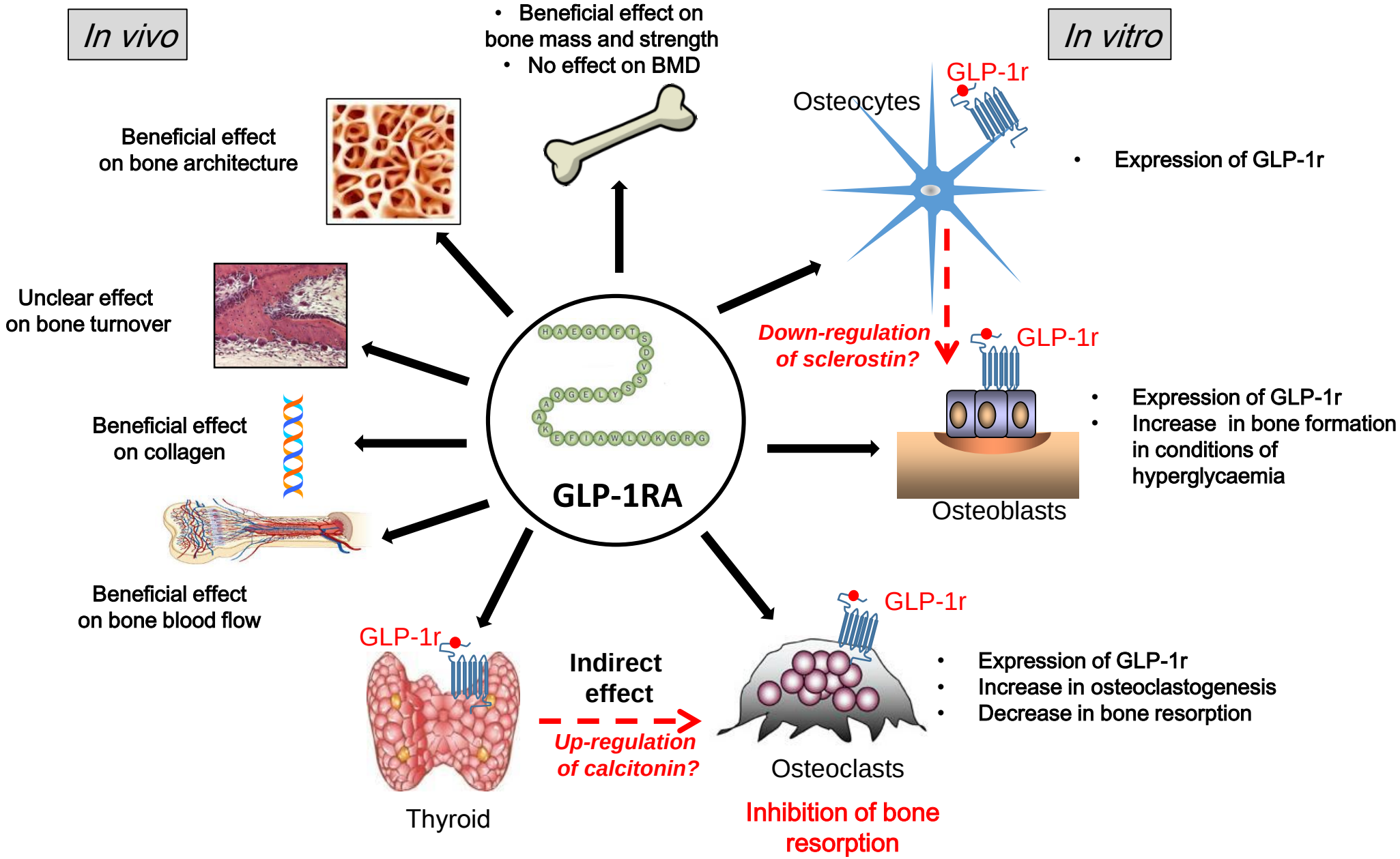
In vivo
• Beneficial effect on bone mass and strength
• No effect on BMD
Beneficial effect on bone architecture
Unclear effect on bone turnover
Beneficial effect on collagen
Beneficial effect on bone blood flow
GLP-1RA
In vitro
Osteocytes
GLP-1r
• Expression of GLP-1r
Down-regulation of sclerostin?
GLP-1r
Osteoblasts
• Expression of GLP-1r
• Increase in bone formation in conditions of hyperglycaemia
GLP-1r
Indirect effect
Up-regulation of calcitonin?
Thyroid
GLP-1r
Osteoclasts
• Expression of GLP-1r
• Increase in osteoclastogenesis
• Decrease in bone resorption
Inhibition of bone resorption

Table 1: Summary of approved GLP-1RAs for the treatment of type 2 diabetes mellitus

Active compound	Drug name	Marketed by	Approved in	Approved dose range
Exenatide (or Exendin-4)	Byetta	Astra Zeneca AB	2006	5-10 µg twice daily
Liraglutide	Victoza	Novo Nordisk A/S	2009	0.6-1.8 mg once daily
Lixisenatide	Lyxumia	Sanofi Aventis Groupe	2013	10-20 µg once daily
Exenatide (or Exendin-4) long acting release	Bydureon	Astra Zeneca AB	2011	2 mg once weekly
Albiglutide	Eperzan	GlaxoSmithKline Trading Services Ltd	2014	30-50 mg once weekly
Dulaglutide	Trulicity	Eli Lilly Nederland B.V.	2014	0.75-1.5 mg once weekly