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

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16

17 **Short title:** GLP-1 receptor agonists and bone

18 **Abstract**

19 Type 2 Diabetes Mellitus (T2DM) leads to bone fragility and predisposes to increased risk of
20 fracture, poor bone healing and other skeletal complications. In addition, some anti-diabetic
21 therapies for T2DM can have notable detrimental skeletal effects. Thus an appropriate
22 therapeutic strategy for T2DM should not only be effective in re-establishing good glycaemic
23 control but also in minimising skeletal complications. There is increasing evidence that
24 Glucagon-like peptide-1 receptor agonists (GLP-1RAs), now greatly prescribed for the
25 treatment of T2DM, have beneficial skeletal effects although the underlying mechanisms
26 are not completely understood. This review provides an overview of the direct and indirect
27 effects of GLP-1RAs on bone physiology, focusing on bone quality and novel mechanisms of
28 action on the vasculature and hormonal regulation. The overall experimental studies
29 indicate significant positive skeletal effects of GLP-1RAs on bone quality and strength
30 although their mechanisms of actions may differ according to various GLP-1RAs and clinical
31 studies supporting their bone protective effects are still lacking. The possibility that GLP-
32 1RAs could improve blood supply to bone, which is essential for skeletal health, is of major
33 interest and suggests that GLP-1 anti-diabetic therapy could benefit the rising number of
34 elderly T2DM patients with osteoporosis and high fracture risk.

35

36 **Lay Abstract**

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

41

42 **1 Introduction**

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

49 Type 2 diabetes mellitus (T2DM) is by far the most common form of DM and is characterised
50 by chronic hyperglycaemia and hyperinsulinaemia mostly caused by insulin resistance (IR) in
51 peripheral tissues such as the liver and muscle. The aetiology of bone fragility in T2DM is
52 unclear. Indeed, bone mineral density is normal or slightly elevated in T2DM despite an
53 increase risk of femoral neck fracture, suggesting alterations of bone "quality" rather than
54 bone mass (Vestergaard et al., 2005, Schwartz et al., 2011, Napoli et al., 2016). Bone quality
55 is an umbrella term that regroups factors such as bone microarchitectures, tissue material
56 properties and bone toughness (Chappard et al., 2011). Another important contributor for
57 bone fracture is represented by an increased risk in falls in this population (Schwartz et al.,
58 2002, Schwartz et al., 2008). At the cellular and molecular levels, T2DM is characterised by a
59 reduction in bone turnover suggesting modifications of bone cell behaviours (Vestergaard,
60 2007). Furthermore, low testosterone and vitamin D levels, and high plasma sclerostin, are
61 common features observed in T2DM patients (Sellmeyer et al., 2016).

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

67 Endogenously, GLP-1 is produced by post-translational processing of the glucagon gene in
68 enteroendocrine cells, mainly L-cells (Habib et al., 2012). Two forms of GLP-1 are produced
69 in the intestine, GLP-1_{7-36NH₂} and GLP-1₇₋₃₇ although the major circulating form is GLP-1_{7-36NH₂}
70 (Orskov et al., 1994). L-cells are an open type endocrine cells highly polarised with secretory
71 granules at their basolateral pole ready to be released in the capillary network running

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

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

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

93

94 **2 Pathogenesis of bone fragility in diabetes**

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

98

99 **2.1 Alterations in bone microarchitecture and bone material properties**

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

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

113

114 **2.2 Advanced glycation endproducts**

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

122

123 **2.3 Bone turnover markers and circulating sclerostin levels**

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

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

136

137 **3 Skeletal effects of GLP-1RAs: direct and/or indirect mechanisms of action**

138 **3.1 Clinical studies**

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

148 Two meta-analyses have also been performed on the use of GLP-1RAs and the possible
149 effects of these medications on fracture risk. They showed divergent effects on bone
150 fractures and differences among GLP-1RAs. It was demonstrated that liraglutide significantly
151 reduced the risk of bone fractures whereas exenatide treatment was associated with an
152 elevated risk of incident bone fractures (Su et al., 2015). The other meta-analysis however
153 found neutral effect of both liraglutide and exenatide as compared with other anti-diabetic
154 medications (Mabilleau et al., 2014). Interestingly, Driessen and colleagues (Driessen et al.,
155 2015b, Driessen et al., 2015a) investigated in the British and Danish populations the
156 incidence of bone fracture in GLP-1RA takers as compared with non-takers. No significant

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

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

- 161 • Bone fractures were not the principal end-points and as such are often disclosed as a
162 serious adverse event, although this represents only a fraction of all fractures
- 163 • There is a lack of information on bone status (bone mineral density,
164 microarchitecture, bone quality) and calcium and phosphorus metabolism at
165 baseline and at the end of studies that could highlight the possible action of GLP-
166 1RAs on bone strength
- 167 • The duration of studies may not be long enough to allow for improvement in bone
168 quality independently of bone turnover markers
- 169 • The incidence of GLP-1RAs on falls, and hence a possible mechanism of action to
170 reduce fracture, is very scarce.

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

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

185

186 **3.2 Effect of DPP-4 inhibitors on the skeleton**

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

191 Data regarding the effects of DPP-4 inhibitors on human skeletal health are quite scarce. A
192 meta-analysis carried out on 28 trials suggests a reduced fracture risk with DPP-4 inhibitors,
193 dependent on the treatment duration (Monami et al., 2011). However, not all studies are
194 showing a positive effect of DPP-4 inhibitors on fracture risk, BMD and bone turnover
195 (Monami et al., 2011, Driessen et al., 2014). Recent preclinical studies showed protective
196 effect of DPP-4 inhibitors on the skeleton of diabetic rats (Glorie et al., 2014, Eom et al.,
197 2016) while others have shown no effect (Gallagher et al., 2014). *In vitro* studies have also
198 indicated neutral effects of DPP-4 inhibitors on bone formation (Gallagher et al., 2014).
199 Therefore, DPP4 inhibitors could have a possible protective effect mediated by an increase
200 of the circulating concentrations of GLP-1 or no adverse effect. Overall, although the
201 interest in this new anti-diabetic treatment effect on bone is high, unfortunately to date
202 data on DPP-4 inhibitors do not allow the stating of recommendations.

203

204 **3.3 Experimental studies**

205 The first understanding of GLP-1 actions in skeletal physiology arises from *Glp1-r* KO mouse.
206 At 10 weeks of age, these mice exhibited a small reduction in bone mass associated with an
207 increased number of osteoclasts and eroded surfaces (Yamada et al., 2008). On the other
208 hand, the mineral apposition and bone formation rates appeared unaffected by GLP-1r
209 inactivation (Yamada et al., 2008). Similarly, observations in the same KO model at 16 weeks
210 of age and in the double incretin receptor knockout model at 26 weeks of age corroborated
211 these findings (Mabilleau, 2017, Mieczkowska et al., 2015). Taken together these results
212 suggested a control of bone resorption (osteoclast differentiation and/or action) by the GLP-
213 1r. According to the literature, this effect on resorption seems to be indirect through a

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

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

233

234 **3.4 *In vitro* studies**

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

243 We recently demonstrated that GLP-1 might directly affect bone cells via a GLP-1r identified
244 in primary mouse osteoblasts isolated from calvaria and bone marrow-derived osteoclasts
245 (Pereira et al., 2015) and this was confirmed *in situ* using a GLP-1r antibody (abcam).
246 Similarly, other studies showed that mouse osteoblast-like MC3T3-E1 cells express a
247 functional receptor for GLP-1 (Aoyama et al., 2014). In contrast, expression of the
248 pancreatic-type GLP-1r mRNA was identified in human osteoblastic cell lines deriving from
249 osteosarcomas, but its expression was dependent on the stage of osteoblastic development
250 (Pacheco-Pantoja et al., 2011). However, other study failed to demonstrate the presence of
251 GLP-1r at the mRNA level in primary murine osteoblasts or osteoclasts (Mabilleau et al.,
252 2013). Similarly, the presence of the pancreatic GLP-1r in osteocytic cells was controversial
253 as it has been reported in some cell lines, but not all (Pereira et al., 2015, Kim et al., 2013),
254 as well as in osteocytes in rat femurs (Kim et al., 2013).

255 The presence of GLP-1r in bone cells *in vitro* and *in situ* implies that GLP-1RAs could have
256 direct effects on bone cells. A study has indeed identified potential skeletal beneficial effects
257 of 10 nM of exenatide by promoting osteoblastogenesis and restraining adipogenesis
258 through a β -catenin pathway, during BMMSC differentiation (Meng et al., 2016). Despite
259 increased osteoblastogenesis, no direct effect of GLP-1RAs on bone nodule mineralisation *in*
260 *vitro* was shown with up to 100 μ M of exenatide and 1000nM of liraglutide (Ma et al., 2013,
261 Pereira et al., 2015). It is well established that exposure of primary osteoblast cells to high
262 glucose levels inhibits *in vitro* bone nodule formation (Pereira et al., 2016, Balint et al.,
263 2001). Interestingly, despite no effect of exenatide on bone formation in normal glucose
264 conditions, unpublished results from our group demonstrate that it can reduce the
265 deleterious effect of glucose on bone formation *in vitro*, in a dose-dependent manner. This
266 could be due to upregulated GLP-1r expression in high glucose conditions, which could in
267 turn magnify the effect of GLP-1RAs (Aoyama et al., 2014). Regarding the effects of GLP-
268 1RAs on osteoclastogenesis *in vitro*, we showed that both liraglutide and exenatide
269 increased osteoclastogenesis, while decreasing the area resorbed per osteoclast, suggesting
270 that GLP-1RAs stimulate osteoclastic differentiation but impair their resorptive activity
271 (Pereira et al., 2015).

272

273 **3.5 GLP-1RA effects on the balance between adipogenesis and**
274 **osteogenesis and adipocytes**

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

292

293 **3.6 Potential signalling mechanisms of GLP-1 in bone**

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

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

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

308

309 **4 GLP-1RAs and bone quality**

310 Unfortunately, as neither peripheral quantitative computed tomography (pQCT) nor iliac
311 crest bone biopsy are part of the usual care in diabetic clinical trials, human data on the
312 effects of GLP-1RAs on all aspects of bone quality are presently missing. As such, the
313 following summary of action of GLP-1RA is based on pre-clinical data obtained in animal
314 models. Several animal models of either osteoporosis or T2DM have been used to assess the
315 effects of two GLP-1RAs, exenatide and liraglutide, on bone quality and strength. However,
316 data concerning potential bone effects of other GLP-1RAs, and especially those
317 administered once weekly, are currently missing. Mice presenting a deletion of GLP-1r have
318 also been generated and represented a suitable model to investigate the role of the GLP-
319 1/GLP-r pathway in bone.

320

321 **4.1 Effects of GLP-1RAs on bone strength**

322 Our knowledge of the effects of the GLP-1/GLP-1r pathway on bone strength has been
323 markedly improved by the use of *Glp-1r* KO mice. Indeed, although these animals are not
324 diabetic, they exhibited a significant reduction in bone strength represented by lower
325 ultimate load and stiffness (Mabilleau et al., 2013). Bone strength in response to the GLP-
326 1RA exenatide has also been investigated in osteoporotic animal models generated either
327 by ovariectomy or disuse. In ovariectomy-induced osteoporosis, the use of exenatide at a
328 concentration as low as 1 µg/kg/day for 16 weeks, led to improvement in maximum load
329 and stiffness as well as Young's modulus and ultimate stress, suggesting amelioration in
330 bone microarchitecture and/or tissue material properties (Ma et al., 2013). In the rat tail
331 suspension model, the administration of exenatide (4.2 µg/kg/day) for 4 weeks resulted in

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

335

336 **4.2 Effects of GLP-1RAs on bone microarchitecture**

337 In *Glp-1r* KO animals, unpublished observations from our group, revealed that these animals
338 presented with a reduction in cancellous bone volume associated with a lower trabeculae
339 numbers and higher trabecular spacing. These data have been confirmed by the elegant
340 study of Yamada et al, (Yamada et al., 2008) who reported a significant reduction in
341 cancellous bone mineral density in the same transgenic animal model. Alterations of cortical
342 bone in this mouse model were also evidenced with lower outer bone diameter and cortical
343 thickness (Mabilleau et al., 2013). Exenatide and liraglutide have been used as a treatment
344 option in pre-clinical animal models of osteoporosis. They demonstrated positive effects on
345 trabecular bone microarchitecture in the axial and appendicular skeleton evidenced by
346 amelioration of structural parameters in lumbar vertebra and long bones as early as 4 weeks
347 treatment. Indeed, both liraglutide and exenatide treatments resulted in higher bone
348 volume/total volume (BV/TV) values (24% to 148%, depending on dose and treatment
349 duration) and higher values for trabecular number (Tb.N), thickness (Tb.Th) and reduction in
350 separation (Tb.Sp) (Ma et al., 2013, Meng et al., 2016, Pereira et al., 2015, Sun et al., 2016,
351 Lu et al., 2015). When comparing the effects of both GLP-1RAs, liraglutide (0.3mg/kg/day)
352 was more potent than exenatide (10µg/kg/day) (Pereira et al., 2015). The effects of GLP-
353 1RAs on cortical microarchitecture were only observed after a minimum of 8 weeks
354 treatment with exenatide or liraglutide, but highlighted significant increases in cortical
355 thickness with 20µg/kg/day of exenatide or 0.6mg/kg/day of liraglutide (Lu et al., 2015, Sun
356 et al., 2016).

357 In opposition to what is commonly observed in humans, animal models of T2DM exhibit
358 significant alteration of trabecular and cortical microarchitectures. The effects of GLP-1RAs
359 in diabetic animal models have also been reported. The use of exenatide at a regimen of
360 10µg/kg/day for 3 days in T2DM animals resulted in improvement in trabecular
361 microarchitecture at the femur and lumbar spine (Nuche-Berenguer et al., 2011, Nuche-

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

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

370

371 **4.3 Effects of GLP-1RAs on tissue material properties**

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

389

390 **5 GLP-1RAs and blood flow to bone**

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

405 Incretin-based therapy seems very promising for the prevention of diabetic vascular
406 complications (Mima, 2016). The potential for GLP-1RAs to enhance vascular function has
407 been demonstrated in several studies (Nystrom et al., 2004, Zhou et al., 2015, Sufiun et al.,
408 2015, Smits et al., 2015). The improvement of vascular endothelial function restores
409 impaired glucose tolerance by ameliorating insulin resistance in skeletal muscle (Kubota et
410 al., 2011). Interestingly, two weeks administration of 0.5µg/kg/d exenatide was shown to
411 accelerate diabetic wound healing by increasing angiogenesis in the wound and the number
412 of circulating EPCs (Roan et al., 2017). Our recent, not yet published, work also
413 demonstrates that 10µg/kg exenatide can have beneficial effects on bone vascularisation in
414 diabetic bone by acutely increasing blood flow to bone in db/db mice. This suggests that the
415 increased bone formation induced by exenatide treatment in diabetic mice could be
416 attributed in part to this increased skeletal perfusion. No study has yet examined the effect
417 of liraglutide on bone blood flow. More work is therefore needed to examine whether
418 skeletal perfusion is linked to bone formation in diabetic bone and if GLP-1RAs could be
419 used as treatment to increase vascularisation in diabetic patients with poor fracture healing.

420

421 **6 GLP-1RAs and hormones that regulate bone metabolism**

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

427

428 **6.1 Sclerostin**

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

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

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

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

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

469

470 **6.2 Osteocalcin**

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

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

492

493 **6.3 Calcitonin**

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

509 **7 Differences in mechanisms of action between liraglutide and exenatide**

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

528

529 **8 Conclusion**

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

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

542

543 **Declaration of interest**

544 There is no conflict of interest that could be perceived as prejudicing the impartiality of the
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546

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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.

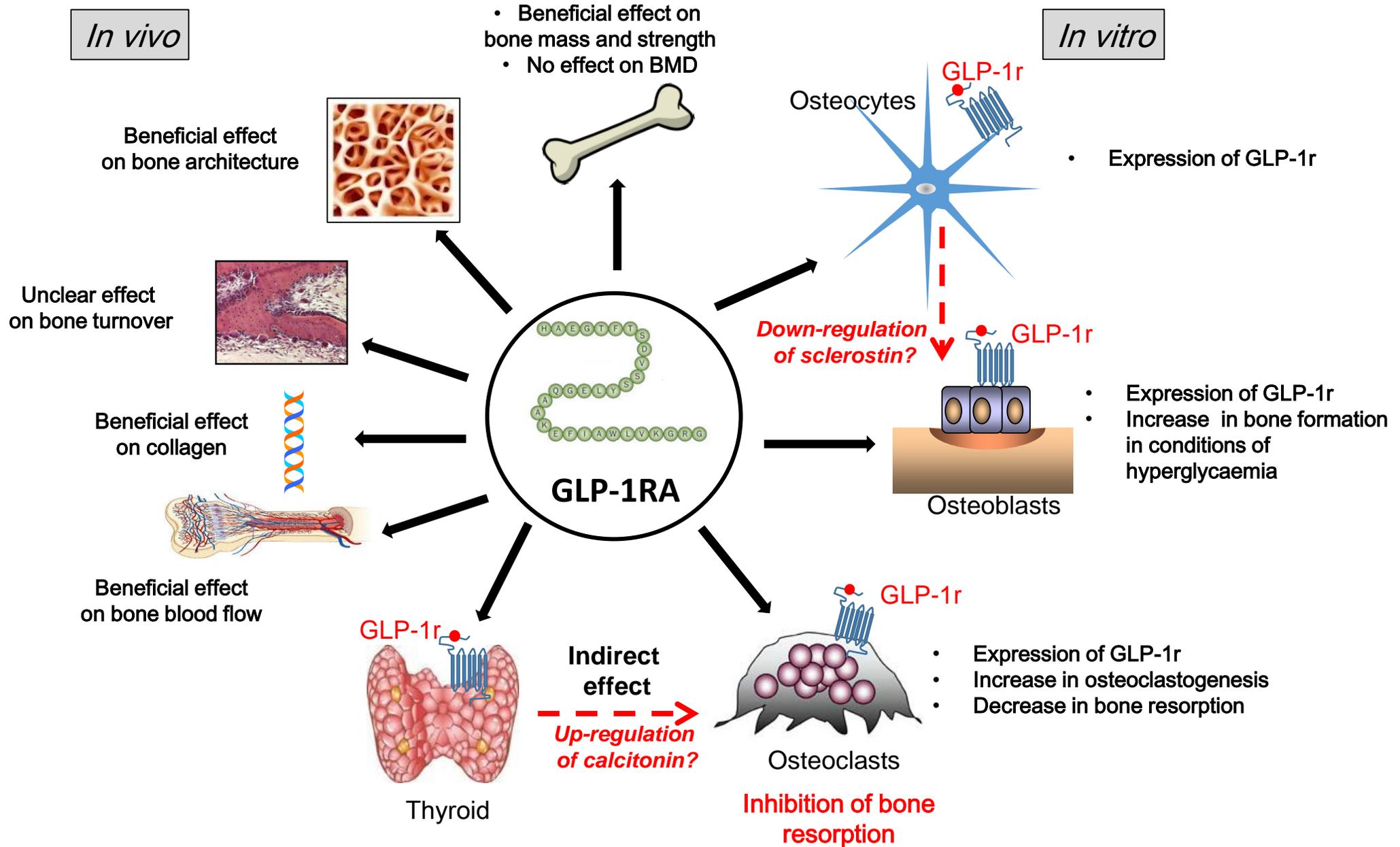


Table 1: Summary of approved GLP-1RAs for the treatment of type 2 diabetes mellitus

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