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

2 agonists

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- 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 effects of GLP-1RAs on bone physiology, focusing on bone quality and novel mechanisms of 27 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.

42 **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 cardiovascular events, nephropathy, retinopathy, neuropathy and bone fragility that dampen the 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., 2002, Schwartz et al., 2008). At the cellular and molecular levels, T2DM is characterised by a 58 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.

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-1_{7-36NH2} and GLP-1₇₋₃₇ although the major circulating form is GLP-1_{7-36NH2}
(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).

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).

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.

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94 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.

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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).

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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.

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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, 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.

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

However, interpretation of the above clinical studies and meta-analyses/observationalstudies 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.

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.

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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 guite 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.

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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

reduction in calcitonin gene expression in GLP-1r-deficient animals (Yamada et al., 2008) but
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 μ g/kg/day and 4.2 μ g/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 μ g/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 by bone cells, or indirect, via an increase in calcitonin production by the thyroid C-cell which 239 240 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 241 242 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[®]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).

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3.5 GLP-1RA effects on the balance between adipogenesis and 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).

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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).

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 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.

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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.

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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 μ g/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

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.

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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 volume/total volume (BV/TV) values (24% to 148%, depending on dose and treatment 348 349 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).

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).

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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, GIp-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).

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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 delayed in diabetic patients, one of the main reasons being the impairment in 393 394 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 $0.5\mu g/kg/d$ 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 $10\mu g/kg$ 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).

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).

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

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).

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 (lepsen 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.

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 mailnly 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 545 research presented

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 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