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1 **Optimal bone mechanical and material properties require a functional**
2 **GLP-1 receptor**

3
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13 **Running title: Bone strength in GLP-1R KO mice**
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20 **ABSTRACT**

21 Bone is permanently remodeled by a complex network of local, hormonal and neuronal factors that
22 affect osteoclast and osteoblast biology. Among these factors a role for gastro-intestinal hormones has
23 been proposed based on evidence that bone resorption dramatically falls after a meal. Glucagon like
24 peptide-1 (GLP-1) is one of these gut hormones and despite several reports suggesting an anabolic
25 effect of GLP-1, or its stable analogues, on bone mass, little is known about the effects of the GLP-
26 1/GLP-1 receptor on bone strength. In the present study we investigated by three-point bending,
27 quantitative x-ray microradiography, microCT, qBEI and FTIRI bone strength and bone quality in male
28 GLP-1R knockout (GLP-1R KO) mice as compared with control wild-type (WT) animals. Animals with
29 a deletion of the GLP-1R presented with a significant reduction of ultimate load, yield load, stiffness,
30 total absorbed and post-yield energies as compared with WT animals. Furthermore, cortical thickness
31 and bone outer diameter were significantly decreased in deficient animals. The mineral quantity and
32 quality were not significantly different between GLP-1R KO and WT animals. On the other hand, the
33 maturity of the collagen matrix was significantly reduced in deficient animals and associated with
34 lowered material properties. Taken together, these data support a positive effect of the GLP-1R on
35 bone strength and quality.

36

37 INTRODUCTION

38 Bone is a living mineralized material, highly complex and constantly remodeled in mass and
39 architecture to adapt and repair the damage induced by growth, ageing and mechanical stress. In
40 order to maintain a constant bone mass, bone remodeling necessitates a spatio-temporal coupling
41 between osteoclasts, the bone-resorbing cells, and osteoblasts, the bone-forming cells. Bone
42 remodeling is a complex process regulated by various stimuli that may affect osteoclast and osteoblast
43 physiologies. A role for the gastro-intestinal tract in bone remodeling has been suggested with
44 evidence that modulation of serum markers of bone resorption is mirrored with the profile of gut
45 hormone release after a meal (Elnenaï, et al. 2010; Henriksen, et al. 2003).

46 Glucagon-like peptide-1 (GLP-1) is a gut hormone synthesized and secreted into the blood stream by
47 intestinal endocrine L cells in response to a variety of stimuli (Wu, et al. 2010). Biologically active GLP-
48 1 is secreted as a 7-37 or 7-36 amide peptide which is rapidly inactivated to GLP-1(9-36) by cleavage
49 at the site of the second N-terminal alanine amino acid residue by the aminopeptidase, dipeptidyl
50 peptidase IV (DPP-IV) (Drucker and Nauck 2006). GLP-1 action is tightly controlled by DPP-IV as
51 more than 50% of GLP-1 is inactivated before it reaches the systemic circulation and the half-life of
52 circulating GLP-1 is less than 2 min (Baggio and Drucker 2007). Nevertheless, GLP-1 binds to its
53 receptor, the GLP-1R, widely expressed in pancreatic islets, kidney, lung, heart, stomach, intestine
54 and the central nervous system and this interaction results in activation of intracellular signaling
55 (Bullock, et al. 1996; Campos, et al. 1994).

56 Although uncertainty persists to clearly demonstrate expression of the GLP-1R in bone cells,
57 administration of GLP-1 or its enzyme resistant analogue exendin-4 for three days in normal and
58 diabetic rats results in increased trabecular bone mass and augmentation of the expression of
59 osteoblast markers in these animals, suggesting a possible anabolic effect of GLP-1 on trabecular
60 bone (Nuche-Berenguer, et al. 2009; Nuche-Berenguer, et al. 2010). Using a model of GLP-1
61 signaling deficiency, Yamada et al., reported that GLP-1R knockout mice exhibit a trend for lower
62 trabecular bone mass in the proximal metaphysis of the tibia (Yamada, et al. 2008). These authors
63 also reported reduction in cortical BMD as evidenced by an experimental CT-based densitometry.
64 Taken together, all these data support an anabolic role of the GLP-1/GLP-1R pathway in bone.
65 However, despite these observations, little is known about the impact of the GLP-1/GLP-1R on bone
66 strength. Bone strength depends on bone and mineral quantity and also on structural and material
67 properties of the bone matrix. Microarchitecture and material properties including degree of
68 mineralization, mineral maturity and collagen properties are known factors that influence bone strength
69 (Chappard, et al. 2011).

70 The aims of the present study were to investigate bone strength and bone quality in GLP-1R knockout
71 male mice. Our results suggest that GLP-1R-deficient male mice present with significant alterations of
72 the cortical morphology and material properties that undoubtedly resulted in reduced bone strength.
73 These findings support a positive role for the GLP-1/GLP-1R pathway in controlling bone strength.

74

75 MATERIAL AND METHODS

76 **Animals**

77 Sixteen-week-old male mice presenting a deletion of the GLP-1R (kindly provided by Prof. DJ Drucker,
78 Toronto, Canada) were used in this study. The background and generation of GLP-1R-deficient mice
79 used in this study were derived from an in-house breeding colony originally described elsewhere
80 (Hansotia, et al. 2004). Age-matched wild-type (WT) mice bred in-house with the same C57BL/6
81 genetic background were used as controls. A total of 8 deficient and 15 control mice were used in this
82 study. Animals were maintained on a 12h:12h light-dark cycle in a temperature-controlled room ($21.5 \pm$
83 1°C). Animals were individually caged and received food and water *ad libitum*. All experiments were
84 conducted according to United Kingdom Office regulations (UK Animals Scientific Procedures Act
85 1986) and European Union laws. Animals were sacrificed by lethal inhalation of CO_2 and left and right
86 femurs were collected, cleaned of soft tissue. Left femurs were stored in 70% ethanol at 4°C whilst
87 right femurs were frozen in a saline-soaked gauze and stored at -20°C until use. Figure 1 describes
88 the experimental design.

89

90 **Bone mechanical testing**

91 Three-point bending experiment were performed on the right femur. Before mechanical testing, femurs
92 were thaw overnight and rehydrated in saline for 24hrs at room temperature as described elsewhere
93 (Ammann, et al. 2007). Three-point bending strength was measured with a constant span length of 10
94 mm. The press head as well as the two support points were rounded to avoid shear load and cutting.
95 Femurs were positioned horizontally with the anterior surface facing upwards, centered on the support
96 and the pressing force was applied vertically to the midshaft of the bone. Each bone was tested with a
97 loading speed of $2 \text{ mm}\cdot\text{min}^{-1}$ until failure with a 90N load cell. The load-time curve obtained was
98 converted into a load-displacement curve by the MTS testSuite TW software (MTS, Créteil, France).
99 Ultimate load and ultimate displacement were respectively defined as the maximum load and
100 maximum displacement recorded before break-down of the bone. Stiffness was calculated as the
101 slope of the elastic deformation of the bone. The total absorbed energy was defined as the total area
102 under the load-displacement curve and represents the total energy absorbed by the midshaft femur.
103 The yield was defined as the load necessary to initiate the transformation from elastic to plastic
104 deformation. The post-yield energy was defined as the area under the load-displacement curve from
105 yield until failure and represents the energy absorbed by bone during plastic deformation. Material
106 properties such as ultimate strength, ultimate strain, elastic modulus, yield strength, breaking strength
107 and work to fracture have been calculated from mechanical parameters according to previously
108 published equations (Ritchie, et al. 2008; Turner and Burr 1993).

109

110 **Quantitative x-ray microradiograph imaging**

111 Bone mineral content was determined using qXRI. Briefly, digital X-ray images of the left femur were
112 recorded at a $12\text{-}\mu\text{m}$ pixel resolution using a Faxitron MX20 device (Edimex, Angers, France)
113 operating at 26 kV and a 4X magnification. The region of interest was located 6 mm below the growth
114 plate (mid-shaft of the femur) and represented a height of 2 mm. The relative mineral content of

115 calcified tissues was determined as reported by Bassett et al. (Bassett, et al. 2012) with the following
116 modifications. Briefly, a 1.5-mm thick steel plate, a 1.5-mm pure aluminum wire and a 1.5-mm thick
117 polyester plate were used on each microradiograph and served as standards. Before converting the
118 16-bit DICOM images into 8-bit tiff images, the histogram was stretched from the polyester (gray level
119 0) to the steel (gray level 255) standards using ImageJ 1.45s. Increasing gradations of mineralization
120 density were represented in 16 equal intervals using the 16-colours lookup table in ImageJ 1.45s. The
121 frequency of occurrence of an i grey level (F_i) was calculated as follows:

$$F_i = 100 \times \frac{N_i}{N_t}$$

122
123 Where N_i represents the number of pixels with the i grey level and N_t the total number of pixels. The
124 frequency distribution as a function of grey level was plotted and the mean grey level (GL_{mean}) of each
125 bone was deduced from this distribution using the following formula:

$$GL_{mean} = \sum \frac{F_i \times GL_i}{100}$$

126
127 where GL_i represents the value of the i grey level.

128

129 ***X-ray microcomputed tomography (MicroCT)***

130 MicroCT analysis was performed in the proximal left femur with a Skyscan 1172 microtomograph
131 (Bruker MicroCT, Kontich, Belgium) equipped with an X-ray tube working at 69 kV/100 μ A. The
132 pixel size was fixed at 3.75 μ m, the rotation step at 0.25° and exposure was done with a 0.5-mm
133 aluminum filter. The region of interest (VOI) was located 6-mm below the growth plate. External bone
134 diameter (B.Dm in mm), marrow diameter (Ma.Dm in mm), cortical thickness (Ct.Th in μ m), and cross-
135 sectional moment of inertia (CSMI in mm⁴) were measured with a lab-based routine made with ImageJ
136 1.45s (NIH, Bethesda, MD) according to guidelines and nomenclature proposed by the American
137 Society for Bone and Mineral Research (Bouxsein, et al. 2010). Three-D models represent a region of
138 interest of 2 mm centered 6 mm below the growth plate.

139

140 ***Bone Mineral Density Distribution measured by backscattered electron imaging.***

141 Quantitative backscattered electron imaging was employed to determine the bone mineral density
142 distribution (BMDD) as previously reported (Gaudin-Audrain, et al. 2013; Roschger, et al. 1998).
143 Rapidly after 3-point bending, lower portions of the right femur diaphysis were embedded
144 undecalcified in methylmethacrylate at 4°C. Polymethylmethacrylate blocks were then polished to a 1-
145 μ m finish with diamond particles, carbon-coated and observed with a scanning electron microscope
146 (EVO LS10, Carl Zeiss Ltd, Nanterre, France) equipped with a five quadrant semi-conductor
147 backscattered electron detector. The microscope was operated at 20 keV with a probe current of 120
148 pA and a working distance of 8.5 mm. The backscattered signal was calibrated using pure carbon
149 ($Z=6$, mean grey level = 25), pure aluminum ($Z=13$, mean grey level =225) and pure silicium ($Z=14$,
150 mean grey level =253) standards (Micro-analysis Consultants Ltd, St Ives, UK). For these
151 contrast/brightness settings, the BSE grey level histogram was converted into weight percentage of
152 calcium. Eventual changes in brightness and contrast due to instrument instabilities were checked by
153 monitoring the current probe and imaging the reference material (C, Al and Si) every 15 min. The

154 cortical bone area was imaged at a 200X nominal magnification, corresponding to a pixel size of 0.5
155 μm per pixel. The grey levels distribution of each image was analyzed with a lab-made routine using
156 Image J. Three variables were obtained from the bone mineral density distribution: Ca_{peak} is the most
157 frequently observed calcium concentration, Ca_{mean} is the average calcium concentration and Ca_{width} is
158 the width of the histogram at half maximum of the peak.

159

160 ***Nanomechanical testing***

161 Nanoindentation tests evaluated the mechanical properties of the bone matrix. As nanoindentation
162 assesses volume of material at a length scale less than that of individual microstructural features in
163 bone, this technique avoids confounding factors such as bone microarchitecture and porosity that
164 affect tissue properties at larger length scales such as 3-point bending. Tests were performed on the
165 same sample used for qBEI measurements after rapid polishing. Briefly, femurs were rehydrated
166 overnight in saline prior to nanoindentation testing. Eight indents were positioned in cortical bone 6-
167 mm below the growth plate with a NHT-TTX system (CSM, Peseux, Switzerland) equipped with a
168 Berkowitch diamond probe. The indents were done up to a depth of 900 nm with a loading/unloading
169 rate of 40mN/min. At maximum load, a holding period of 15 seconds was applied to avoid creeping of
170 the bone material. Maximum load, indentation modulus, hardness and dissipated energy were
171 determined according to Oliver and Pharr (Oliver and Pharr 1992).

172

173 ***Fourier Transformed InfraRed spectroscopy (FTIR)***

174 After 3 point bending, upper portions of the femur diaphysis were embedded in pMMA at 4°C.
175 Sections of 4 μm thickness were cut dry on a heavy duty microtome equipped with tungsten carbide
176 knives (Leica Polycut S) and sandwiched between BaF₂ optical windows. Spectral analysis were
177 obtained on a Bruker Vertex 70 spectrometer (Bruker optics, Ettlingen, Germany) interfaced with a
178 Bruker Hyperion 3000 infrared microscope equipped with a standard single element Mercury
179 Cadmium Telluride (MCT) detector (750-4000 cm^{-1}). Infrared spectra were recorded at a resolution of
180 4 cm^{-1} , with an average of 32 scans in transmission mode. Background spectral images were collected
181 under identical conditions from the same BaF₂ windows at the beginning and end of each experiment
182 to ensure instrument stability. For FTIR analysis, 10 spectra were acquired approximately 6-mm below
183 the growth plate on cortical bone, corresponding to the fracture zone observed by 3 point bending, and
184 analyzed with the Opus Software (release 5.5, Bruker). The contribution of the embedding
185 polymethylmethacrylate (pMMA) and water vapor were corrected for each spectrum prior to baseline
186 correction. Then individual spectra have been subjected to curve fitting using a commercial available
187 software package (Grams/AI 8.0, Thermofisher scientific, Villebon sur Yvette, France). Briefly, using
188 the Levenberg-Maquardt algorithm every absorption band is characterized by its area. The second
189 derivative spectrum was used to determine the number and the position of the bands constituting
190 every spectral interval. All bands were positioned at maximal intensities with a Gaussian shape.
191 Position, height, width at half intensity and area were obtained. Peaks corresponding to amide I and II
192 (1350-1725 cm^{-1}) and $\nu_1\nu_3$ phosphate (900-1200 cm^{-1}) were considered for further analysis. The
193 evaluated IR spectral parameters were (1) mineral-to-matrix ratio which reflects the degree of

194 mineralization of the bone matrix, calculated from the ratio of integrated areas of the phosphate ν_1 , ν_3
195 band at 900-1200 cm^{-1} to the amide I band at 1585-1725 cm^{-1} ; (2) mineral maturity, which reflects the
196 ratio of apatic vs. nonapatic domains, calculated as the ratio of the relative intensity of subbands at
197 1020 and 1030 cm^{-1} of the phosphate band (Paschalis, et al. 1996);(3) carbonate-to-phosphate ratio
198 was expressed by the ratio of integrated areas of the ν_2 CO_3^{2-} region (850-890 cm^{-1}) to the ν_1 , ν_3
199 phosphate band (900-1200 cm^{-1}) (Boskey, et al. 2005) and (4) collagen maturity, determined as the
200 relative ratio of subbands located at 1660 cm^{-1} and 1690 cm^{-1} of the amide I peak. Although multiple
201 theories exists about the significance of this ratio (pyridinium trivalent to
202 dehydrodihydroxylysinonorleucine divalent collagen cross-links, modifications of secondary structure
203 of collagen molecules), this ratio indicates the maturity of the collagen of the bone matrix (Farlay, et al.
204 2011; Paschalis, et al. 2001).

205

206 **Gene expression**

207 Bone marrow cells were isolated from the long bones of 16 weeks-old WT animals by flushing tibias
208 and femurs with alpha-MEM as previously reported (Mieczkowska, et al. 2012). For osteoblast
209 differentiation, bone marrow cells were plated into a 25 cm^2 culture flask until confluency (~3 days).
210 For osteoclast culture, bone marrow cells were cultured for 24hrs into a 25 cm^2 flask in alpha-MEM
211 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$
212 streptomycin to allow stromal cells attachment. Non adherent cells were then collected and plated in
213 24 well-plate in alpha-MEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml
214 penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 25 ng/ml macrophage-colony stimulating factor (M-CSF-R&D
215 systems, Abingdon, UK) and 50 ng/ml soluble RANKL (peprotech Ltd, London, UK). After seven days
216 of culture, multinucleated osteoclasts were evidenced in culture as previously described (Mabilleau, et
217 al. 2011). Total RNA were extracted from osteoblast or osteoclast ex vivo cultures, lung, pancreatic
218 islets and heart using TriZol, reversed transcribed using iScript cDNA synthesis kit (Bio-Rad) and
219 amplified by real-time PCR using SYBR GREEN PCR master mix (Bio-Rad). The mouse *Glp1r* mRNA
220 transcript was amplified by quantitative PCR using primer pairs 5'-
221 GGGTCTCTGGCTACATAAGGACAAC-3' and 5'-AAGGATGGCTGAAGCGATGAC-3'. The expression
222 level of each sample was normalized against *gapdh* mRNA expression using primer pairs 5'-
223 AATGGATTTGGACGCATTGGT-3' and 5'-TTTGCACTGGTACGTGTTGAT-3'.

224

225 **Statistical analysis**

226 Results were expressed as mean \pm standard error of the mean (SEM). Non-parametric Mann-Whitney
227 U-test was used to compare the differences between the groups using the Systat statistical software
228 release 13.0 (Systat software Inc., San Jose, CA). Correlation between material properties and
229 collagen cross-link ratio was assessed by a least squares linear regression analysis. Differences at
230 $p < 0.05$ were considered significant.

231

232

233 **RESULTS**

234 ***GLP-1R is required for optimum bone strength***

235 Bone strength of the femur was assessed by three-point bending and the results are presented Table
236 1. In GLP-1R KO mice, mechanical parameters such as ultimate load, yield load, stiffness, total
237 absorbed and post-yield energies were reduced significantly by 17 %, 21%, 25 %, 34% and 26%
238 respectively as compared with WT animals. No differences in ultimate displacement were observed
239 between these two groups of animals. Material properties such as work to fracture and yield strength
240 were significantly reduced in GLP-1R-deficient mice by 20%, 20% and 7% respectively as compared
241 with WT mice. No significant differences were observed for ultimate strength, breaking strength,
242 ultimate strain and elastic modulus.

243 ***GLP-1R-deficiency results in altered femoral mineral density and cortical geometry***

244 As represented Figure 2A, the bone mineral content seemed lower in GLP-1R KO animals and indeed,
245 the frequency of occurrence of grey level, representing the bone mineral content, was switched toward
246 the left in GLP-1R KO mice indicating a lower bone mineral content. Furthermore, the mean grey level
247 was significantly reduced by 5.2% in GLP-1R KO mice as compared with WT controls (p=0.021).
248 Histomorphometric analysis of cortical bone (Figure 2B) revealed that B.Dm was reduced significantly
249 by 6% in GLP-1R KO animals as compared with WT controls (p=0.034) whilst Ma.Dm was unchanged
250 (p=0.289). Consequently, Ct.Th and CSMI were lowered significantly by 13% and 25% respectively in
251 deficient animals as compared with WT (p=0.034 and p=0.031 respectively).

252 ***Nanomechanical properties of the bone matrix are reduced in GLP-1R KO mice***

253 As compared with WT animals, GLP-1R-deficient animals exhibited a significant 19% decrease in
254 maximum load, as well as a significant reduction in bone matrix hardness (-19%) (Table 2). The
255 indentation modulus was unchanged between the two groups of animals but the energy dissipated
256 was significantly reduced by 15% in GLP-1R deficient mice.

257 ***Collagen maturation but not mineral maturation is altered in GLP-1R KO mice***

258 The bone mineral density distribution in the bone matrix was investigated by qBEI in WT and GLP-1R
259 KO mice (Table 3). Interestingly, no differences were observed between the two groups of animals in
260 any of the studied parameters, indicating that the profile of mineralization of the bone matrix was
261 similar in both cases. Similarly, the mineral-to-matrix ratio determined from FTIR measurements was
262 not significantly different between WT and GLP-1R *-/-* animals (Figure 3A). The mineral maturity and
263 carbonate content were unchanged in GLP-1R *-/-* animals as compared with WT (p=0.221 and
264 p=0.536 respectively). On the other hand, the ratio of trivalent mature over divalent immature collagen
265 crosslinks was significantly decreased by 15% in GLP-1R *-/-* mice as compared with WT (p=0.007,
266 Figure 3D). As shown in Figure 4, a positive correlation ($R^2=0.62$, $p<0.004$) was found between
267 dissipated energy assessed by nanoindentation and the $1660/1690\text{ cm}^{-1}$ ratio assessed by FTIR.

268 However, no significant correlations were found between maximum load or hardness with the
269 1660/1690 cm⁻¹ ratio (R²=0.33, p=0.067 and R²=0.13, p=0.268, respectively).

270 **GLP-1R is not expressed in bone cells**

271 As expected, a strong expression of GLP-1R was found in lung and pancreatic islets and to a lower
272 extent in heart (Figure 5). On the other hand, *Glp-1r* transcripts were not found in either osteoblasts or
273 osteoclasts.

274

275 **DISCUSSION**

276 In the present study, we investigated bone strength in a mouse model of GLP-1R deficiency to
277 understand how the GLP-1/GLP-1R pathway may affect bone strength. Three-point bending
278 experiments, performed on the midshaft femur, revealed a significant decrease in the total absorbed
279 energy as well as reduction in ultimate load, stiffness, yield load, post-yield energy, work to fracture
280 and yield strength. These results suggest reduced bone strength in these animals. However, several
281 factors might influence the outcome of three-point bending including bone microarchitecture and bone
282 material properties (Turner and Burr 1993). Indeed, material properties calculated from stress-strain
283 curves indicated that work to fracture and yield strength were significantly reduced in deficient animals
284 but not ultimate strength or strain or the elastic modulus. Taken together these data indicate that
285 cortical bone **morphology** and material properties may be altered. In the C57BL/6 mouse strain, at the
286 midshaft femur, bone tissue is composed almost exclusively of cortical bone (Bouxsein, et al. 2005;
287 Judex, et al. 2004). A study conducted by Yamada et al, reported a reduction in cortical BMD in the
288 same GLP-1R *-/-* mice model in the tibia (Yamada et al. 2008). In the present study, cortical bone
289 morphology was investigated by microCT and significant reductions in bone outer diameter, cortical
290 thickness and cross-sectional moment of inertia were observed in GLP-1R *-/-* mice. Interestingly,
291 cortical thickness diminution was associated with a decrease in the outer bone diameter whilst the
292 marrow diameter was unchanged. Furthermore, gene expression analysis revealed that the GLP-1R is
293 not expressed in bone cells suggesting that the decrease in bone strength and quality observed in this
294 animal model may be a consequence of GLP-1R deletion in other tissues rather than a direct effect on
295 bone cells.

296 Indeed, the GLP-1R is expressed in paraventricular, arcuate and dorsomedial nuclei of the
297 hypothalamus (Merchenthaler, et al. 1999). Some of these regions have been implicated in the central
298 control of bone remodeling by targeting osteoblasts and as such, alterations of bone quality could also
299 result from activation/inactivation of specific central relays. Furthermore, these mice present with a
300 mild glucose intolerance represented by fasting hyperglycemia (Ayala, et al. 2010). Regarding the
301 beneficial role of insulin in bone growth and or quality, dysregulation of insulin action in these mice
302 may also represent another mechanism leading to the observed phenotype. Furthermore, alterations
303 of IGF-1R expression have been noted in these mice (Cornu, et al. 2009) and could also participate to
304 the reduction in bone diameter and hence bone quality. The GLP-1R is also expressed in C cells of
305 the thyroid gland and exerts, when activated by GLP-1 or its stable analogues, a stimulating effect on

306 calcitonin secretion in rodents (Bjerre Knudsen, et al. 2010). Calcitonin is one of the most efficient
307 inhibitors of bone resorption in vitro (Moonga, et al. 1992; Zaidi, et al. 1990). Evidence from previous
308 studies suggests that basally, male GLP-1R KO mice present with a 2.5 fold increase in circulating
309 plasma calcitonin (Madsen, et al. 2012). As regard to the inhibitory action of calcitonin on bone
310 resorption, it would be legitimate to hypothesize that bone resorption should be the primary target of
311 calcitonin increase. However, recent evidence made with genetic ablation of either the calcitonin or
312 calcitonin receptor genes highlighted an unexpected phenotype of high bone mass with no or little
313 apparent effect on bone resorption (Dacquin, et al. 2004; Davey, et al. 2008; Hoff, et al. 2002). This
314 unexpected phenotype indicated a possible inhibitory action (direct or indirect) of calcitonin on bone
315 formation without affecting bone resorption. Therefore, the contributing effect of increased circulating
316 calcitonin to bone formation in our GLP-1R KO model remains a possible scenario.

317

318 Another factor that strongly affects bone strength is the quality of the bone matrix (Ammann et al.
319 2007). GLP-1R KO mice presented with a decrease in maximum load (-17%), hardness (-19%) and
320 dissipated energy (-15%). Owing to the nanocomposite composition of the bone matrix, any of the
321 collagen or mineral might be involved in the reduction in material properties. Investigation of the bone
322 mineral, by qBEI and FTIR, revealed that neither the degree of mineralization nor the maturity of the
323 mineral was affected by the GLP-1R deletion. However, it is important to bear in mind that due to
324 instrument limitations, we were not capable of investigating mineral cristallinity as proposed by Farlay
325 et al (Farlay, et al. 2010). On the other hand, the maturity of the collagen matrix, determined as the
326 1660/1690 cm^{-1} ratio, was significantly reduced. Some controversies exist in the interpretation of this
327 ratio as some groups reported that this ratio represent the trivalent over divalent cross-links ratio but
328 recently an elegant study of Farlay and collaborators demonstrated that variation in this parameter
329 could be more related to secondary structure of the collagen matrix after mineralization than a ratio of
330 trivalent over divalent collagen crosslinks (Farlay et al. 2011). Nevertheless, it is an indicator of
331 collagen maturity. The reduction of collagen maturity could itself explain the reduction in material
332 properties, as we found a significant positive association between collagen maturity and dissipated
333 energy. Furthermore, alteration of the collagen matrix often alters the post-yield properties of the bone
334 matrix (Bailey, et al. 1999; Burr 2002; Zioupos, et al. 1999). In our study, the observed reduction in
335 mature trivalent crosslinks could also account for the decreased post-yield energy determined by 3-
336 point bending.

337 In conclusion, mice lacking the GLP-1R appear to have a decrease in bone strength observed at the
338 anatomical level with a decrease in three-point bending resistance and decreased cortical thickness.
339 Bone strength was also reduced at the tissue level and was associated with reductions of collagen
340 crosslinking but no alteration of the bone mineral. Overall these data support a beneficial role for the
341 GLP-1/GLP-1R signaling pathway in bone quality. This is important regarding the introduction of GLP-
342 1 mimetics for the treatment of patients with type 2 diabetes mellitus.

343

344

345 **DECLARATION OF INTEREST**

346 The authors declared no conflict of interest that could be perceived as prejudicing the impartiality of
347 the research reported.

348

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352

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359 **REFERENCES**

- 360 Ammann P, Badoud I, Barraud S, Dayer R & Rizzoli R 2007 Strontium ranelate treatment improves
361 trabecular and cortical intrinsic bone tissue quality, a determinant of bone strength. *J Bone Miner Res*
362 **22** 1419-1425.
- 363 Ayala JE, Bracy DP, James FD, Burmeister MA, Wasserman DH & Drucker DJ 2010 Glucagon-like
364 peptide-1 receptor knockout mice are protected from high-fat diet-induced insulin resistance.
365 *Endocrinology* **151** 4678-4687.
- 366 Baggio LL & Drucker DJ 2007 Biology of incretins: GLP-1 and GIP. *Gastroenterology* **132** 2131-2157.
- 367 Bailey AJ, Sims TJ, Ebbesen EN, Mansell JP, Thomsen JS & Mosekilde L 1999 Age-related changes
368 in the biochemical properties of human cancellous bone collagen: relationship to bone strength. *Calcif*
369 *Tissue Int* **65** 203-210.
- 370 Bassett JH, van der Spek A, Gogakos A & Williams GR 2012 Quantitative X-ray imaging of rodent
371 bone by Faxitron. *Methods Mol Biol* **816** 499-506.
- 372 Bjerre Knudsen L, Madsen LW, Andersen S, Almholt K, de Boer AS, Drucker DJ, Gotfredsen C,
373 Egerod FL, Hegelund AC, Jacobsen H, et al. 2010 Glucagon-like Peptide-1 receptor agonists activate
374 rodent thyroid C-cells causing calcitonin release and C-cell proliferation. *Endocrinology* **151** 1473-
375 1486.
- 376 Boskey AL, DiCarlo E, Paschalis E, West P & Mendelsohn R 2005 Comparison of mineral quality and
377 quantity in iliac crest biopsies from high- and low-turnover osteoporosis: an FT-IR microspectroscopic
378 investigation. *Osteoporos Int* **16** 2031-2038.
- 379 Bouxsein ML, Boyd SK, Christiansen BA, Guldberg RE, Jepsen KJ & Muller R 2010 Guidelines for
380 assessment of bone microstructure in rodents using micro-computed tomography. *J Bone Miner Res*
381 **25** 1468-1486.
- 382 Bouxsein ML, Myers KS, Shultz KL, Donahue LR, Rosen CJ & Beamer WG 2005 Ovariectomy-
383 induced bone loss varies among inbred strains of mice. *J Bone Miner Res* **20** 1085-1092.
- 384 Bullock BP, Heller RS & Habener JF 1996 Tissue distribution of messenger ribonucleic acid encoding
385 the rat glucagon-like peptide-1 receptor. *Endocrinology* **137** 2968-2978.
- 386 Burr DB 2002 The contribution of the organic matrix to bone's material properties. *Bone* **31** 8-11.
- 387 Campos RV, Lee YC & Drucker DJ 1994 Divergent tissue-specific and developmental expression of
388 receptors for glucagon and glucagon-like peptide-1 in the mouse. *Endocrinology* **134** 2156-2164.
- 389 Chappard D, Basle MF, Legrand E & Audran M 2011 New laboratory tools in the assessment of bone
390 quality. *Osteoporos Int* **22** 2225-2240.
- 391 Cornu M, Yang JY, Jaccard E, Poussin C, Widmann C & Thorens B 2009 Glucagon-like peptide-1
392 protects beta-cells against apoptosis by increasing the activity of an IGF-2/IGF-1 receptor autocrine
393 loop. *Diabetes* **58** 1816-1825.
- 394 Dacquin R, Davey RA, Laplace C, Levasseur R, Morris HA, Goldring SR, Gebre-Medhin S, Galson
395 DL, Zajac JD & Karsenty G 2004 Amylin inhibits bone resorption while the calcitonin receptor controls
396 bone formation in vivo. *J Cell Biol* **164** 509-514.

397 Davey RA, Turner AG, McManus JF, Chiu WS, Tjahjono F, Moore AJ, Atkins GJ, Anderson PH, Ma
398 C, Glatt V, et al. 2008 Calcitonin receptor plays a physiological role to protect against hypercalcemia in
399 mice. *J Bone Miner Res* **23** 1182-1193.

400 Drucker DJ & Nauck MA 2006 The incretin system: glucagon-like peptide-1 receptor agonists and
401 dipeptidyl peptidase-4 inhibitors in type 2 diabetes. *Lancet* **368** 1696-1705.

402 Elnenaei MO, Musto R, Alagband-Zadeh J, Moniz C & Le Roux CW 2010 Postprandial bone turnover
403 is independent of calories above 250 kcal. *Ann Clin Biochem* **47** 318-320.

404 Farlay D, Duclos ME, Gineyts E, Bertholon C, Viguet-Carrin S, Nallala J, Sockalingum GD, Bertrand
405 D, Roger T, Hartmann DJ, et al. 2011 The ratio 1660/1690 cm^{-1} measured by infrared
406 microspectroscopy is not specific of enzymatic collagen cross-links in bone tissue. *PLoS One* **6**
407 e28736.

408 Farlay D, Panczer G, Rey C, Delmas PD & Boivin G 2010 Mineral maturity and crystallinity index are
409 distinct characteristics of bone mineral. *J Bone Miner Metab* **28** 433-445.

410 Gaudin-Audrain C, Irwin N, Mansur S, Thorens B, Flatt PR, Basle MF, Chappard D & Mabileau G
411 2013 Glucose-dependent insulinotropic polypeptide receptor deficiency leads to modifications of
412 trabecular bone mass and quality in mice. *Bone* **53** 221-230.

413 Hansotia T, Baggio LL, Delmeire D, Hinke SA, Yamada Y, Tsukiyama K, Seino Y, Holst JJ, Schuit F &
414 Drucker DJ 2004 Double incretin receptor knockout (DIRKO) mice reveal an essential role for the
415 enteroinsular axis in transducing the glucoregulatory actions of DPP-IV inhibitors. *Diabetes* **53** 1326-
416 1335.

417 Henriksen DB, Alexandersen P, Bjarnason NH, Vilsboll T, Hartmann B, Henriksen EE, Byrjalsen I,
418 Krarup T, Holst JJ & Christiansen C 2003 Role of gastrointestinal hormones in postprandial reduction
419 of bone resorption. *J Bone Miner Res* **18** 2180-2189.

420 Hoff AO, Catala-Lehnen P, Thomas PM, Priemel M, Rueger JM, Nasonkin I, Bradley A, Hughes MR,
421 Ordonez N, Cote GJ, et al. 2002 Increased bone mass is an unexpected phenotype associated with
422 deletion of the calcitonin gene. *J Clin Invest* **110** 1849-1857.

423 Judex S, Garman R, Squire M, Donahue LR & Rubin C 2004 Genetically based influences on the site-
424 specific regulation of trabecular and cortical bone morphology. *J Bone Miner Res* **19** 600-606.

425 Mabileau G, Chappard D & Sabokbar A 2011 Role of the A20-TRAF6 axis in lipopolysaccharide-
426 mediated osteoclastogenesis. *J Biol Chem* **286** 3242-3249.

427 Madsen LW, Knauf JA, Gotfredsen C, Pilling A, Sjogren I, Andersen S, Andersen L, de Boer AS,
428 Manova K, Barlas A, et al. 2012 GLP-1 receptor agonists and the thyroid: C-cell effects in mice are
429 mediated via the GLP-1 receptor and not associated with RET activation. *Endocrinology* **153** 1538-
430 1547.

431 Merchenthaler I, Lane M & Shughrue P 1999 Distribution of pre-pro-glucagon and glucagon-like
432 peptide-1 receptor messenger RNAs in the rat central nervous system. *J Comp Neurol* **403** 261-280.

433 Mieczkowska A, Basle MF, Chappard D & Mabileau G 2012 Thiazolidinediones induce osteocyte
434 apoptosis by a G protein-coupled receptor 40-dependent mechanism. *J Biol Chem* **287** 23517-23526.

435 Moonga BS, Alam AS, Bevis PJ, Avaldi F, Soncini R, Huang CL & Zaidi M 1992 Regulation of
436 cytosolic free calcium in isolated rat osteoclasts by calcitonin. *J Endocrinol* **132** 241-249.

437 Nuche-Berenguer B, Moreno P, Esbrit P, Dapia S, Caeiro JR, Cancelas J, Haro-Mora JJ & Villanueva-
438 Penacarrillo ML 2009 Effect of GLP-1 treatment on bone turnover in normal, type 2 diabetic, and
439 insulin-resistant states. *Calcif Tissue Int* **84** 453-461.

440 Nuche-Berenguer B, Moreno P, Portal-Nunez S, Dapia S, Esbrit P & Villanueva-Penacarrillo ML 2010
441 Exendin-4 exerts osteogenic actions in insulin-resistant and type 2 diabetic states. *Regul Pept* **159** 61-
442 66.

443 Oliver WC & Pharr GM 1992 An improved technique for determining hardness and elastic modulus
444 using load and displacement sensing indentation experiments. *J Mater Res* **7** 1564-1583.

445 Paschalis EP, DiCarlo E, Betts F, Sherman P, Mendelsohn R & Boskey AL 1996 FTIR
446 microspectroscopic analysis of human osteonal bone. *Calcif Tissue Int* **59** 480-487.

447 Paschalis EP, Verdellis K, Doty SB, Boskey AL, Mendelsohn R & Yamauchi M 2001 Spectroscopic
448 characterization of collagen cross-links in bone. *J Bone Miner Res* **16** 1821-1828.

449 Ritchie RO, Koester KJ, Ionova S, Yao W, Lane NE & Ager JW, 3rd 2008 Measurement of the
450 toughness of bone: a tutorial with special reference to small animal studies. *Bone* **43** 798-812.

451 Roschger P, Fratzl P, Eschberger J & Klaushofer K 1998 Validation of quantitative backscattered
452 electron imaging for the measurement of mineral density distribution in human bone biopsies. *Bone* **23**
453 319-326.

454 Turner CH & Burr DB 1993 Basic biomechanical measurements of bone: a tutorial. *Bone* **14** 595-608.

455 Wu T, Rayner CK, Jones K & Horowitz M 2010 Dietary effects on incretin hormone secretion. *Vitam*
456 *Horm* **84** 81-110.

457 Yamada C, Yamada Y, Tsukiyama K, Yamada K, Udagawa N, Takahashi N, Tanaka K, Drucker DJ,
458 Seino Y & Inagaki N 2008 The murine glucagon-like peptide-1 receptor is essential for control of bone
459 resorption. *Endocrinology* **149** 574-579.

460 Zaidi M, Datta HK, Moonga BS & MacIntyre I 1990 Evidence that the action of calcitonin on rat
461 osteoclasts is mediated by two G proteins acting via separate post-receptor pathways. *J Endocrinol*
462 **126** 473-481.

463 Zioupos P, Currey JD & Hamer AJ 1999 The role of collagen in the declining mechanical properties of
464 aging human cortical bone. *J Biomed Mater Res* **45** 108-116.

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468 **FIGURE LEGENDS**

469 **Figure 1:** Schematic representation of the experimental design used in this study

470 **Figure 2:** Bone mineral content and cortical bone morphology is altered in GLP-1R KO animals. (A),
471 bone mineral content as determined by quantitative X-ray microradiograph is reduced in
472 GLP1-R KO animals (Black line and bar) as determined by a shift to the left of the
473 frequency of occurrence of grey pixel and lower value of GLmean as compared with WT
474 (grey line and bar). (B) 3-D models of WT and GLP-1R KO femurs and (C)
475 histomorphometric analysis of cortical bone in WT and GLP-1R KO mice. *: p<0.05 vs. WT
476 animals and **: p<0.01 vs. WT animals

477 **Figure 3:** FTIR assessment of bone matrix properties. (A) Mineral-to-matrix ratio, (B) mineral
478 maturity, (C) carbonate content and (D) collagen maturity. *: p<0.05 vs. WT animals and **:
479 p<0.01 vs. WT animals

480 **Figure 4:** Regression analysis between maximum load (A), hardness (B) and dissipated energy (C)
481 and 1660/1690 cm⁻¹ ratio. Open circles represent WT, close circles represent GLP-1R KO
482 mice. **: p<0.01 vs. WT

483 **Figure 5:** Expression of *Glp1r* gene in mice. RT-PCR analysis was performed using RNA prepared
484 from lung, pancreatic islets, heart and ex vivo culture of osteoblasts and osteoclasts from
485 WT mice. Results were normalized relative to levels of *gapdh* mRNA transcripts in the
486 same sample. Data are mean ± SEM, n=3.

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489 **TABLES**

490

491 **Table 1:** Three-point bending parameters in WT and GLP-1R KO mice

	WT (n=12)	GLP-1R KO (n=8)	p value
Ultimate load (N)	14.47 ± 0.35	11.95 ± 0.27 *	0.034
Ultimate displacement (mm)	0.31 ± 0.006	0.30 ± 0.033	1.000
Stiffness (N.mm ⁻¹)	42.68 ± 0.85	31.92 ± 5.45 *	0.05
Total absorbed energy (N.mm)	2.33 ± 0.09	1.54 ± 0.24 *	0.021
Yield load (N)	11.83 ± 0.45	9.37 ± 0.42 *	0.021
Post-yield energy (N.mm)	1.52 ± 0.05	1.12 ± 0.12 *	0.048
Ultimate strength (MPa)	84.2 ± 2.0	82.9 ± 2.0	0.513
Breaking strength (MPa)	80.9 ± 3.4	79.2 ± 7.3	0.783
Ultimate strain	0.013 ± 0.001	0.011 ± 0.002	0.714
Elastic modulus (GPa)	3.0 ± 0.2	3.0 ± 0.5	0.513
Work to fracture (KJ.m ⁻²)	0.211 ± 0.007	0.168 ± 0.017*	0.047
Yield strength (MPa)	69.3 ± 1.7	64.2 ± 0.5 *	0.05

492 *: p<0.05 vs. WT animals

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495 **Table 2:** Nanomechanical properties of the bone matrix

	WT (n=12)	GLP-1R KO (n=8)	p value
Maximum load (mN)	11.9 ± 0.6	9.9 ± 0.4 *	0.037
Hardness (MPa)	627.8 ± 34.4	510.7 ± 28.9 *	0.028
Indentation modulus (GPa)	12.9 ± 0.6	12.1 ± 0.4	0.203
Dissipated energy (mN.nm)	3189.7 ± 176.7	2708.6 ± 130.9 *	0.028

496 *: p<0.05 vs. WT animals

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499 **Table 3:** Bone mineral density distribution in WT and GLP-1R deficient mice

	WT (n=12)	GLP-1R KO (n=8)	p value
Ca _{peak} (%)	26.9 ± 0.7	26.8 ± 0.7	1.00
Ca _{mean} (%)	26.3 ± 0.6	26.3 ± 0.7	0.936
Ca _{width} (%)	2.7 ± 0.1	2.9 ± 0.2	0.337

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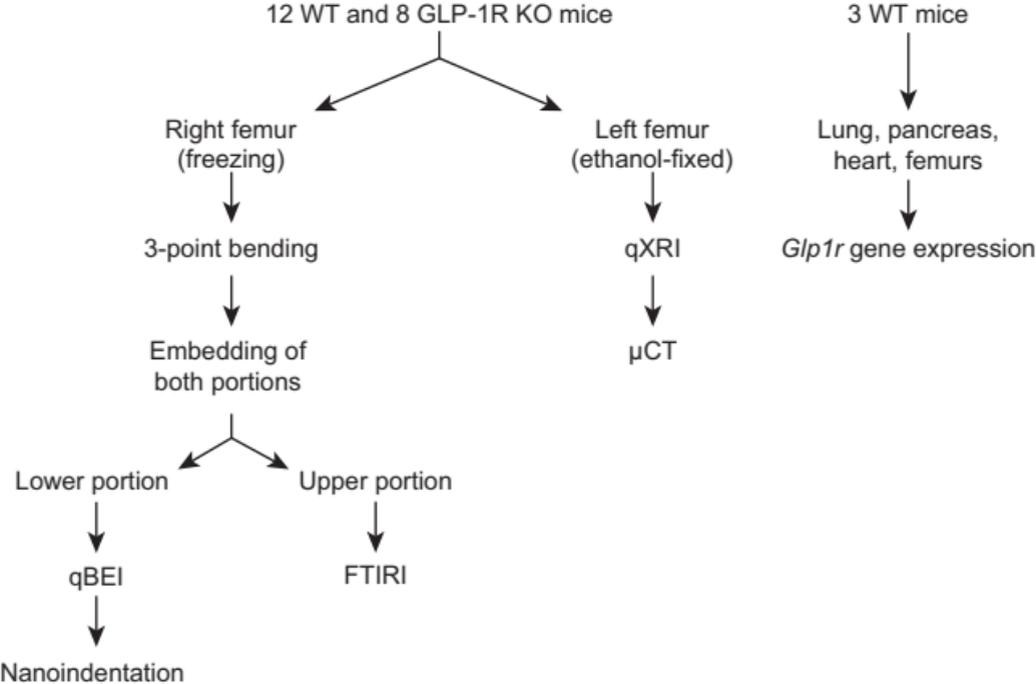
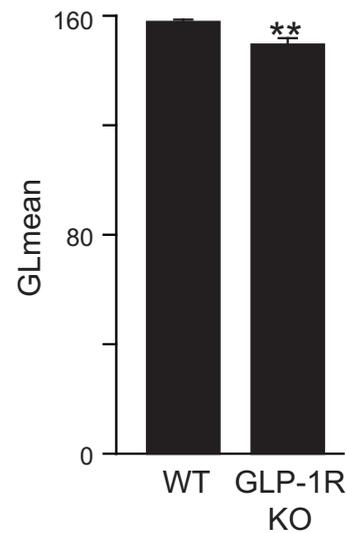
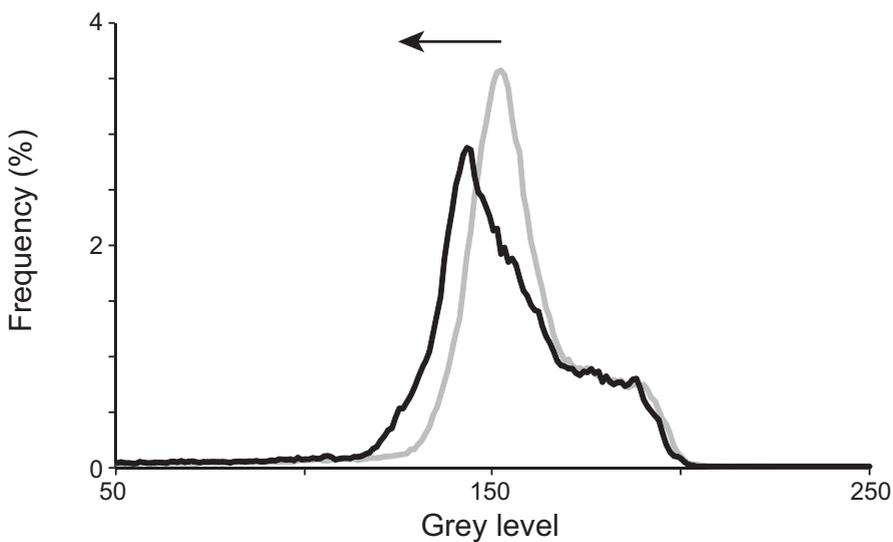
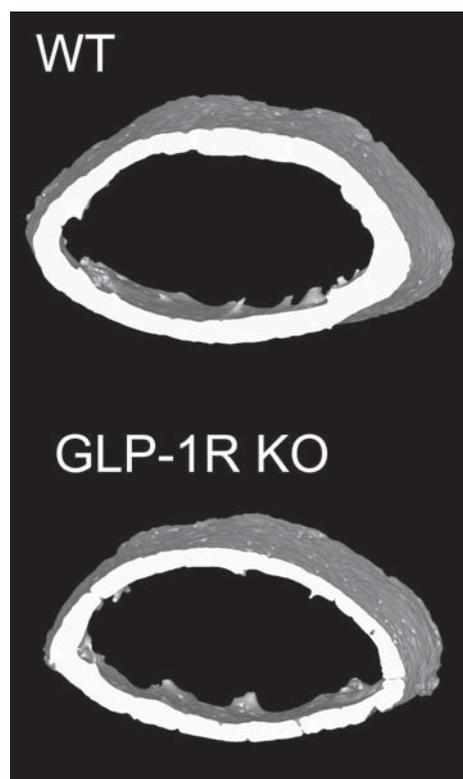


Figure 1

(A)



(B)



(C)

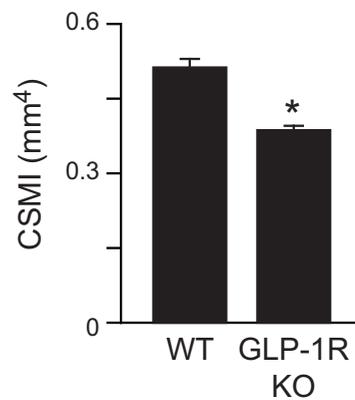
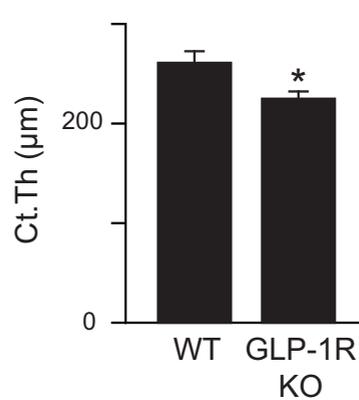
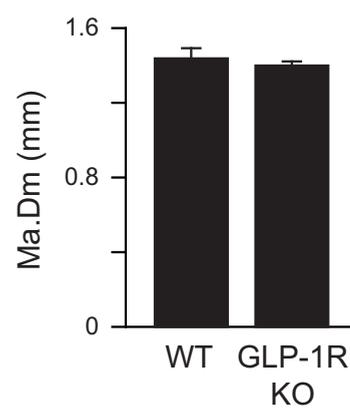
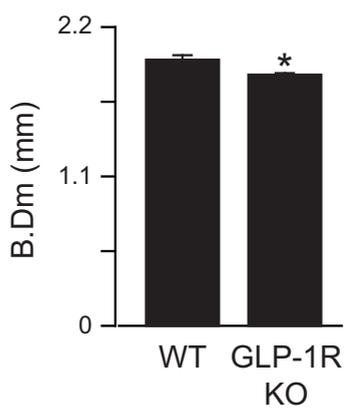


FIGURE 2

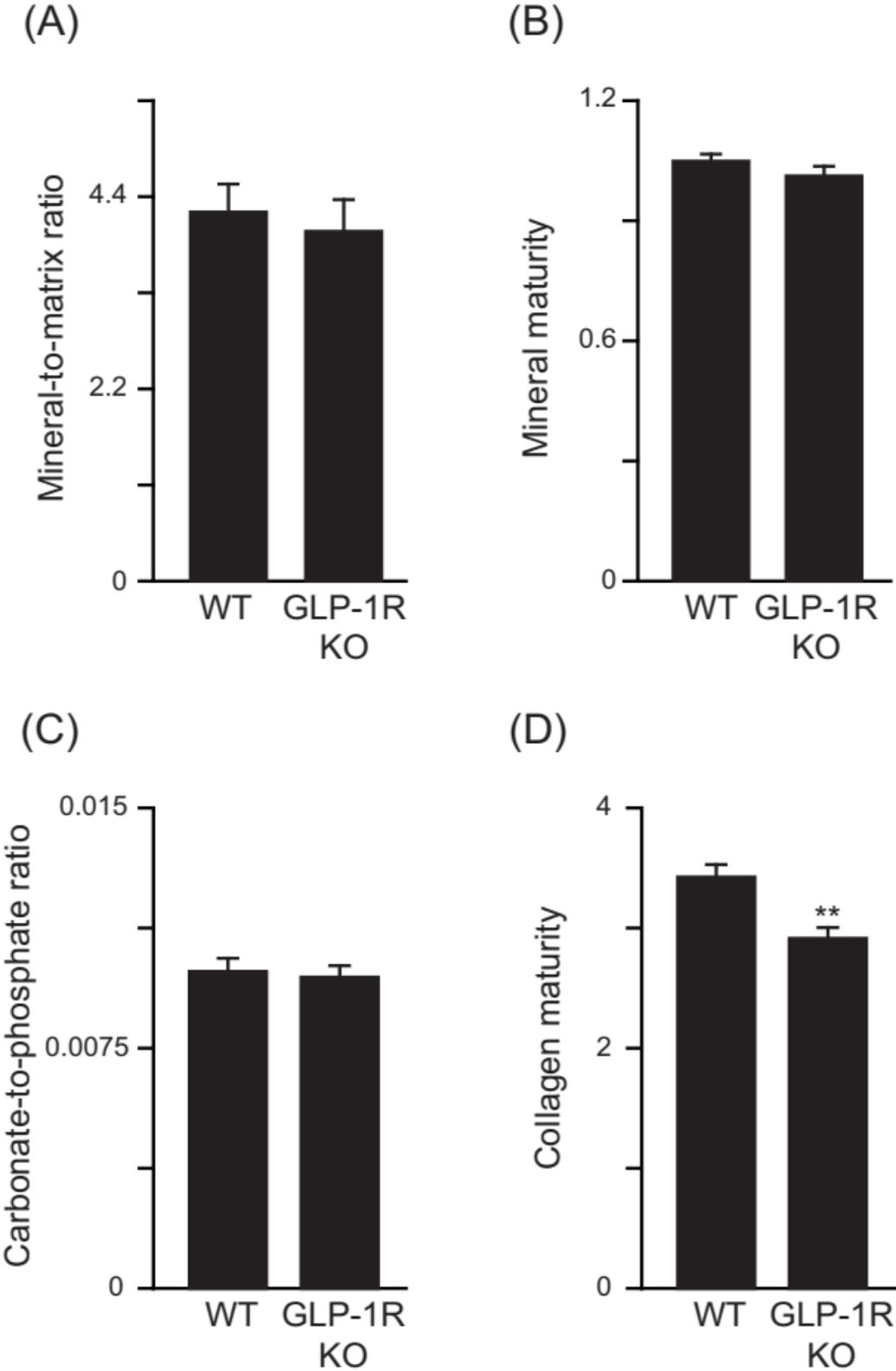
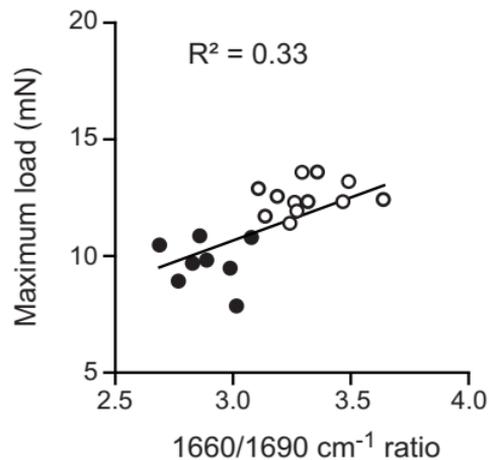
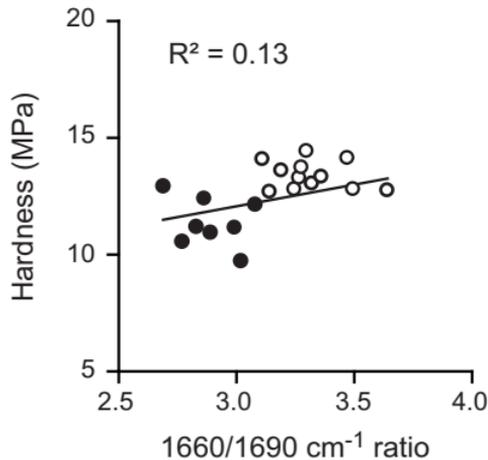


FIGURE 3

(A)



(B)



(C)

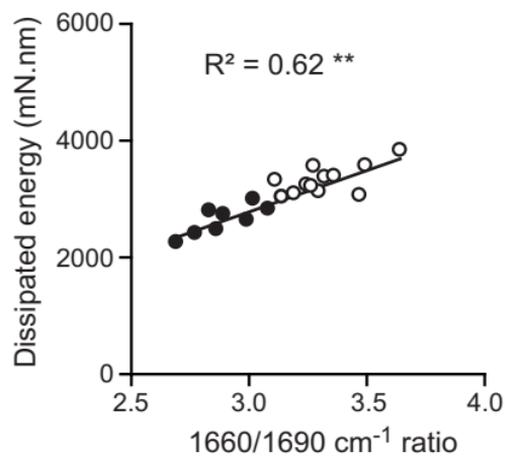


FIGURE 4

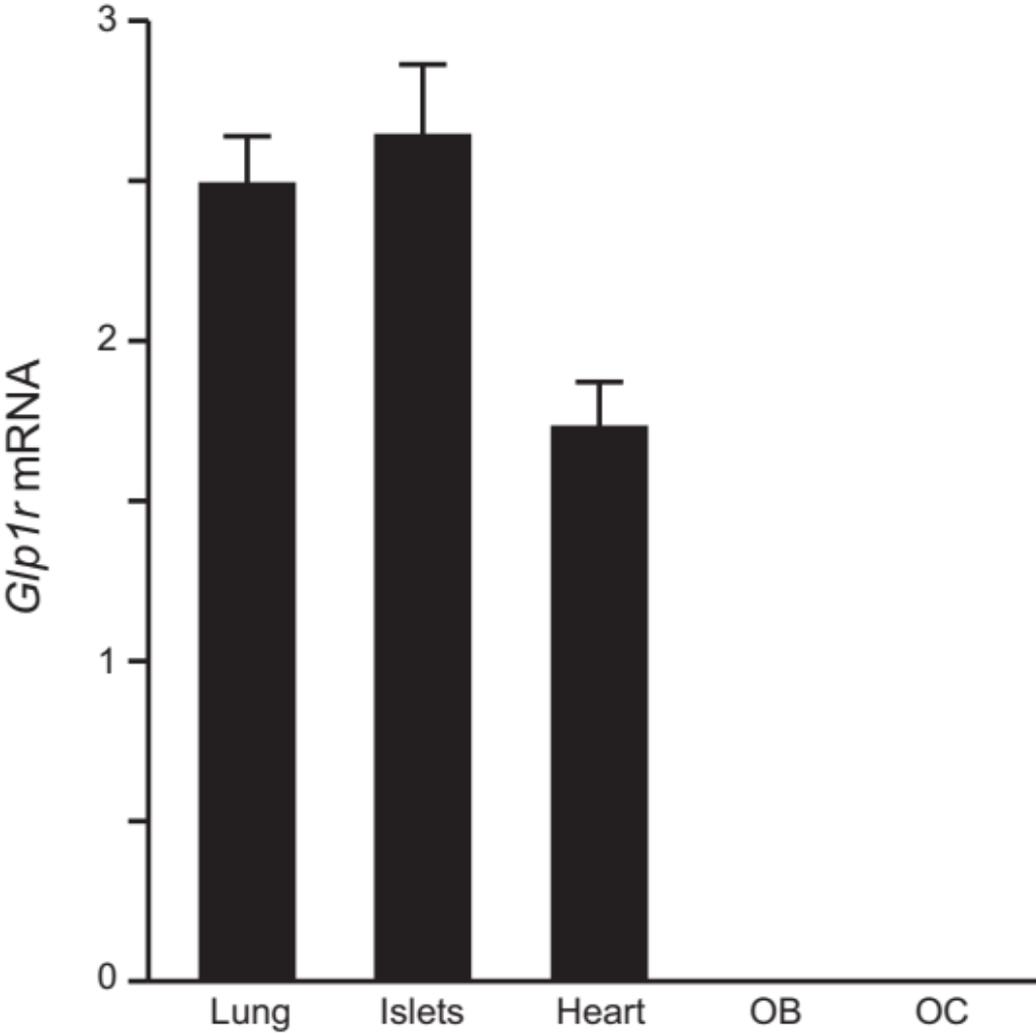


Figure 5