1 **TITLE**

2 **Effect of lysophosphatidic acid receptor inhibition on bone changes of**

3 **ovariectomized mice**

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21   **Abstract**

22   Pharmacological inhibition of signaling through lysophosphatidic acid (LPA) receptors

23   reduces bone erosions in an experimental model of arthritis by mechanisms involving

24   reduced osteoclast differentiation and bone resorption and increased differentiation of

25   osteoblasts and bone mineralization. These results led us to hypothesize that LPA

26   receptor inhibition will be beneficial in osteoporosis. Our aim has been to test this

27   hypothesis with the LPA receptor antagonist, Ki16425 in ovariectomized mice, a model

28   of postmenopausal osteoporosis.

29   Ovariectomized mice treated with Ki16425 showed bone loss similar to the observed in

30   the controls. Osteoblast markers, *Alpl, Bglap* and *Col1a1*, were increased at the mRNA

31   level but not any changes were detected in serum. Not any additional difference was

32   observed in the Ki16425 treated mice relative to the ovariectomized controls in the

33   osteoclast function markers or in the assays of matrix mineralization or osteoclast

34   differentiation. Then, pharmacological inhibition of LPA receptor was not beneficial for

35   preventing bone loss in ovariectomized mice indicating that its favorable effect on bone

36   remodeling is less general than hypothesized.

## Introduction

Lysophosphatidic acid (LPA) is a multifunctional phospholipid messenger mainly produced by cleavage of lysophosphatidylcholine in the extracellular fluids by the lysophospholipase D enzyme, also called autotaxin (ATX). LPA is recognized by six G-protein coupled receptors (LPA1-6) and it is involved in regulating proliferation, apoptosis, differentiation, motility and chemotaxis [1]. Recently, we have identified multiple beneficial effects of inhibiting signaling through its receptors in an experimental model of rheumatoid arthritis including reduction of inflammation, apoptosis of fibroblast-like synovial cells (FLS) and bone erosion [2]. Both, siRNA suppression and pharmacological inhibition of LPA1 receptor led to these changes. Prominent among the improvements was decreased number of osteoclasts, as observed in joint histology, and reduction in bone erosions. These changes were accompanied by impaired osteoclast differentiation and function and, on the contrary, increased osteoblast differentiation and function [2]. These effects are of special relevance because it is very unusual to find molecules uncoupling regulation of osteoclasts and osteoblasts. They make of LPA$_1$ a good target for the treatment of diseases with unbalanced bone remodeling as osteoporosis. Additional studies have also shown a role of LPA in osteoclast survival [3] and activity and in the progression of bone metastasis [4,5] reinforcing its involvement in regulating bone resorption.

Osteoporosis results from an imbalance between increased bone resorption and insufficient bone formation. Bone resorption is carried out by osteoclasts and bone formation is performed by osteoblast. The two bone remodeling processes are coupled because osteoclast differentiation is induced by macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor kB ligand (RANKL), which are expressed by osteoblasts; and osteoblastic bone formation is done in response to bone

62    resorption [6,7]. In osteoporosis the uncoupling towards increased resorption is most

63    often due to estrogen deficiency during menopause and leads to a reduction of mineral

64    bone density and increased risk of fractures. It is a major health problem in developed

65    countries with an elevated socioeconomic cost and increasing prevalence due to

66    population aging [8,9]. Most available treatments aim to restore the balance by

67    inhibiting osteoclast function, only PTH is aimed to promote osteoblastic function [10].

68    The beneficial effects of $LPA_1$ receptor inhibition inhibiting osteoclasts and potentiating

69    osteoblasts could mean a new and potentially very powerful way of treating

70    osteoporosis.

71    In the present study, we have tested if inhibition of the LPA pathway in an ovariectomy

72    model of postmenopausal osteoporosis is able to prevent loss of bone mass.

73    Unfortunately, treatment with Ki16425 [11], a specific antagonist of $LPA_1$ (Ki 0.34μM)

74    and $LPA_3$ (Ki 0.93 μM) receptors, did not produce the hypothesized beneficial effects.

75

## Material and Methods

### Mice

C57BL/6OlaHsd female mice were obtained from Harlan Laboratories B.V. Mice were housed under specific pathogen-free conditions in the animal facility of the University of Santiago de Compostela (USC). Animal care was in compliance with Spanish regulations on the protection of animals used for experimental and other scientific purposes (Real Decreto 1201/2005), and European Directives (Council Directive 86/609/EEC). The experimental protocols were approved by the Research Committee of the Complejo Hospitalario Universitario de Santiago (CHUS).

Mice were bilaterally ovariectomized under Ketolar-Domtor anaesthesia at 6 weeks of age. Other mice were sham-operated, the ovaries were identify and left intact. Two weeks later, Ki16425 (Selleck Chemicals, Munich, Germany) at 20 mg/Kg in 30% DMSO/PBS was started with subcutaneous administration 5 times a week for 5 weeks. Eight ovariectomized (OVX) were treated in this way. Twelve OVX and 12 sham-OVX mice received vehicle following the same protocol as controls.

### Micro-computed tomography analysis

Right femurs were obtained from 7 mice of the three groups, OVX and treated, OVX controls and sham-OVX controls. They were kept in gauzes soaked in 0.9% NaCl at -80ºC until analysis. Femurs were scanned with a SkyScan 1172 micro-computed tomography (micro-CT) equipment (Bruker microCT NV, Kontich, Belgium). The X-ray source energy selected was 50 KV and 200 µA. The acquisitions were made with a 5.5 µm image pixel size, a rotation step of 0.3° and with the use of a 0.5 mm in thickness aluminum filter. CT images were reconstructed using the modified Feldkamp algorithm and analyzed with the software SkyScan CT-Analyzer version 1.10.0.2.

5

100    Analyses of trabecular bone were carried out in a 1.5 mm region starting 0.25 mm from

101    the growth plate of the distal metaphysis (secondary spongiosa area, rich in high-

102    turnover trabecular bone). The region of interest in cortical bone started 2.5 mm from

103    the growth plate and extended 1.0mm in proximal direction.

104    **Osteoblast cultures and bone matrix mineralization assay**

105    Primary osteoblasts were obtained from left femurs of OVX mice, both treated and

106    control. Bones were removed; their diaphyses were cut into small pieces and cultured in

107    α-MEM (α-Minimum Essential Medium) supplemented with 10% FBS, 10 mM β-

108    glycerophosphate and 0.28 mM ascorbic acid (Sigma Aldrich, St Louis, MO, USA.

109    Throughout the process of culturing, the cells were not treated with Ki16425. After 5

110    days of differentiation, assessment of matrix mineralization was done. Formation of

111    mineralized nodules was analyzed by von Kossa staining after washing, decellularizing

112    and fixing the plates. Briefly, staining was done with 5% silver nitrate solution,

113    followed by exposure to UV light for 1 h and incubation with 1% pyrogallol (Merck

114    KGaA, Darmstadt, Germany). Micrographs were captured with VisionWorks LS

115    software and analyzed using the Image J analysis system.

116    *In vitro* **osteoclastogenesis**

117    Left femurs of OVX mice, both treated and control were dissected and bone marrow

118    cells were removed. They were cultured in 96-well plates at $1.5 \times 10^4$ cells/well and

119    differentiated in α-MEM containing 10% v/v FBS (both from PAA), 1% penicillin-

120    streptomycin, 1% L-glutamine (Sigma Aldrich) and 40 ng/mL MCSF (eBioscience,

121    Peprotech) for 4 days. Thereafter, cells were cultured in α-MEM medium containing

122    100 ng/mL RANKL (eBioscience) and 40 ng/mL MCSF for 4 additional days.

123    Throughout the process of culturing, the cells were not treated with Ki16425. TRAP

124  staining was performed to evaluate osteoclast differentiation using the leukocyte acid

125  phosphatase kit (Sigma-Aldrich).

126  **Real-time PCR analysis**

127  Total RNA was obtained from tibias of OVX or sham-OVX mice using Trizol

128  (Invitrogen) and the NucleoSpin RNA/Protein kit (Macherey-Nagel GmbH & Co. KG,

129  Düren, Germany). Quantitative real-time PCR was performed in duplicate in an

130  Mx3005P Real-Time PCR system (Strategene, La Jolla, CA, USA), using Brilliant

131  SYBR Green Single Step QRT-PCR Master Mix (Stratagene). Gene expression was

132  quantified by the comparative $2^{-\Delta\Delta Ct}$ method using as normalization control the β-actin

133  gene: where $\Delta C_t = C_t$ target $- C_t$ β-actin; $\Delta\Delta C_t = [C_t$ target$-C_t$ β-actin] control$-[C_t$

134  target$- C_t$ β-actin] experiment. For control, $2^{-\Delta\Delta Ct} = 1$. For experiment, the value $2^{-\Delta Ct}$

135  indicates gene expression relative to β-actin and $2^{-\Delta\Delta Ct}$ indicates the fold change in gene

136  expression relative to the control. Melting curves and agarose gel electrophoresis were

137  used to check for PCR specificity.

138  **EIA assays**

139  Osteocalcin (Biomedical Technologies Inc, Stoughton, MA, USA), bone alkaline

140  phosphatase (BALP) (BMASSAY, KaiCheng, China), pyridinoline cross-links (PYD)

141  (Metra PYD EIA, Quidel, San Diego, CA, USA) and tartrate-resistant acid phosphatase

142  form 5b (TRACP 5b) (Immunodiagnostic Systems Ltd, Tyne & Wear, UK) were

143  determined in serum obtained at time of sacrifice of mice, by enzyme immunoassays,

144  according to the manufacturer's instructions.

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147 **Statistical analysis**

148 Differences between experimental groups were assessed by the Mann-Whitney U test. $P$

149 values below 0.05 were considered significant.

150

151 **RESULTS**

152 **Effect of Ki16425 treatment on bone microstructure of ovariectomized (OVX) mice**

153 Micro-computed tomography of bone did not show differences between OVX mice

154 treated with Ki16425 and their controls receiving only vehicle. These analyses were

155 done both in trabecular bone and in cortical bone from the distal femur. In trabecular

156 bone, OVX mice showed reduction of bone volume fraction (BV/TV), bone surface

157 density (BS/TV), trabecular number (Tb.N) and bone mineral density (vBMD) relative

158 to the sham-OVX mice (Figure 1). No differences were observed between OVX

159 Ki16425-treated mice and OVX control mice (Figure 1). Micro-CT analysis of cortical

160 bone revealed a reduction of cortical thickness (Ct.Th), cortical volume (Ct.V) and

161 cross-sectional thickness (Cs.Th) after ovariectomy (Figure 2) without differences

162 between Ki16425-treated mice and OVX controls.

163 **Bone remodeling markers in OVX mice after LPA receptor inhibition**

164 We analyzed the mRNA expression of $LPA_{1-3}$ receptors in bone tissue from sham-OVX

165 and OVX treated with vehicle and OVX mice treated with Ki16425. Expression of

166 $LPA_1$ was higher than $LPA_2$ or $LPA_3$ receptors in all groups of mice (Figure 3a).

167 Interestingly, a significant reduction of expression of the three LPA receptors was

168 observed after ovariectomy, which was partially recovered after Ki16425 treatment

169 (Figure 3a). Next, we analyzed the effect of Ki16425 treatment on bone remodeling

170 markers in OVX mice. As shown in Figure 3b, no differences were observed in the

171 expression of *Tnfrsf11a* (encoding RANK), *Tnfsf11* (encoding RANKL) and *Csf1*

172 (encoding MCSF) between different groups of mice. In addition, whereas the

173 expression of the resorption marker, *Ctsk* (encoding cathepsin K) was no different

174 between sham-OVX and OVX mice treated with vehicle, increased expression was

175    observed in OVX mice treated with Ki16425 as compared with control sham-OVX

176    mice, although no differences was found between both groups of OVX mice. The

177    expression of *Acp5* (encoding tartrate-resistant acid phosphatase type 5) was higher in

178    both groups of OVX mice as compared with sham-OVX mice; however Ki16425

179    treatment did not reduce the expression of *Acp5* in OVX mice (Figure 3c).

180    Osteoblast differentiation and function markers *Alpl* (alkaline phosphatase, Figure 4a),

181    *Col1a1* (collagen I, Figure 4b) and *Bglap* (osteocalcin, Figure 4c) were decreased in

182    OVX mice compared to sham-OVX mice whereas Ki16425 induced a significant

183    increased expression in OVX mice. However, the increase in bone of the three

184    osteoblast markers at the level of mRNA did not led to an increase in serum of the same

185    proteins, alkaline phosphatase (BALP) and osteocalcin (OCN) in mice treated with

186    Ki16425, (Figure 5a). In the same way, no differences were observed for the two bone

187    resorption markers, tartrate-resistant acid phosphatase type 5 (TRACP 5b) and

188    pyridinoline cross-links (PYD), in serum (Figure 5b).

189    ***In vitro* analysis of osteoblast and osteoclast function**

190    Finally, in vitro assays of osteoblast function and osteoclast differentiation were

191    performed. No differences between OVX mice treated with Ki16425 and controls were

192    detected either in the assay assessing extracellular mineralization with von Kossa

193    staining (Figure 6a) or in the differentiation of bone marrow precursors toward

194    osteoclasts as evaluated by counting TRAP+ multinucleated cells, i.e. mature

195    osteoclasts (Figure 6b).

196

197 **DISCUSSION**

198 In the present study we analyzed the effect of the LPA receptor antagonist, Ki16425 in

199 the ovariectomy mouse model of postmenopausal osteoporosis. Microtomographic

200 scanning showed a significant reduction of bone density in ovariectomized mice

201 compared with control sham-ovariectomized. Contrary to our hypothesis, no

202 improvement in bone density was obtained by inhibiting LPA signaling in the

203 ovariectomized mice. Lack of effect did not offer doubts because sensitive micro-

204 computed tomography did not detect differences in any of the parameters analyzed

205 measuring cortical and trabecular bone microstructure.

206 The analysis of mRNA expression of remodeling markers in bone showed that

207 ovariectomy reduced the expression of bone formation markers and induced a relative

208 increase of bone resorption markers, according to previously published (). However, the

209 LPA receptor antagonist did not induced changes in resorption markers or in *in vitro*

210 differentiation of osteoclasts and only modest increase in the expression of osteoblast

211 function genes were observed. These latter changes showed an increase of the

212 osteoblastic function that is concordant with our hypothesis and previous results in the

213 arthritis model. However, the induction of osteoblast function seems modest and of very

214 little consequence because it was not reflected in protein changes when assessed in

215 serum or in increased mineralized matrix formation *in vitro*, and was not translated in

216 changes of bone microstructure.

217 The absence of a more relevant effect of blocking LPA signaling cannot be ascribed to

218 insufficient inhibition of the $LPA_1$ receptor because we have administered the same

219 dose of Ki16425 used in the arthritis study [2]. In addition, this dose has been

220 administered for 5 weeks whereas only 4 injections were given to the mice with

11

221 arthritis, and the latter showed a marked reduction of bone erosions, reduced osteoclast

222 differentiation and bone resorption and increased differentiation of osteoblasts and bone

223 mineralization [2].

224 A bone protective role of LPA signalling has previously been shown in addition to the

225 arthritis model [2] in bone metastasis of breast cancer [4, 5] and in studies showing how

226 it promotes differentiation and survival of primary osteoclasts [3,12]. On the contrary,

227 the study of Gennero $et$ $al.$ [13] reported that $LPA_1$ KO has low bone mass accompanied

228 by decreased osteoblast differentiation and matrix mineralization. However,

229 interpretation of the results from the $LPA_1$ KO is complicated by other anomalies

230 present in these mice that could led to the bone phenotype with independence of a direct

231 effect on remodeling including impaired suckling, neurological abnormalities and

232 defects in cartilage formation and endochondral ossification, as has been already

233 pointed out [14].

234 Hence, discrepancies of the effects of LPA receptor inhibition between arthritis and

235 bone metastasis on one side and the results described here, in osteoporosis model on the

236 other have not known mechanism although some possibilities can be suggested. In

237 arthritis, there is a significant increase in the production and levels of LPA [14] as well

238 as in the expression of $LPA_1$ receptor in joint tissue [15]. In a similar way, tumor cells

239 co-opt platelets to greatly increase levels of LPA contributing to progression of

240 osteolitic bone metastasis [4,5]. In contrast, here we have observed that ovariectomy

241 down regulates the expression of LPA receptors, which could lead to a reduced efficacy

242 of the antagonist. In addition, there are important differences between bone resorption in

243 arthritis and metastasis in one side and osteoporosis on the other: the first take place at

244 specific locations that are heavily influenced by cells foreign to the bone, synovial cells

245 or tumor cells, that produce cytokines and other extracellular mediators, making of bone

246    resorption a very targeted phenomenon [5,16,17]. In contrast, bone resorption in

247    osteoporosis is stochastic and widespread, not influenced for foreign cells or localized

248    mediators, but for systemic factors like estrogen, PTH and vitamin D3 [18]. As a

249    consequence of these differences cytokines play a major role in the first two forms of

250    bone resorption compared with osteoporosis, where hormones are much more

251    important. Also, there are pieces of evidence suggesting that osteoclasts in inflammation

252    and metastasis degrade bone mainly through the action of metalloproteinases (MMPs)

253    and less by the action of acid and cathepsin K [18-23], whereas cathepsin K is the

254    dominant mechanism in osteoporosis [19, 24]. These differences could affect the

255    strength with which the LPA signaling pathway impinges in osteoclast or osteoblast

256    function and in the final balance of bone remodeling.

257    **Conclusions**

258    This study indicates that pharmacological inhibition of LPA receptor in ovariectomized

259    mice did not led to reduction of bone loss, excluding LPA receptor as a therapeutic

260    target in postmenopausal osteoporosis.

261

262 **Competing interests**

263 The authors declare that they have not competing interests.

264

265

266 **ACKNOWLEDGMENTS**

273

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343        postmenopausal women with low bone density. J Bone Miner Res 25(5): 937-

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348    **FIGURE LEGENDS**

349    **Figure 1. Lack of effect of lysophosphatidic acid (LPA) receptor inhibition on**

350    **trabecular bone structure in ovariectomized (OVX) mice.** Trabecular architecture of

351    distal femoral metaphysis was measured by micro-CT in 7 OVX mice treated with

352    Ki16425, 7 sham-OVX and 7 OVX mice treated with vehicle as controls. A. Results are

353    shown as bone volume/total volume (BV/TV), bone surface density (BS/TV), trabecular

354    spacing (Tb.Sp), trabecular number (Tb.N), and bone mineral density (vBMD). Values

355    expressed as mean ± SE of the mean (SEM); **$p$=0.005 and ***$p$=0.0005, by Mann-

356    Whitney U test. B. Representative three-dimensional images of trabecular bone from the

357    different groups of mice. Ki: Ki16425, Vh: Vehicle.

358    **Figure 2. Lack of effect of Ki16425 treatment on cortical bone structure in OVX**

359    **mice.** A. Data of cortical architecture analysis shown as cross-sectional thickness

360    (Cs.Th), cortical thickness (Ct.Th) and cortical volume (Ct.V). B. Representative

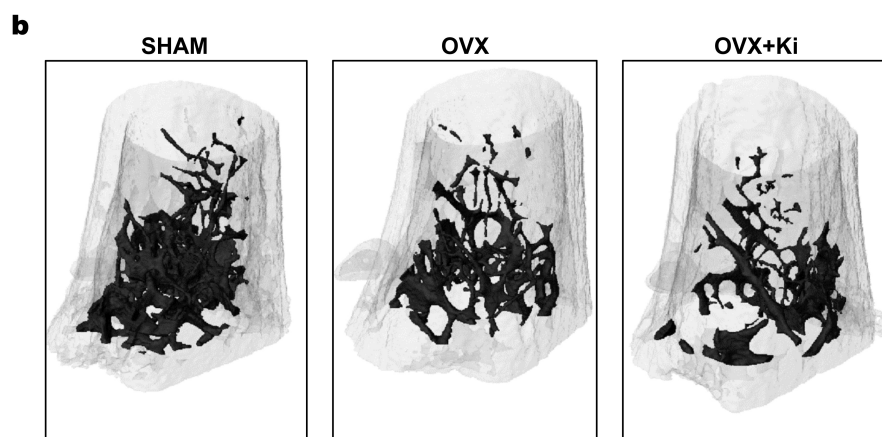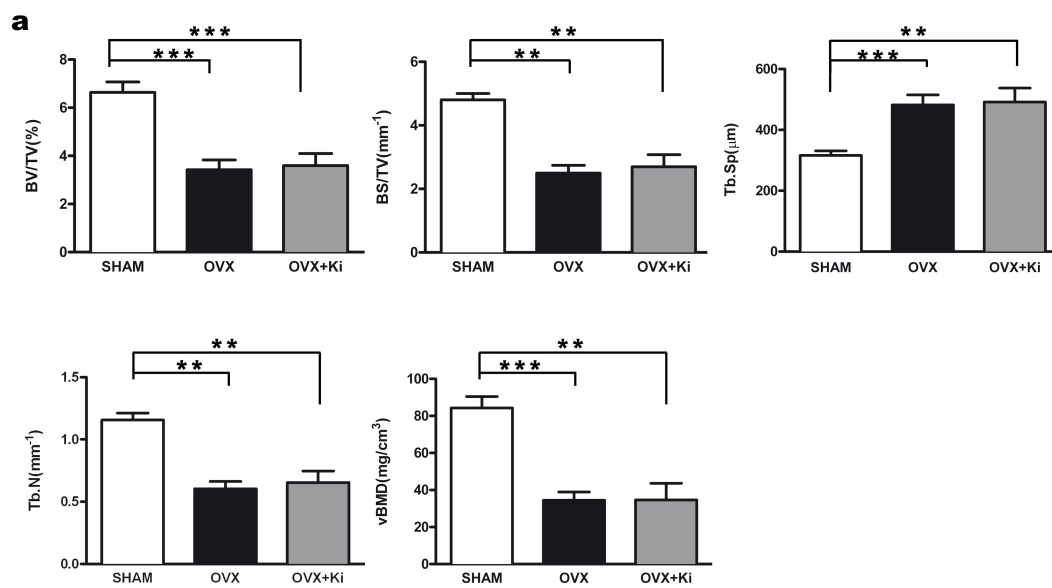361    images are shown. Groups of mice and analysis are as in Figure 1.

362    **Figure 3. Lack of effect of Ki16425 treatment on LPA receptor and osteoclastic**

363    **markers in OVX mice. (**A). Levels of mRNA for lysophosphatidic acid receptors

364    (LPA) relative to that for β-actin were determined in bone tissue by real-time PCR.

365    Levels of mRNA for *Tnfrsf11a, Tnfsf11* and *Csf1* (B) and *Ctsk* and *Acp5* (C) relative to

366    that for β-actin on bone tissue from vehicle-treated sham-OVX and OVX mice and

367    Ki16425-treated OVX mice (7-12 mice per group). Values expressed as mean ± SEM.

368    **Figure 4. Increased expression of osteoblast differentiation markers in**

369    **ovariectomized mice treated with Ki16425.** Comparisons of *Alpl* (A), *Col1a1* (B) and

370    *Bglap* (C) mRNA levels between vehicle-treated sham-OVX and OVX mice and
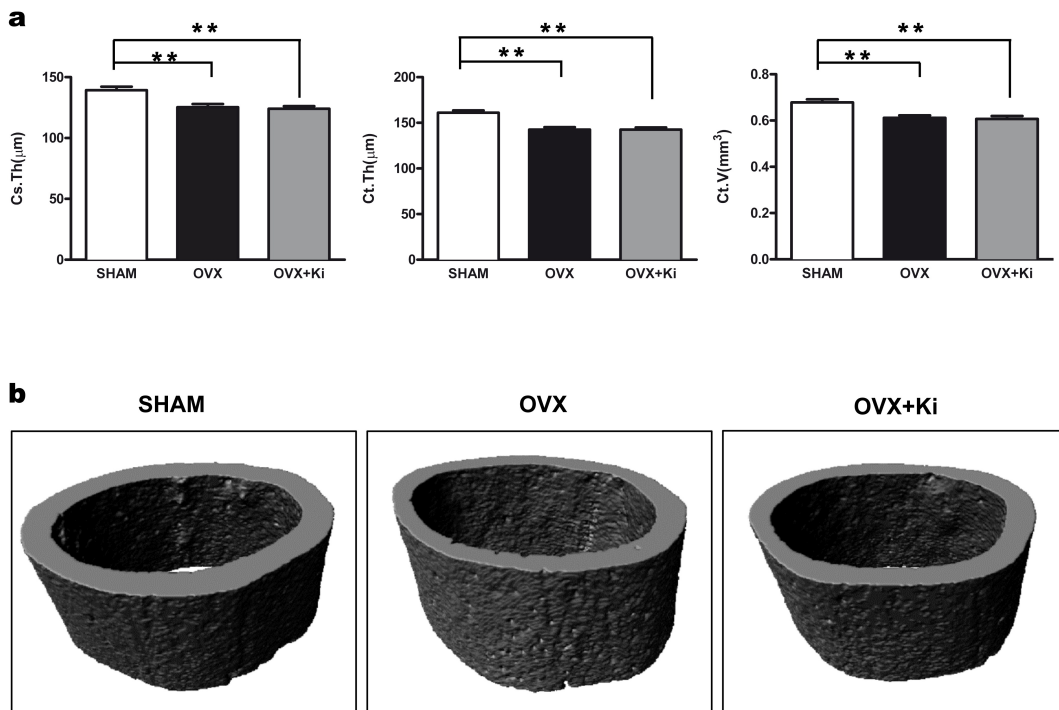
19

371  Ki16425-treated OVX mice (7-12 mice per group). Values expressed as mean ± SEM;

372  *$p$=0.05 and **$p$=0.005, by Mann-Whitney U test.

373  **Figure 5. Effect of lysophosphatidic acid (LPA) receptor inhibition on bone**

374  **remodelling markers in sera from ovariectomized mice.** Sera levels of the bone

375  formation markers, BALP and osteocalcin (A) and the bone resorption markers,

376  TRACP5b and PYD (B) in vehicle-treated and Ki16425-treated OVX mice. Values are

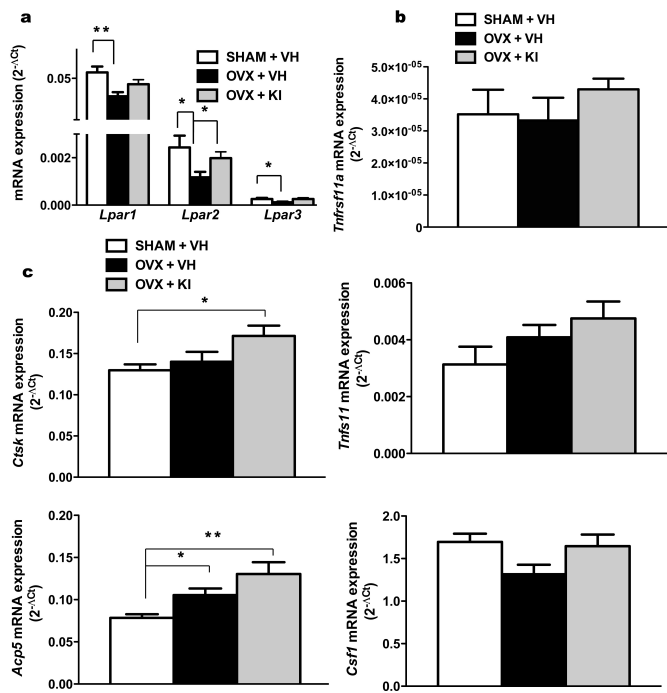377  mean ± SEM from 7-12 mice per group.

378  **Figure 6. Effect of Ki16425 treatment of OVX mice on *in vitro* matrix**

379  **mineralization and osteoclasts differentiation.** A. Mineralized area produced by

380  osteoblasts isolated from vehicle-treated and Ki16425-treated OVX mice. B. Number of

381  fully differentiated osteoclasts from bone marrow precursors assessed by counting

382  TRAP+ multinucleated cells relative to the total number of TRAP+ cells. Values are
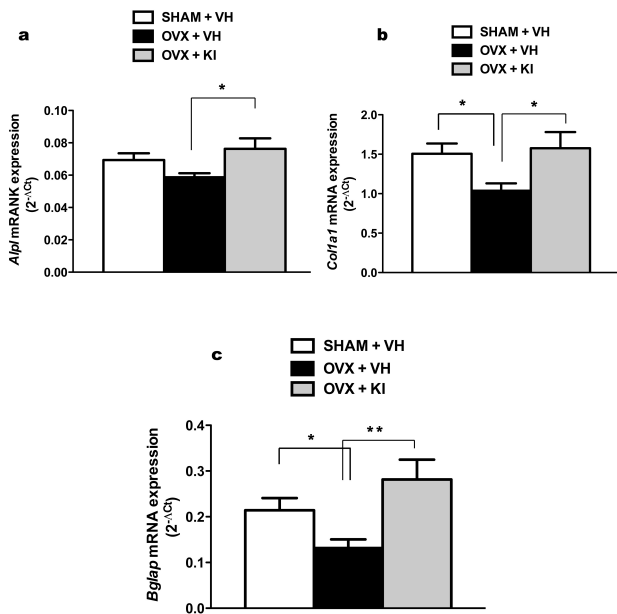
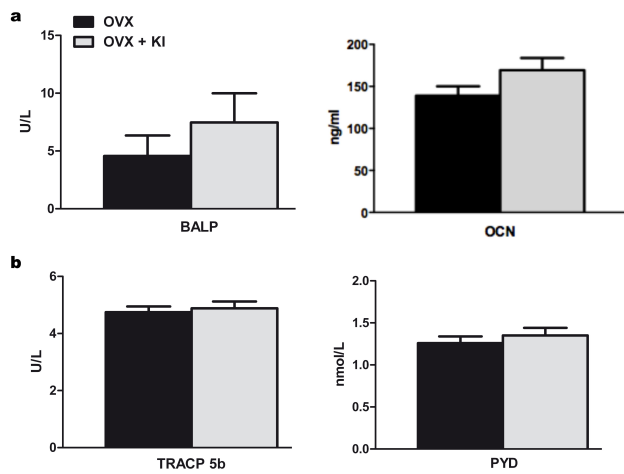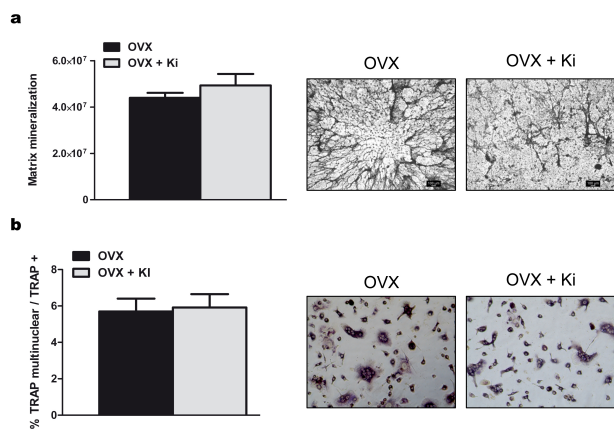383  mean ± SEM from 7-11 mice per group.

Figure 1

**a**



**b**

SHAM | OVX | OVX+Ki

**Figure 2**

**a**

mRNA expression ($2^{-\Delta Ct}$)

SHAM + VH
OVX + VH
OVX + KI

$**$
$*$
$*$
$*$

0.05
0.002
0.000

*Lpar1*   *Lpar2*   *Lpar3*

**b**

SHAM + VH
OVX + VH
OVX + KI

*Tnfrsf11a* mRNA expression ($2^{-\Delta Ct}$)

$5.0\times10^{-05}$
$4.0\times10^{-05}$
$3.0\times10^{-05}$
$2.0\times10^{-05}$
$1.0\times10^{-05}$
0

**c**

SHAM + VH
OVX + VH
OVX + KI

*Ctsk* mRNA expression ($2^{-\Delta Ct}$)

$*$

0.20
0.15
0.10
0.05
0.00

*Tnfs11* mRNA expression ($2^{-\Delta Ct}$)

0.006
0.004
0.002
0.000

*Acp5* mRNA expression ($2^{-\Delta Ct}$)

$**$
$*$

0.20
0.15
0.10
0.05
0.00

*Csf1* mRNA expression ($2^{-\Delta Ct}$)

2.0
1.5
1.0
0.5
0.0

**Figure 3**

Figure 4

**a**

OVX
OVX + KI

BALP

OCN

**b**

TRACP 5b

PYD

**Figure 5**

**a**



**b**



**Figure 6**