

1 **Bile acids and bilirubin effects on osteoblastic gene profile.**

2 **Implications in the pathogenesis of osteoporosis in liver diseases.**

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5 Silvia Ruiz-Gaspà<sup>1</sup>, Nuria Guañabens<sup>1,2</sup>, Susana Jurado González<sup>1</sup>, Marta Dubreuil<sup>1</sup>,  
6 Andres Combalia<sup>2</sup>, Pilar Peris<sup>1,2</sup>, Ana Monegal<sup>2</sup>, Albert Parés<sup>1,3</sup>

7  
8 <sup>1</sup>Centro de Investigación Biomédica en Red en Enfermedades Hepáticas y Digestivas  
9 (CIBERehd),

10 <sup>2</sup>Metabolic Bone Diseases Unit, Department of Rheumatology, Hospital Clínic,  
11 IDIBAPS, University of Barcelona,

12 <sup>3</sup>Liver Unit, Hospital Clínic, IDIBAPS, University of Barcelona, Barcelona, Spain.  
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19 **List of abbreviations:**

20  
21 ALPL, gene encoding the tissue-nonspecific alkaline phosphatase; ATCC, American  
22 Type Culture Collection; BGLAP, osteocalcin or bone gamma-carboxyglutamic acid-  
23 containing protein; BMPs, Bone Morphogenetic Proteins; CALCR, calcitonin receptor;  
24 CASP1, Caspase 1; CASP9, Caspase 9; COL10A1, Collagen X alpha-1; COL15A1,  
25 collagen XV alpha-1; COL19A1, Collagen XIX Alpha-1; COL7A1, collagen VII alpha-  
26 1; CSF2, colony stimulating factor 2; CYP24A1, 1,25-dihydroxyvitamin D3 24-  
27 hydroxylase; DKK1, dickkopf-related protein 1; DMEM, Dubelcco's modified Eagle  
28 medium; DSPP, dentin sialophosphoprotein; FBS, fetal bovine serum; HBSS, Hanks  
29 Balanced Salt Solution; IGF1, Insulin-like growth factor 1; LCA, lithocholic acid;  
30 MGP, matrix Gla protein ; PBC, primary biliary cholangitis; RANKL, receptor activator  
31 of nuclear factor-kappaB ligand; RIN, RNA integrity number; RUNX2, runt-related  
32 transcription factor 2; Saos-2, human osteosarcoma cells; SD, standard deviation;

33 SMAD 6, decapentaplegic homolog 6; SP7, osterix transcription factor; SPOCK3,  
34 osteonectin; SPP1, osteopontin; TGFB1, Transforming growth factor beta-1;  
35 UDCA, Ursodeoxycholic acid.

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49 **Corresponding Author:**

50 Núria Guañabens, MD, PhD  
51 Department of Rheumatology  
52 Hospital Clinic  
53 Villarroel 170  
54 08036-Barcelona  
55 Spain  
56 Phone: +34932271735  
57 [nguanabens@ub.edu](mailto:nguanabens@ub.edu)

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## 61 **Abstract**

62 Osteoporosis in advanced cholestatic and end-stage liver disease is related to low bone  
63 formation. Previous studies have demonstrated the deleterious consequences of  
64 lithocholic acid (LCA) and bilirubin on osteoblastic cells. These effects are partially or  
65 completely neutralized by ursodeoxycholic acid (UDCA). We have assessed the  
66 differential gene expression of osteoblastic cells under different culture conditions. The  
67 experiments were performed in human osteosarcoma cells (Saos-2) cultured with LCA  
68 (10  $\mu$ M), bilirubin (50  $\mu$ M) or UDCA (10 and 100  $\mu$ M) at 2 and 24 hours. Expression of  
69 87 genes related to bone metabolism and other signalling pathways were assessed by  
70 TaqMan micro fluidic cards. Several genes were up-regulated by LCA, most of them pro-  
71 apoptotic (*BAX*, *BCL10*, *BCL2L13*, *BCL2L14*), but also *MGP* (matrix Gla protein),  
72 *BGLAP* (osteocalcin), *SPPI* (osteopontin) and *CYP24A1*, and down-regulated bone  
73 morphogenic protein genes (*BMP3* and *BMP4*) and *DKK1* (Dickkopf-related protein 1).  
74 Parallel effects were observed with bilirubin, which up-regulated apoptotic genes and  
75 *CSF2* (colony-stimulating factor 2) and down-regulated antiapoptotic genes (*BCL2* and  
76 *BCL2L1*), *BMP3*, *BMP4* and *RUNX2*. UDCA 100  $\mu$ M had specific consequences since  
77 differential expression was observed, up-regulating *BMP2*, *BMP4*, *BMP7*, *CALCR*  
78 (calcitonin receptor), *SPOCK3* (osteonectin), *BGLAP* (osteocalcin) and *SPPI*  
79 (osteopontin), and down-regulating pro-apoptotic genes. Furthermore, most of the  
80 differential expression changes induced by both LCA and bilirubin were partially or  
81 completely neutralized by UDCA. *Conclusion*: Our observations reveal novel target  
82 genes, whose regulation by retained substances of cholestasis may provide additional  
83 insights into the pathogenesis of osteoporosis in cholestatic and end-stage liver diseases.

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87 **Key words:** Osteoporosis; Bone turnover; Cholestasis; Bile acids; Primary biliary

88 cholangitis; Osteoblasts.

89

## 90 **Introduction**

91

92 Osteoporosis is a skeletal disease characterized by low bone mass and micro-architectural  
93 deterioration of bone tissue, leading to an increased fragility and susceptibility to fracture.

94 It is a common complication of liver diseases, particularly in chronic cholestasis and  
95 especially in those patients with primary biliary cholangitis (PBC) [1-4]. Low bone  
96 formation as a consequence of a deficient osteoblast activity is the main cause for bone  
97 loss [1], but an increased resorption has been described as well [5].

98 Different studies have found that high concentrations of bilirubin and bile acid can  
99 contribute to the abnormal osteoblast function [6], as both bilirubin and lithocholic acid  
100 (LCA) in addition to serum from jaundiced patients have detrimental effects on these  
101 bone-forming cells [7].

102 Ursodeoxycholic acid (UDCA), the standard treatment for patients with PBC has greatly  
103 changed the natural history of the disease [8-11]. Moreover, in bone cells UDCA  
104 increases survival and improves differentiation of human osteoblasts, neutralizing the  
105 detrimental effects of LCA and bilirubin on osteoblast survival, differentiation and  
106 mineralization [12]. Lastly, while bilirubin and LCA act as pro-apoptotic agents in human  
107 osteoblasts, UDCA has anti-apoptotic effects and neutralizes the apoptosis induced by  
108 LCA and bilirubin in osteoblastic cells [12].

109 In previous studies a down-regulation of RUNX2 gene by bilirubin and an up-regulation  
110 of the RANKL/OPG expression ratio by jaundiced sera in osteoblastic cells were  
111 demonstrated [7]. However, more data on the influence of the retained substances of  
112 cholestasis in osteoblastic cell gene expression is lacking. To gain new insights into  
113 cholestatic-induced osteoporosis, we have assessed whether the damaging effects of the

114 retained substances such as bilirubin and bile acids can modify the gene expression  
115 profiling of osteoblastic cells.

116

## 117 **Materials and methods**

118

### 119 *Materials*

120 Dubelcco's modified Eagle medium (DMEM), fetal bovine serum (FBS), Hanks  
121 Balanced Salt Solution (HBSS), L-glutamine and trypsin were purchased from Invitrogen  
122 (Grand Island, NY, USA); LCA, UDCA and bilirubin were from Sigma Chemical Co.  
123 (St. Louis, MO, USA); Penicillin-streptomycin was from LabClinics (Barcelona, Spain).

### 124 *Cell culture and incubation*

125 The experiments were performed with human osteosarcoma cell line Saos-2. The cell line  
126 Saos-2 was obtained from the American Type Culture Collection (ATCC) (Rockville,  
127 MD, USA) (HTB85; ATCC) and cultured as a monolayer in DMEM containing 10%  
128 FCS, 100 U/mL penicillin and 100 mg/mL streptomycin. Cells were incubated at 37 °C  
129 in a humidified atmosphere of 5% CO<sup>2</sup> in air.

### 130 *Administrated treatments*

131 Saos-2 cells were cultured for 2 and 24 h in different conditions: (a) LCA (10 μM),  
132 bilirubin (50 μM) and UDCA 10 μM and 100 μM. (b) To analyze the interaction of  
133 UDCA with LCA and bilirubin, cells were incubated with a steady concentration of LCA  
134 (10 μM) or bilirubin (50 μM) and two concentrations of UDCA (10 μM and 100 μM).

135 All treatment concentrations used were selected based on the cytotoxicity assays carried  
136 out in previous studies [7,13].

#### 137 ***Experimental bilirubin solution preparation***

138 Bilirubin (Sigma) stock solution of 1600  $\mu\text{M}$  was prepared just before use by dissolving  
139 bilirubin in 10 ml 0.01N NaOH under dim light as previously described [12,15-18]. It was  
140 filtered through a sterile filter (0.22  $\mu\text{m}$  pore size) and adjusted to pH 7.2-7.4 with 0.1N  
141 HCl, if necessary. The bilirubin stock solution was added to a final concentration of 50  
142  $\mu\text{M}$  in the culture medium. The cell cultures were kept in dark conditions to prevent  
143 bilirubin light degradation. Control cells were treated with vehicle (NaOH 0.1N).

#### 144 ***RNA isolation and quantification***

145 Total cellular RNA was extracted from cultured cells using an acid guanidinium-phenol-  
146 chloroform method (Trizol reagent; Invitrogen, Grand Island, NY, USA) according to the  
147 manufacturer's protocols. RNA integrity was determined by a microfluidics-based  
148 electrophoresis system using a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).  
149 RNA integrity number (RIN) from automated analysis software allows classification of  
150 RNA in a numeric system with one for complete degradation and ten for optimal  
151 intactness. Both analyses displayed highly intact RNA, with RIN values of 9.8–10.

#### 152 ***Expression analysis with TaqMan microfluidic cards***

153 cDNA synthesis was performed with the High-Capacity cDNA Reverse Transcription Kit  
154 (Applied Biosystems, Foster City, CA, USA) with a Master Mix containing 2.5 U/ $\mu\text{l}$  of  
155 MultiScribe Reverse Transcriptase and 1  $\mu\text{g}$  of total RNA. The reaction mixture was  
156 incubated at 25°C for 10 min, followed by 120 min at 37°C and then by heat inactivation

157 of the enzyme at 85°C for 5 sec. Next, we mixed 2 µl of single-stranded cDNA (equivalent  
158 to around 100 ng of total RNA) with 48 µl of nuclease-free water and 50 µl of TaqMan  
159 Universal PCR Master Mix. After loading 100 µl of the sample-specific PCR mixture into  
160 one sample port of the microfluidic cards (Human ABC Transporter Panel; Applied  
161 Biosystems), the cards were centrifuged twice for 1 min at 280g and sealed to prevent  
162 well-to-well contamination. The cards were placed in the microfluidic card Sample Block  
163 of an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). The  
164 thermal cycling conditions were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles  
165 of 30 sec at 97°C and 1 min at 59.7°C. The assay for each gene on the microfluidic card  
166 was carried out in triplicate, due to the design of this specific panel. The calculation of  
167 the threshold cycle (Ct) values were performed using the SDS 2.2 software (Applied  
168 Biosystems), after automatically setting the baseline and the threshold.

#### 169 ***Gene selection***

170 A total of eighty-seven genes were selected to investigate the profile gene expression  
171 under bilirubin, LCA and UDCA treatment. Genes were chosen according to their  
172 relevant function in cellular processes and signaling pathways related to bone metabolism  
173 (Table 1).

#### 174 ***Statistics***

175 Significant differences between two groups were determined by Student's t-test or Mann-  
176 Whitney U-test. When multiple groups were compared, ANOVA was utilized, followed  
177 by a Tukey's multiple contrast test, when applicable. A p-value  $\leq 0.05$  was considered  
178 significant. All analyses were performed using the PASW Statistics 20 (SPSS, Chicago,  
179 IL, USA).



## 180 **Results**

### 181 *Effect of LCA and bilirubin on gene profiles*

182 As compared with controls the effects of LCA at 10 $\mu$ M and bilirubin at 50 $\mu$ M were  
183 observed after 2 hours of treatment, but frequently were more apparent after 24 hours.  
184 The most relevant results are shown in figures 1A and 1B.

185 The apoptosis-related genes were the ones most affected after treatments with LCA and  
186 bilirubin, resulting in severe gene expression changes. LCA and bilirubin significantly  
187 up-regulated the expression of some pro-apoptotic genes and down-regulated some anti-  
188 apoptotic genes ( $p < 0.05$ ). Caspase 1 (*CASP1*) was overexpressed under LCA treatment  
189 and bilirubin decreased the expression of caspase 9 (*CASP9*).

190 When assessing bone morphogenetic proteins (BMPs), both LCA and bilirubin  
191 diminished the expression of some of these genes, mainly *BMP3* and *BMP4* (Figures 1A  
192 and 1B). These effects were observed after just two hours of treatment and were more  
193 evident under LCA treatment (figure 2).

194 The expression of collagen X alpha-1 (*COL10A1*), collagen XIX alpha-1 (*COL19A1*) and  
195 collagen VII alpha-1 (*COL7A1*) was down-regulated by bilirubin after 2 hours of  
196 treatment. However, collagen XV alpha-1 (*COL15A1*) was up-regulated after 24 hours.  
197 No significant changes were observed with LCA on the different evaluated collagen  
198 genes.

199 The analysis of selected osteogenic genes displayed substantial overexpression of  
200 osteocalcin or bone gamma-carboxyglutamic acid-containing protein (*BGLAP*) and  
201 osteopontin (*SPP1*) ( $p < 0.001$ ) under LCA treatment. Moreover, a significant decrease of

202 alkaline phosphatase expression (*ALPL*) was found under LCA treatment ( $p < 0.001$ ) and  
203 under bilirubin treatment at 24 hours ( $p < 0.05$ ).

204 Regarding the specific transcription factors, SP7 (osterix transcription factor) expression  
205 was down-regulated after 24 hours under LCA treatment, while it was up-regulated after  
206 24 hours with bilirubin treatment. The runt-related transcription factor 2 (*RUNX2*) was  
207 down-regulated by bilirubin. This effect was observed at 2 and 24 hours.

208 With respect to genes involved in osteoclast differentiation and bone resorption, LCA up-  
209 regulated the receptor activator of nuclear factor-kappaB ligand (*RANKL*) and the  
210 calcitonin receptor (*CALCR*) after 2 hours. This effect was not evident at 24 hours.

211 Similarly, LCA induced a constant overexpression of the matrix Gla protein (*MGP*). No  
212 significant effects of bilirubin were observed in the expression of these genes.  
213 Conversely, the colony stimulating factor 2 (*CSF2*) ( $p < 0.001$ ) was significantly down-  
214 regulated after 2 hours under LCA, but upregulated by bilirubin at 24 hours.

215 Within the growth factor family, LCA increased Insulin-like growth factor 1 (*IGF1*)  
216 expression levels after 2 hours, and bilirubin significantly increased transforming growth  
217 factor beta-1 (*TGFBI*) after 24 hours ( $p = 0.03$ ).

218 LCA significantly up-regulated 1,25-dihydroxyvitamin D<sub>3</sub> 24-hydroxylase (*CYP24A1*)  
219 gene expression, an effect which was observed from the first two hours and sustained  
220 throughout of the experiment ( $p < 0.001$ )

221 Finally, expression levels of both dentin sialophosphoprotein (*DSPP*) ( $p < 0.01$ ) and  
222 decapentaplegic homolog 6 (*SMAD 6*) ( $p < 0.02$ ) were down-regulated after 2 hours under  
223 bilirubin treatment, whereas LCA produced the same effect on the Wnt signaling pathway  
224 by down-regulating the dickkopf-related protein 1 (*DKK1*) gene expression. This effect  
225 was observed after 2 hours of treatment and was much higher at 24 hours ( $p = 0.01$ ).

226

227 ***Effect of UDCA and interaction with LCA and bilirubin on gene profiles***

228 As compared with controls, UDCA has a significant effect, mainly on three big family  
229 genes: apoptosis (figure 3A), bone morphogenetic proteins (BMPs) (figures 2 and 3B)  
230 and bone specific genes (osteoblastic transcription factors and specific osteogenic factors)  
231 (figure 3C). UDCA resulted in specific gene expression changes by itself or by modifying  
232 the effects of LCA and bilirubin. Accordingly, UDCA 10 $\mu$ M and 100 $\mu$ M diminished the  
233 gene expression of pro-apoptotic genes *BAX*, *BCL10*, *BCL2L13*, *BCL2L14* and *BCL3*,  
234 and counteracted both the LCA and bilirubin effects. UDCA increased the expression of  
235 the anti-apoptotic genes *BCL2*, *BCL2A1*, *BCL2L1* and *BCL2L2*, being significant for  
236 *BCL2A1* (p=0.03). Moreover, this UDCA anti-apoptotic function neutralized the LCA  
237 and bilirubin effects by abolishing the significant decrease produced by them on these  
238 anti-apoptotic genes (p<0.03). No significant changes were found in the expression of  
239 caspase family genes under UDCA (figure 3A).

240 UDCA significantly increased *BMP2*, *BMP3*, *BMP4* and *BMP7* gene expression from 2  
241 hours of treatment at 10 $\mu$ M and 100 $\mu$ M and neutralized the down-regulation induced by  
242 LCA and bilirubin on BMPs expression (p<0.001) (Figures 2 and 3B).

243 Furthermore, UDCA significantly increased gene expression of the bone resorption  
244 marker *CALCR* and of the osteoblastic specific bone markers *BGLAP*, *SPOCK3* and  
245 *SPPI*, when cells were treated with UDCA (10 $\mu$ M or 100 $\mu$ M) or when cells were treated  
246 at the same time with 10 $\mu$ M LCA or 50 $\mu$ M bilirubin (Figure 3C).

247

248 **Discussion**

249 The effects of substances retained in chronic cholestasis on bone cells have been  
250 previously described, suggesting direct harmful effects of bilirubin and LCA on human  
251 osteoblastic cells. Janes et al. observed that plasma with high concentrations of bilirubin  
252 resulted in decreased human osteoblast-like cells proliferation [19]. Similarly, bilirubin  
253 induced a marked dose-dependent inhibition of avian chondrocytes proliferation [20].  
254 Previous studies from our group have shown a decrease of human osteoblastic cells  
255 viability and differentiation when bilirubin and sera from jaundiced patients was added  
256 to the culture media [7]. Also, an increase in apoptosis was observed in both bilirubin and  
257 LCA-treated osteoblastic cells [24]. However, there are scarce data on the targets of these  
258 substances in osteoblasts [7,24]. The current study provides new insights in the effects of  
259 bilirubin and LCA on the expression of relevant groups of genes, as well as on the  
260 involved molecular pathways.

261

262 The essential impact of bilirubin on the expression of genes involved in apoptosis in  
263 human osteoblasts is confirmed in this study. It should be noted that bilirubin at 50  $\mu$ M  
264 dramatically increases the pro-apoptotic related genes and decreases the anti-apoptotic  
265 genes. Both phenomena occur after only few hours of treatment, suggesting that bilirubin  
266 could play a major role in the regulation of the apoptotic related genes. This observation  
267 leads us to confirm our earlier observations about the pro-apoptotic role of bilirubin on  
268 osteoblastic cells [24] and other cell types and tissues [15-16,25,26]. Likewise, the  
269 relationship between LCA and apoptosis in different tissues and cell types has been  
270 widely demonstrated. Previous studies observed LCA-induced apoptosis mediated by the  
271 nuclear receptor Nur77 expression in both human liver and colon cancer cells as well as  
272 in mouse hepatocytes [27]. LCA has also a pro-apoptotic effect in human colon  
273 adenocarcinoma cell lines [28], in human neuroblastoma cells [29] and in cultured

274 syncytiotrophoblast cells [30]. Finally, LCA induces apoptosis in human osteoblasts,  
275 increasing DNA fragmentation, caspase-3 activity and producing an up-regulation and a  
276 down-regulation of *BAX* and *BCL2* respectively [24]. Accordingly, the current study  
277 describes the apoptotic action of LCA through the increase of pro-apoptotic and the  
278 decrease of anti-apoptotic genes.

279

280 A new finding described in this study is the relationship between BMPs gene expression  
281 under the effects of LCA, bilirubin and UDCA (figures 2 and 3B). BMPs are members of  
282 the transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily and a subset of BMPs possess the  
283 ability to induce bone and cartilage formation and enhance osteogenesis and fracture  
284 healing [31-33]. BMPs are extracellular cytokines, originally isolated from bone extract  
285 and are produced in nearly all skeletal cells [34,35]. Accordingly, *BMP2* vastly increases  
286 osteocalcin, and a short-term expression of *BMP2* is necessary and sufficient to  
287 irreversibly induce bone formation [36,37]. Additionally, *BMP7* accelerates calcium  
288 mineralization and induces the expression of osteoblastic differentiation markers such as  
289 ALP activity [38,39], and loss of both *BMP2* and *BMP4* results in severe impairment of  
290 osteogenesis [40]. The decrease of *BMP3* and *BMP4* expression observed in our study  
291 under LCA and bilirubin treatments open up new approaches to illustrate their mechanism  
292 of action. On the other hand, the current study clearly demonstrates that UDCA causes an  
293 important up-regulation of *BMP2*, *BMP3*, *BMP4* and *BMP7* genes, and in addition  
294 counteracts the effects of LCA and bilirubin induced down-regulation of these genes.

295 The runt-related transcriptional factor 2 (RUNX2) [41] is the key transcription factor  
296 involved in osteoblasts differentiation under the guidance of BMPs signaling, since its  
297 expression can be induced by both *BMP2* and *BMP7* [42]. The increased expression of

298 BMPs under UDCA treatment leads us to relate it with the expression levels of the  
299 transcriptional factor RUNX2, which are down-regulated after bilirubin 50 $\mu$ M  
300 administration on cells treated for a short time and maintained steadily for 24 hours. These  
301 results are consistent with those published in previous studies, in which a down-regulation  
302 of RUNX2 with a consequent decrease in osteoblast differentiation was produced by the  
303 cell exposure to 50 $\mu$ M of bilirubin [7] and under low concentrations of bilirubin (3 and  
304 30 $\mu$ M) in rat osteoblasts primary culture with osteogenic medium for 3 or 14 days [43].  
305 The potential beneficial effects of UDCA on bone cells may be partially explained the  
306 BMPs up-regulation, which in turn induces RUNX2 expression. These effects were not  
307 clearly observed in these experiments, although UDCA partially attenuates the RUNX2  
308 down-regulation induced by bilirubin.

309 Despite the fact that new insights into gene profiling induced by bilirubin, LCA and  
310 UDCA have been described in this study, some limitations should be taken into account.  
311 The main concern is that the experiments were carried-out using the human osteosarcoma  
312 cell line Saos-2, which although it is most similar cell line to human primary osteoblasts,  
313 its behavior could be different. Our next approach will then be to check the gene  
314 expression of the most relevant results of our current study in primary human cultures,  
315 particularly those related to BMPs and some transcription factors such as RUNX2.

316 In summary, the current study shows that accumulated products of cholestasis decrease  
317 the expression of some BMPs, which are mostly strong osteogenic agents and synergize  
318 with osteogenic transcriptional factors such as RUNX2, that was down-expressed as well.  
319 Furthermore, the addition of biliary acids and bilirubin up-regulated pro-apoptotic and  
320 down-regulated anti-apoptotic genes. These changes in the apoptosis pathways involving  
321 the Saos-2 cells could modify the rate of bone formation and, therefore, be considered as

322 a truthful pathogenic mechanism of osteoporosis in cholestatic and in end-stage liver

323 diseases.

324

## 326 **References**

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328 [1]. Guañabens N, Pares A, Mariñoso L, Brancós MA, Piera C, Serrano S, et al. Factors  
329 influencing the development of metabolic bone disease in primary biliary cirrhosis.  
330 *Am J Gastroenterol* 1990;85:1356-1362.

331 [2]. Eastell R, Dickson ER, Hodgson SF, Wiesner RH, Porayko MK, Wahner HW, et  
332 al. Rates of vertebral bone loss before and after liver transplantation in women with  
333 primary biliary cirrhosis. *Hepatology* 1991;14:296-300.

334 [3]. Menon KV, Angulo P, Weston S, Dickson ER, Lindor KD. Bone disease in primary  
335 biliary cirrhosis: independent indicators and rate of progression. *J Hepatol*  
336 2001;35:316-323.

337 [4]. Guañabens N, Parés A, Ros I, Caballería L, Pons F, Vidal S, et al. Severity of  
338 cholestasis and advanced histological stage but not menopausal status are the major  
339 risk factors for osteoporosis in primary biliary cirrhosis. *J Hepatol* 2005;42:573-  
340 577.

341 [5]. Guichelaar MM, Malinchoc M, Sibonga J, Clarke BL, Hay JE. Bone metabolism in  
342 advanced cholestatic liver disease: analysis by bone histomorphometry. *Hepatology*  
343 2002; 36:895-903.

344 [6]. Janes CH, Dickson ER, Okazaki R, Bonde S, McDonagh AF, Riggs BL. Role of  
345 hyperbilirubinemia in the impairment of osteoblast proliferation associated with  
346 cholestatic jaundice. *J Clin Invest* 1995;95:2581-2586.

347 [7]. Ruiz-Gaspà S, Martinez-Ferrer A, Guañabens N, Dubreuil M, Peris P, Enjuanes A,  
348 et al. Effects of bilirubin and sera from jaundiced patients on osteoblasts:



- 349 contribution to the development of osteoporosis in liver diseases. *Hepatology*  
350 2011;54:2104-2113.
- 351 [8]. Lee YM, Kaplan MM. The natural history of PBC: has it changed? *Semin Liver*  
352 *Dis* 2005;25:321-326.
- 353 [9]. Parés A, Caballeria L, Rodés J. Excellent long-term survival in patients with  
354 primary biliary cirrhosis and biochemical response to ursodeoxycholic acid.  
355 *Gastroenterology* 2006;130:715-720.
- 356 [10]. Beuers U, Boberg KM, Chapman RW, Chazouillères O, Invernizzi P, Jones DE, et  
357 al. EASL clinical practice guidelines: management of cholestatic liver diseases. *J*  
358 *Hepatol* 2009;51:237-267
- 359 [11]. Paumgartner G, Beuers U. Ursodeoxycholic acid in cholestatic liver disease:  
360 mechanisms of action and therapeutic use revisited. *Hepatology* 2002;36:525-531.
- 361 [12]. Dubreuil M, Ruiz-Gaspà S, Guañabens N, Peris P, Alvarez L, Monegal A, et al.  
362 Ursodeoxycholic acid increases differentiation and mineralization and neutralizes  
363 the damaging effects of bilirubin on osteoblastic cells. *Liver Int* 2013;33:1029-  
364 1038.
- 365 [13]. Ruiz-Gaspà S, Guañabens N, Enjuanes A, Peris P, Martinez-Ferrer A, Martinez de  
366 Osaba MJ, et al. Lithocholic acid downregulates vitamin D effects in human  
367 osteoblasts. *Eur J Clin Invest* 2010;40:25-34.
- 368 [14]. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time  
369 quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; 25:402-408.

- 370 [15]. Yahia S, Shabaan AE, Gouida M, El-Ghanam D, Eldeglia H, El-Bakary A, et al.  
371 Influence of hyperbilirubinemia and phototherapy on markers of genotoxicity  
372 and apoptosis in full-term infants. *Eur J Pediatr* 2015;174:459-464.
- 373 [16]. Kosar NM, Tosun M, Polat C, Kahraman A, Arikan Y. Hepatocyte apoptotic index  
374 and p53 expression in obstructive jaundice rats. *Bratisl Lek Listy* 2014;115:352-6.
- 375 [17]. Bilbe G, Roberts E, Birch M, Evans DB. PCR phenotyping of cytokines, growth  
376 factors and their receptors and bone matrix proteins in human osteoblast-like cell  
377 lines. *Bone* 1996;19:437-445.
- 378 [18]. Pautke C, Schieker M, Tischer T, Kolk A, Neth P, Mutschler W, et al.  
379 Characterization of osteosarcoma cell lines MG-63, Saos-2 and U-2 OS in  
380 comparison to human osteoblasts. *Anticancer Res* 2004;24:3743-3748.
- 381 [19]. Janes CH, Dickson ER, Okazaki R, Bonde S, McDonagh AF, Riggs BL. Role of  
382 hyperbilirubinemia in the impairment of osteoblast proliferation associated with  
383 cholestatic jaundice. *J Clin Invest* 1995;95:2581-2586.
- 384 [20]. Vassilopoulou-Sellin R, Rey-Bear N, Oyedeji CO. Bilirubin as an inhibitor of  
385 cartilage metabolism: effect on avian chondrocyte proliferation in cell culture. *J*  
386 *Bone Miner Res* 1990; 5:769–774.
- 387 [21]. Stocker R, Ames BN. Potential role of conjugated bilirubin and copper in the  
388 metabolism of lipid peroxides in bile. *Proc Natl Acad Sci U S A* 1987;84:8130-  
389 8134.
- 390 [22]. Doré S, Snyder SH. Neuroprotective action of bilirubin against oxidative stress in  
391 primary hippocampal cultures. *Ann N Y Acad Sci* 1999;890:167-172.

- 392 [23]. Jansen T1, Hortmann M, Oelze M, Opitz B, Steven S, Schell R, et al. Conversion  
393 of biliverdin to bilirubin by biliverdin reductase contributes to endothelial cell  
394 protection by heme oxygenase-1-evidence for direct and indirect antioxidant  
395 actions of bilirubin. *J Mol Cell Cardiol* 2010;49:186-195.
- 396 [24]. Ruiz-Gaspà S, Dubreuil M, Guañabens N, Combalia A, Peris P, Monegal A, et al.  
397 Ursodeoxycholic acid decreases bilirubin-induced osteoblast apoptosis. *Eur J Clin*  
398 *Invest* 2014;44:1206-1214.
- 399 [25]. NaveenKumar SK, Thushara RM, Sundaram MS, Hemshekhar M, Paul M,  
400 Thirunavukkarasu C, et al. Unconjugated Bilirubin exerts Pro-Apoptotic Effect on  
401 Platelets via p38-MAPK activation. *Sci Rep* 2015;5:15045. doi:  
402 10.1038/srep15045.
- 403 [26]. Yueh MF, Chen S, Nguyen N, Tukey RH. Developmental onset of bilirubin-  
404 induced neurotoxicity involves Toll-like receptor 2-dependent signaling in  
405 humanized UDP-glucuronosyltransferase1 mice. *J Biol Chem* 2014;289:4699-  
406 4709.
- 407 [27]. Hu Y, Chau T, Liu HX, Liao D, Keane R, Nie Y, et al. Bile acids regulate nuclear  
408 receptor (Nur77) expression and intracellular location to control proliferation  
409 and apoptosis. *Mol Cancer Res* 2015;13:281-292.
- 410 [28]. Brossard D, Lechevrel M, El Kihel L, Quesnelle C, Khalid M, Moslemi S, et al.  
411 Synthesis and biological evaluation of bile carboxamide derivatives with pro-  
412 apoptotic effect on human colon adenocarcinoma cell lines. *Eur J Med*  
413 *Chem* 2014;86:279-290.

- 414 [29]. Goldberg AA, Beach A, Davies GF, Harkness TA, Leblanc A, Titorenko VI.  
415 Lithocholic bile acid selectively kills neuroblastoma cells, while sparing normal  
416 neuronal cells. *Oncotarget* 2011;2:761-782.
- 417 [30]. Du Q, Zhang Y, Pan Y, Duan T. Lithocholic acid-induced placental tumor necrosis  
418 factor- $\alpha$  upregulation and syncytiotrophoblast cell apoptosis in intrahepatic  
419 cholestasis of pregnancy. *Hepato Res* 2014;44:532-541.
- 420 [31]. Beederman M, Lamplot JD, Nan G, Wang J, Liu X, Yin L, et al. BMP signaling in  
421 mesenchymal stem cell differentiation and bone formation. *J Biomed Sci Eng*  
422 2013;6:32-52.
- 423 [32]. Wan M, Cao X. BMP signaling in skeletal development. *Biochem Biophys Res*  
424 *Commun* 2005;328:651-657.
- 425 [33]. Miyazono K, Maeda S, Imamura T. BMP receptor signaling: transcriptional targets,  
426 regulation of signals, and signaling cross-talk. *Cytokine Growth Factor Rev*  
427 2005;16:251-263.
- 428 [34]. Javed A, Chen H, Ghorri FY. Genetic and transcriptional control of bone formation.  
429 *Oral Maxillofac Surg Clin North Am* 2010;22:283-293.
- 430 [35]. James AW. Review of signaling pathways governing MSC osteogenic and  
431 adipogenic differentiation. *Scientifica (Cairo)* 2013;2013:684736.
- 432 [36]. Huang Z, Ren PG, Ma T, Smith RL, Goodman SB. Modulating osteogenesis of  
433 mesenchymal stem cells by modifying growth factor availability. *Cytokine* 2010;  
434 51:305-310.
- 435 [37]. Noël D, Gazit D, Bouquet C, Apparailly F, Bony C, Ponce P, et al. Short-term BMP-  
436 2 expression is sufficient for in vivo osteochondral differentiation of mesenchymal  
437 stem cells. *Stem Cells* 2004;22:74-85.

- 438 [38]. Gu K, Zhang L, Jin T, Rutherford RB. Identification of potential modifiers of  
439 Runx2/Cbfa1 activity in C2C12 cells in response to bone morphogenetic protein-7.  
440 Cells Tissues Organs 2004;176:28-40.
- 441 [39]. Shen B, Wei A, Whittaker S, Williams LA, Tao H, Ma DD, et al. The role of BMP-7  
442 in chondrogenic and osteogenic differentiation of human bone marrow multipotent  
443 mesenchymal stromal cells in vitro. J Cell Biochem 2010;109:406-416.
- 444 [40]. Bandyopadhyay A, Tsuji K, Cox K, Harfe BD, Rosen V, Tabin CJ. Genetic analysis  
445 of the roles of BMP2, BMP4, and BMP7 in limb patterning and skeletogenesis.  
446 PLoS Genet 2006;2:e216.
- 447 [41]. Miyazono K, Maeda S, Imamura T. Coordinate regulation of cell growth and  
448 differentiation by TGF-beta superfamily and Runx proteins. Oncogene  
449 2004;23:4232-4237.
- 450 [42]. Sharff KA, Song WX, Luo X, Tang N, Luo J, Chen J, et al. Hey1 basic helix-loop-  
451 helix protein plays an important role in mediating BMP9-induced osteogenic  
452 differentiation of mesenchymal progenitor cells. J Biol Chem 2009;284:649-659.
- 453 [43]. Lin TH, Tang CH, Hung SY, Liu SH, Lin YM, Fu WM, et al. Upregulation of heme  
454 oxygenase-1 inhibits the maturation and mineralization of osteoblasts. J Cell  
455 Physiol 2010;222:757-768.
- 456
- 457

Table 1. Selected genes.

<b>Apoptosis</b>	<b>Proapoptotics</b>	
	BAX BCL10 BCL2L13 BCL2L14 BCL3	BCL2 Associated X, Apoptosis Regulator BCL10, Immune Signaling Adaptor BCL2 Like 13 BCL2 Like 14 BCL3, Transcription Coactivator
	<b>Antiapoptotics</b>	
	BCL2 BCL2A1 BCL2L1 BCL2L2	BCL2, Apoptosis Regulator BCL2 Related Protein A1 BCL2 Like 1 BCL2 Like 2
	<b>Caspases</b>	
CASP1 CASP3 CASP5 CASP6 CASP9	Caspase 1 Caspase 3 Caspase 5 Caspase 6 Caspase 9	
<b>Transcription factors</b>	MSX1 MSX2 RUNX2 SP7	Msh Homeobox 1 Msh Homeobox 2 Runt Related Transcription Factor 2 Sp7 Transcription Factor
<b>BMPs</b>	BMP1 BMP2 BMP3 BMP4 BMP5 BMP7 BMPRIA	Bone Morphogenetic Protein 1 Bone Morphogenetic Protein 2 Bone Morphogenetic Protein 3 Bone Morphogenetic Protein 4 Bone Morphogenetic Protein 5 Bone Morphogenetic Protein 7 Bone Morphogenetic Protein Receptor Type 1A
<b>Osteogenics</b>	ALPL BGLAP CDH11 SPOCK SPP1	Alkaline Phosphatase, Biomineralization Associated Bone Gamma-Carboxyglutamate Protein Cadherin 11 SPARC (Osteonectin), Cwcv And Kazal Like Domains Proteoglycan 1 Secreted Phosphoprotein 1
<b>Osteoclast differentiation</b>	CSF CSF2 CSF3	Colony Stimulating Factor 1 Colony Stimulating Factor 1 Colony Stimulating Factor 3
<b>Bone resorption</b>	CALCR PTH1R OPG RANKL	Calcitonin Receptor Parathyroid Hormone 1 Receptor TNF Receptor Superfamily Member 11b (osteoprotegerin) TNF Superfamily Member 11
<b>Mineralization</b>	DMP1 DSPP MGP	Dentin Matrix Acidic Phosphoprotein 1 Dentin Sialophosphoprotein Matrix Gla Protein
<b>Collagen</b>	COL1A2 COL2A1 COL4A1 COL4A4 COL7A1 COL10A1 COL11A1 COL15A1 COL17A1 COL19A1	Collagen Type I Alpha 2 Chain Collagen Type II Alpha 1 Chain Collagen Type IV Alpha 1 Chain Collagen Type IV Alpha 4 Chain Collagen Type VII Alpha 1 Chain Collagen Type X Alpha 1 Chain Collagen Type XI Alpha 1 Chain Collagen Type XIV Alpha 1 Chain Collagen Type XVII Alpha 1 Chain Collagen Type XIX Alpha 1 Chain
<b>Growth factors</b>	EGFR FGF1 FGF2 FGFR2 IGF1 IGF2 IRS1 TGFB1	Epidermal Growth Factor Receptor Fibroblast Growth Factor 1 Fibroblast Growth Factor 2 Fibroblast Growth Factor Receptor 2 Insulin Like Growth Factor 1 Insulin Like Growth Factor 2 Insulin Receptor Substrate 1 Transforming Growth Factor Beta 1
<b>MAPKs</b>	MAPK8	Mitogen-Activated Protein Kinase 8
<b>Vitamin D metabolism</b>	CYP24A1	Cytochrome P450 Family 24 Subfamily A Member 1

	CYP27B1 VDR	Cytochrome P450 Family 27 Subfamily B Member 1 Vitamin D Receptor
<b>Wnt pathway</b>	DKK1 LRP5 SOST	Dickkopf WNT Signaling Pathway Inhibitor 1 LDL Receptor Related Protein 5 Sclerostin
<b>Metalloproteinases</b>	MMP2 MMP8 MMP10 MMP13	Matrix Metalloproteinase 2 Matrix Metalloproteinase 8 Matrix Metalloproteinase 10 Matrix Metalloproteinase 13
<b>SMADs</b>	SMAD1 SMAD3 SMAD6 SMAD7	SMAD Family Member 1 SMAD Family Member 3 SMAD Family Member 6 SMAD Family Member 7
<b>Coagulation</b>	PRLR	Prolactin Receptor
<b>Glucolysis</b>	HK2	Hexokinase 2
<b>Cell Cycle</b>	CDK2 CDK4	Cyclin Dependent Kinase 2 Cyclin Dependent Kinase 4
<b>Oncogens</b>	BRCA1 CDH11 FOS MYC TP53	BRCA1, DNA Repair Associated Cadherin 11 Fos Proto-Oncogene, AP-1 Transcription Factor Subunit MYC Proto-Oncogene, BHLH Transcription Factor Tumor Protein P53
<b>Embryonic development</b>	HOXA1	Homeobox A1
<b>Vascular</b>	VEGFA VEGFB VEGFC	Vascular Endothelial Growth Factor A Vascular Endothelial Growth Factor B Vascular Endothelial Growth Factor C

459

461 **Figure legends**

462

463 **FIG. 1.** Heat map of the effects of lithocholic acid (A) and bilirubin (B) on gene expression  
464 profiles in Saos-2 osteoblastic cells. The red colour corresponds to genes that are up-  
465 regulated, and green colour corresponds to genes that are down-regulated as compared to  
466 controls without LCA or bilirubin. \* indicates significant differences vs non-treated cells.

467

468 **FIG. 2.** Percentage changes in bone morphogenetic protein (BMP3, BMP4 and BMP7)  
469 gene expression at 2 hours (dark grey bars) and 24 hours (light grey bars), in different  
470 culture conditions (LCA, Bilirubin, and UDCA) and the effect of UDCA on cells treated  
471 with LCA or bilirubin. (\*p<0.001 vs controls; ^p<0.01 vs controls; ~p<0.05 vs controls;  
472 # p<0.001 vs UDCA treated cells; @ p<0.01 vs UDCA treated cells).

473

474 **FIG. 3.** Heat map of the differential gene expression induced by UDCA and neutralizing  
475 effects on osteoblastic cells cultured with lithocholic acid (LCA) and bilirubin. A) UDCA  
476 apoptotic profile; B) UDCA BMPs profile, and C) UDCA transcription factors and  
477 osteogenic markers profiles.

478 The red colour corresponds to genes that are up-regulated, and green colour corresponds  
479 to genes that are down-regulated as compared to controls. #indicates significant  
480 differences with respect to non-treated cells. In the experiments with lithocholic acid  
481 (LCA) or bilirubin # significant differences of UDCA treated cells with respect to LCA  
482 or bilirubin treated cells, or with respect to controls.



483

484

485

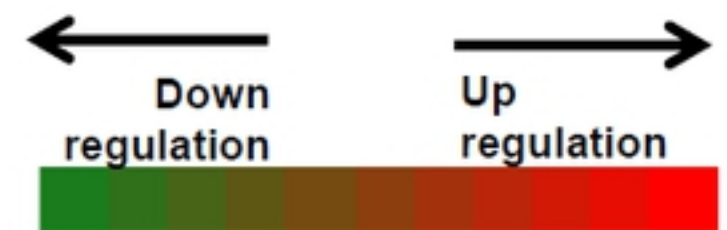
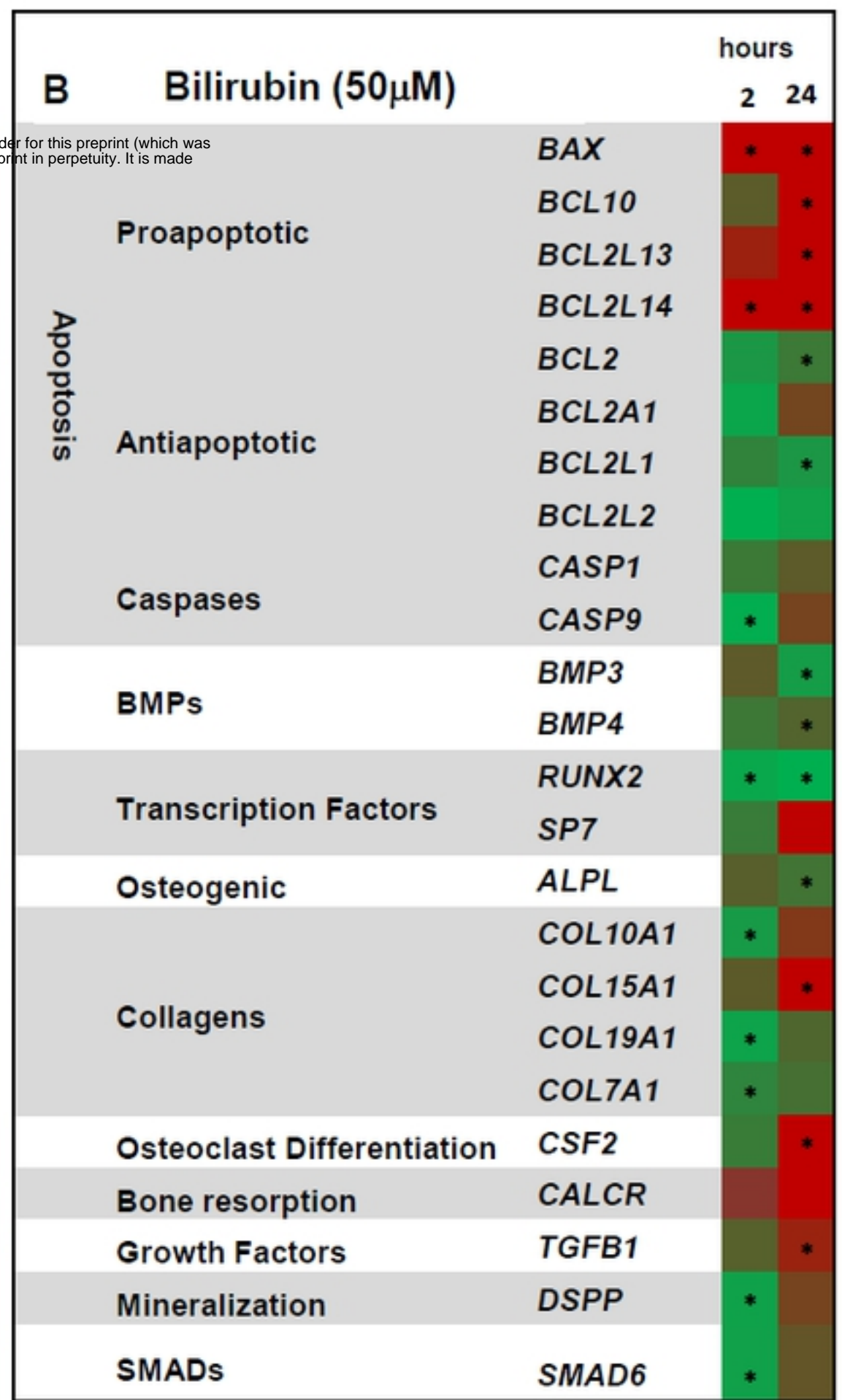
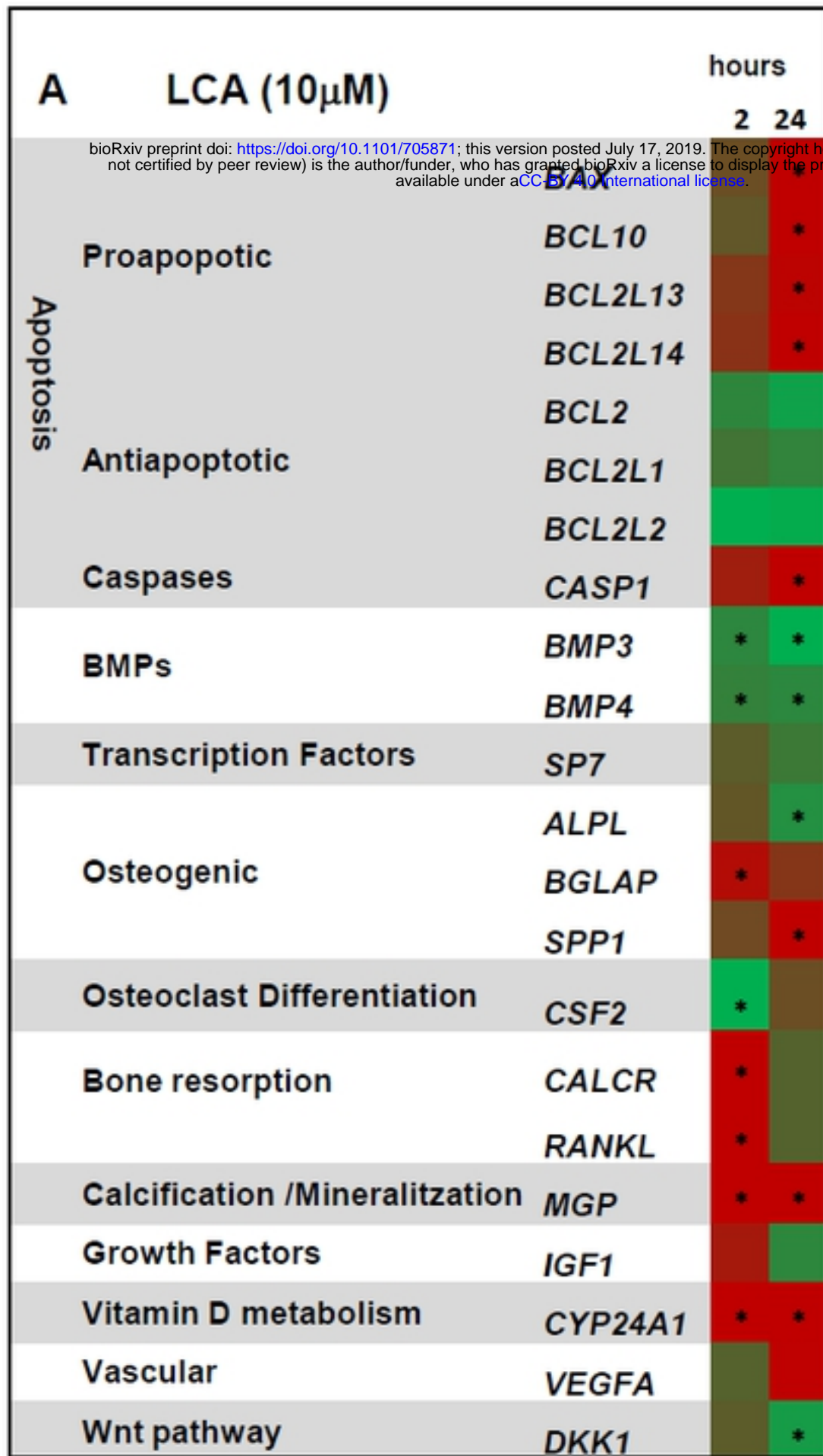
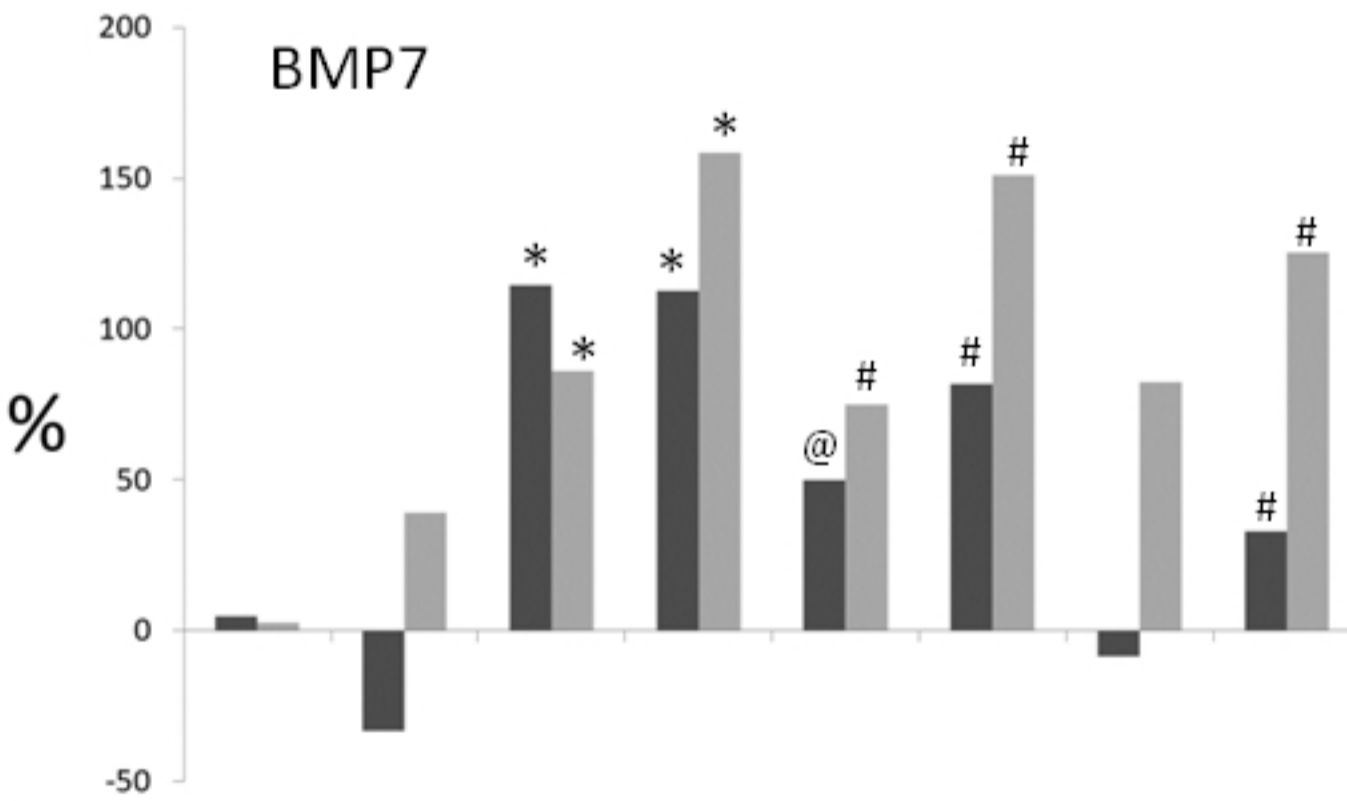
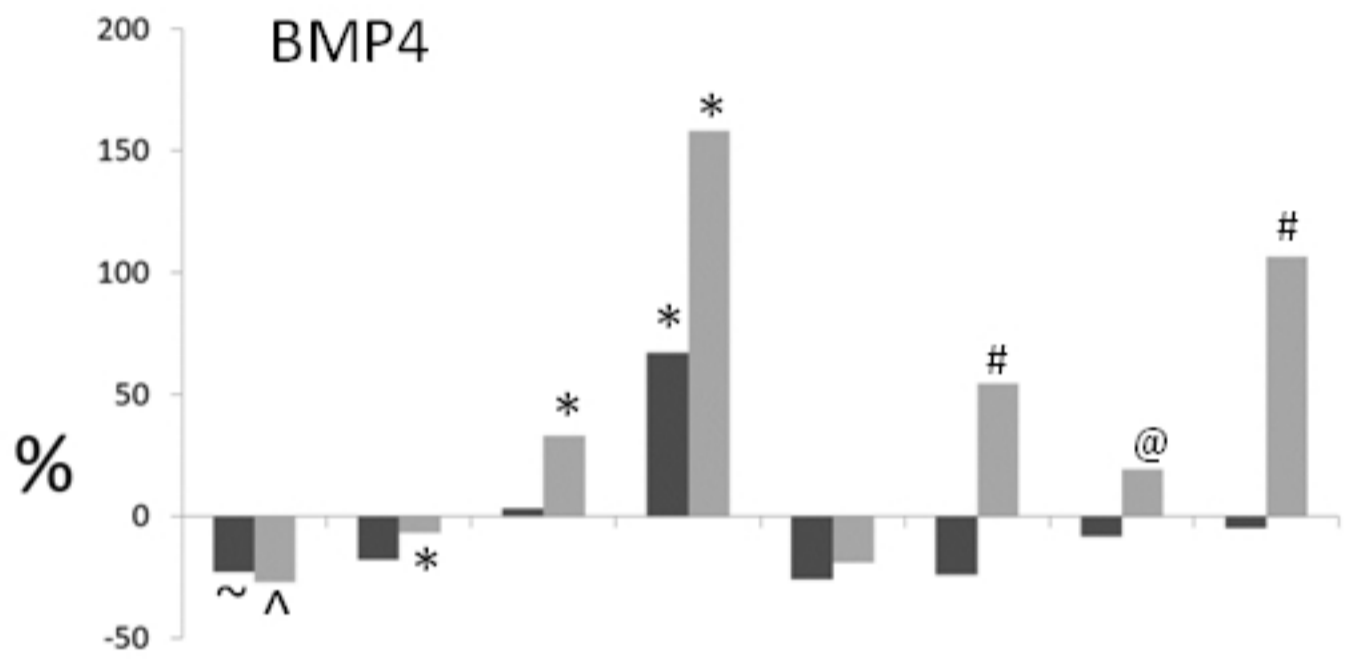
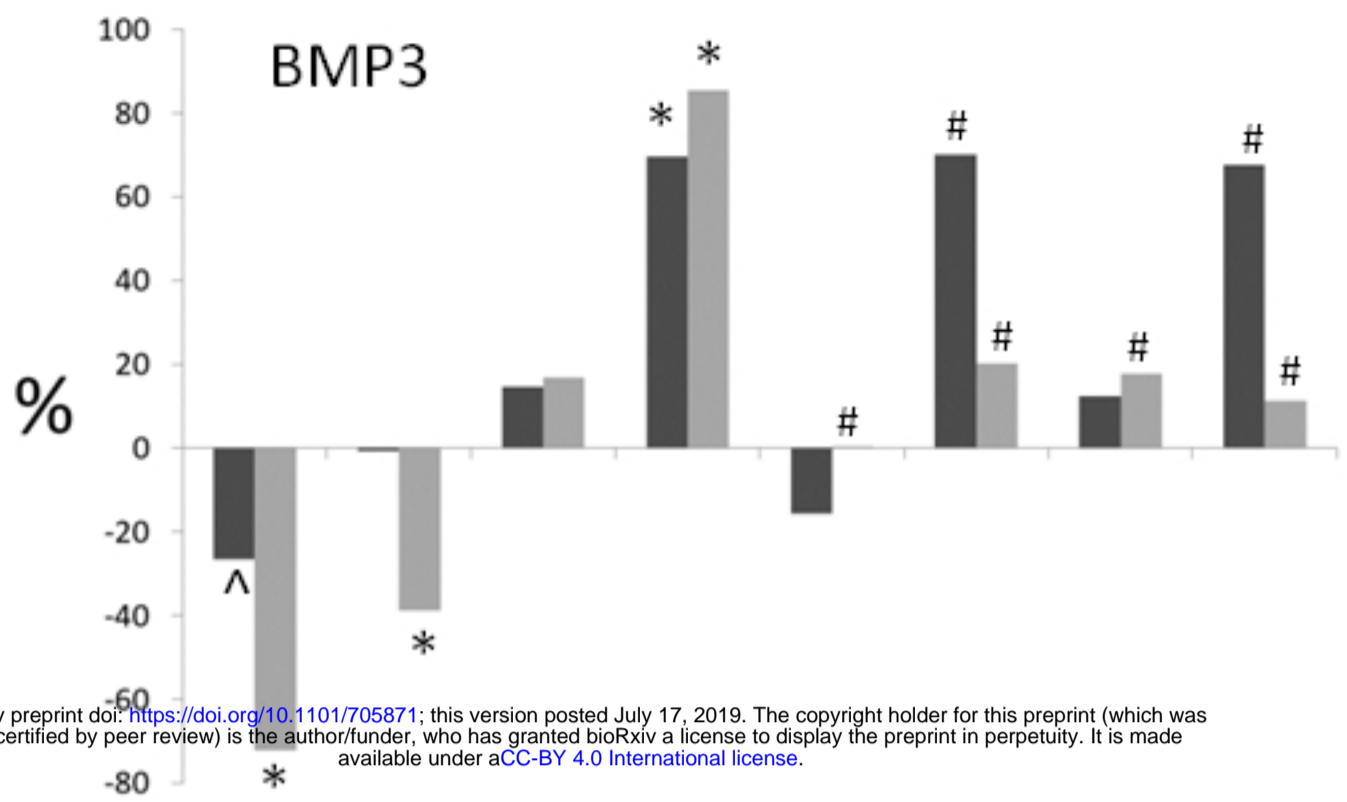


Figure 1

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LCA 10 μM	+	-	-	-	+	+	-	-
Bil 50 μM	-	+	-	-	-	-	+	+
UDCA 10 μM	-	-	+	-	+	-	+	-
UDCA 100 μM	-	-	-	+	-	+	-	+

Figure 2

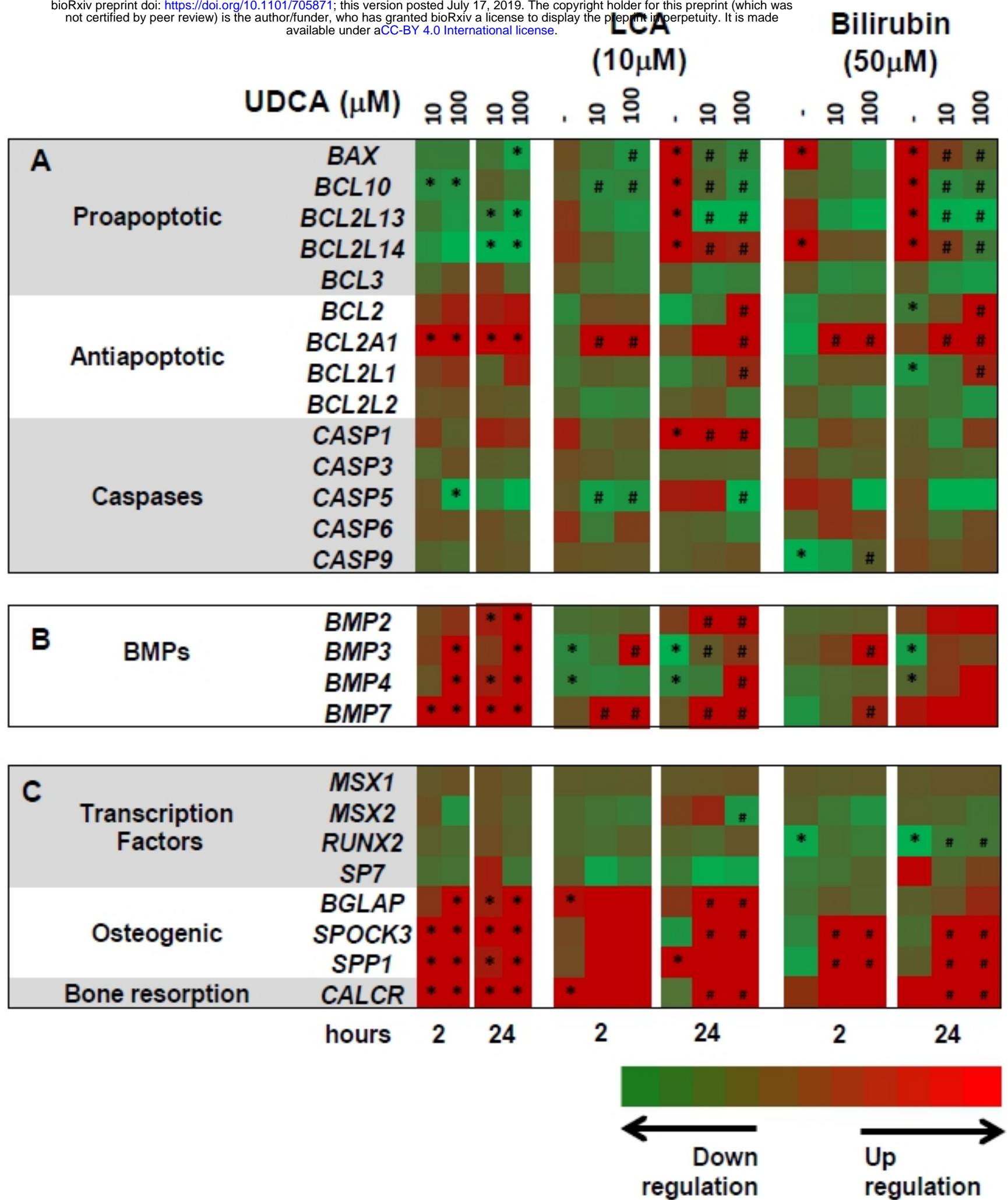


Figure 3