

1                    **Hyocholic acid species and the risk of type 2 diabetes**

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30

31 **ABSTRACT**

32 Hyocholic acid (HCA) and its derivatives are found in only trace amounts in human blood,  
33 but constitute approximately 76 % of the bile acid (BA) pool in the pig, a species known  
34 for its exceptional resistance to type 2 diabetes mellitus (T2DM). Here we show that  
35 HCA species play a crucial role in maintaining glucose homeostasis and preventing  
36 T2DM. We found that in two cohort studies (n=1,213), both obesity and diabetes were  
37 associated with lower serum concentrations of HCA species. Serum HCA levels in  
38 apparently healthy individuals (n=132) were found to be strong predictors for metabolic  
39 health 10 years later. Oral administration of HCA increased serum fasting GLP-1, to a  
40 greater extent than metformin, in healthy and diabetic mouse models. HCA upregulated  
41 GLP-1 secretion in intestinal enteroendocrine cells via simultaneously activating G-  
42 protein-coupled BA receptor, TGR5, and inhibiting farnesoid X receptor, a unique  
43 mechanism that is not found in other BA species.

44

## 45 INTRODUCTION

46 Bile acids (BAs) have long been regarded as digestive detergents for cholesterol  
47 elimination, but are emerging as important signaling molecules that regulate the  
48 metabolism of triglyceride, cholesterol, and glucose <sup>1,2</sup>, and thus, are critically involved in  
49 the development of type 2 diabetes mellitus <sup>3,4</sup>. Glucagon-like peptide-1 (GLP-1) is an  
50 incretin hormone that enhances insulin secretion and decreases blood glucose. The  
51 expression and secretion of GLP-1 in enteroendocrine L-cells is regulated by two BA  
52 receptors, i.e., cell membrane G-protein-coupled BA receptor TGR5 <sup>5,6</sup> and nuclear  
53 farnesoid X receptor (FXR) <sup>7</sup>, suggesting that BAs and BA analogs may be used to  
54 improve glucose homeostasis. In support of this view, altered BA profiles were found in  
55 patients who underwent bariatric surgery for weight and T2DM control <sup>8</sup>. Increases in the  
56 BA pool size and individual BA species occurred rapidly after the surgery, even before  
57 there was significant weight loss <sup>9,10</sup>.

58 The composition of the BA profile varies markedly among mammalian species. A  
59 recent study reported that hyocholic acid (HCA, also known as 3 $\alpha$ ,6 $\alpha$ ,7 $\alpha$ -trihydroxy-5 $\beta$ -  
60 cholanic acid, and gamma-muricholate) and its glycine- and taurine-conjugated  
61 derivatives constituted ~ 42 % of total BAs in pig plasma, but comprised only ~1 % in the  
62 plasma of human and rat <sup>11</sup>. Pigs are routinely raised on obesogenic diets and have little  
63 physical activity, which represent a typical diabetogenic condition for humans. However,  
64 pigs are resistant to the spontaneous development of T2DM, even after induction with  
65 high fat, high fructose and high carbohydrate diets <sup>12,13</sup>. Because of this metabolic  
66 feature, pigs have been used to study hypoglycemia <sup>14</sup>. We suspected that the distinct  
67 BA profile, i.e., the high abundance of HCA and its derivatives in pigs, may play a role in  
68 regulating glucose homeostasis leading to their exceptional resistance to metabolic  
69 disorders.

70 To test this hypothesis, we measured the concentrations of HCA species in the  
71 serum and feces of diabetic patients and healthy controls and evaluated the predictive  
72 value of HCA species for future metabolic outcome for patients. We then validated the  
73 effect of HCA species in three mouse models and one pig model. Finally, we assessed  
74 the effects of HCA species on GLP-1 expression and secretion in intestinal  
75 enteroendocrine L-cells, and the roles of TGR5 and FXR in HCA species-mediated GLP-  
76 1 upregulation. This study underscores a critical role of HCA species in maintaining  
77 glucose homeostasis in human and other mammalian species, and suggests potential  
78 pharmaceutical applications of this group of BAs.

79

## 80 **RESULTS**

### 81 **Lower levels of serum HCAs in diabetes**

82 To evaluate the association between HCA species and diabetes, we conducted a  
83 targeted serum BA profiling in a cohort consisting of 1,107 participants (610 men and  
84 497 women) selected from the Shanghai Obesity Study <sup>15</sup>. The participants were  
85 separated into three groups: healthy lean (HL, n=585), healthy overweight/obese (HO,  
86 n=419), and overweight/obese with newly diagnosed T2DM (OD, n=103). Key clinical  
87 metabolic markers were significantly different between any 2 of the 3 groups (Table S1).  
88 Although the 3 groups had similar total BA (TBA) levels in all, men, and women groups  
89 (Fig S1), total concentration of HCA species, i.e. the concentration summation of HCA,  
90 hyodeoxycholic acid (HDCA), glycohyodeoxycholic acid (GHDCA), and glycohyocholic  
91 acid (GHCA), was the highest in HL and lowest in OD (Fig S1). In addition, the HCA,  
92 HDCA, GHDCA, and GHCA concentrations (Figs S1, S2) were decreased in HO and  
93 even more so, in OD relative to HL. Pairwise Spearman correlation analysis showed  
94 that total and individual HCA species inversely correlated with fasting and post-load  
95 glucose, insulin levels and insulin resistance shown by HOMA-IR (Fig S3).

96 From HL to HO to OD, the participants had increasingly older age, higher body  
97 mass index (BMI), and a lower ratio of men/women (although the sex ratios were not  
98 significantly different among groups) (Table S1). To eliminate the confounding effects of  
99 age, sex, and BMI, we selected 103 older participants with higher BMI, and more women  
100 from the HL and HO groups to better match the 103 participants in the OD group. After  
101 this selection, all 3 groups had matched age and sex ratios while HO and OD also had  
102 matched BMI (Table S2). The 3 groups had similar TBA levels (Fig 1a) and gradually  
103 decreased levels of total (Fig 1b) and individual HCA species (Figs 1c, 1k-1n) after this  
104 selection, with the fold changes of HO/HL and OD/HL for total HCA species (0.75 and  
105 0.55, respectively), HCA (0.82 and 0.45), HDCA (0.81 and 0.47), GHCA (0.68 and 0.57),  
106 and GHDCA (0.72 and 0.60). HCA species remained inversely correlated with fasting  
107 and post-load levels of glucose and insulin as well as, insulin resistance after the  
108 selection (Figs 1d-1j). The results suggest that obesity (HO+OD vs. HL) and diabetes  
109 (OD vs. HO) were associated with lower concentrations of total and individual HCA  
110 species in serum.

111

### 112 **Development of diabetes associated with depleted HCA levels**

113 To confirm the findings above in a separate cohort, and to evaluate the association  
114 between fecal HCA and diabetes, we recruited a second cohort of 106 participants (44  
115 men and 62 women), which included 32 healthy, 34 pre-diabetic and 40 diabetic  
116 individuals. The HbA1c and fasting and post-load blood glucose levels of pre-diabetic  
117 and diabetic patients were significantly higher than those of healthy controls (Tables S3,  
118 4). No significant group differences were found in serum and fecal total BAs (Figs 2a, c).  
119 Compared with healthy controls, the pre-diabetic and diabetic groups had lower levels of  
120 total HCA species in both serum and feces, in the groups, all, men, and women (Figs 2b,  
121 d and Tables S5, 6). The group differences were greater in feces than in serum. As  
122 expected, individual HCA species showed similar group differences (Figs 2e-j). The  
123 concentrations of fecal GHCA and GHCA are not shown as they were below the  
124 detection limit. Total and individual HCA species in feces had stronger inverse  
125 correlations with fasting and post-load blood glucose levels than serum levels of HCA  
126 species (Figs 2k-m, adjusted for age, sex and BMI).

127

#### 128 **HCAs were predictors for metabolic outcome**

129 To evaluate the association between HCA species and future metabolic health, we  
130 selected 132 subjects (36 men and 96 women) from the Shanghai Diabetes Study<sup>16</sup>. All  
131 of them were metabolic healthy (MH, defined in the Method Section) at their enrollment.  
132 After 10 years, 86 participants became metabolically unhealthy (MU, defined in the  
133 Method Section), and 46 remained MH. At baseline, the future MU group were older, had  
134 higher BMI and more men than the future MH group (although group differences of sex  
135 ratio did not reach statistical significance), however, the major metabolic markers were  
136 similar between the two groups (Table S7). To eliminate the confounding effects of age,  
137 sex and BMI, we chose 46 younger participants with lower BMI and comprised of more  
138 women, from the MU group to match the 46 participants in the MH group (Table S8).  
139 When samples from all participants were considered, the concentrations of total BAs in  
140 serum were comparable between the MH and MU groups, but the concentrations of total  
141 and individual HCA species were significantly lower in the MU than the MH group (Fig  
142 S4 and Table S9). Age-, sex- and BMI-matched samples yielded similar results as all  
143 samples did (Figs 3a-f, Table S10), suggesting that the baseline differences of HCA  
144 species between MH and MU groups were independent of age, sex, and BMI. Binary  
145 logistic regression analysis of all samples showed that the association between HCA  
146 species and future MU outcome were (odds ratio (95 % CI) 0.89 (0.86, 0.93), 0.91 (0.87,

147 0.94), 0.90 (0.84, 0.96), 0.92 (0.85, 0.99), 0.52 (0.40, 0.69) and 0.90 (0.86, 0.94)) for  
148 total HCA species, HCA, GHCA, HDCA, and GHDCa, respectively ( $p < 0.05$  for all,  
149 adjusted for age, sex, and BMI) (Fig S5). The receiver operating characteristic (ROC)  
150 curve analysis showed that total HCA species (red line in Fig 3g) had the highest area  
151 under curve (AUC) of 0.83, and the AUCs of individual HCA species ranged from 0.63 to  
152 0.79, providing supporting evidence for using total and individual HCA species as  
153 predictors for future metabolic outcome.

154

### 155 **Gastric bypass surgery increased serum HCAs**

156 We further studied the changes of HCA species in diabetic patients after Roux-en-Y  
157 gastric bypass (RYGB) surgery. Thirty-eight obese diabetic patients who received RYGB  
158 were examined before and at 1, 3, 6, and 12 months post-surgery (Table S11). Serum  
159 concentrations of total BAs gradually increased after RYGB surgery, and became  
160 significantly higher than baseline at 12 months post-operation (Fig 3h). The  
161 concentrations of total and individual HCAs in the serum increased drastically 1 month  
162 after the surgery (FC = 2.52, 2.75, 3.86, 2.28, and 2.33 for total HCA species, HCA,  
163 HDCA, GHCA, and GHDCa, respectively) and maintained minor increases afterwards  
164 (Figs 3i-j, Table S12). Improvements of BMI, fasting and post-load blood glucose levels,  
165 HbA1c, and insulin resistance occurred throughout the 12 months (Fig 3k). ROC  
166 analysis showed that the AUCs of the 12-month changes of total HCA species, HCA,  
167 GHCA, HDCA, and GHDCa were 0.80, 0.70, 0.66, 0.76, and 0.72, respectively (Fig 3l),  
168 evidence for potential prediction capability for the metabolic outcome of RYGB surgery.

169

### 170 **HCAs regulated blood glucose and GLP-1 in animal models**

171 To understand the potential role of HCA species in regulating glucose homeostasis  
172 in pigs, we compared the BA profiles in the sera of pig ( $n=6$ , 3 males and 3 females),  
173 human (from the first cohort study,  $n=1,107$ , 610 men and 497 women), and mouse  
174 (wildtype C57BL/6J,  $n=10$ , 5 males and 5 females). Fig 4a shows that the HCA species  
175 accounted for the majority of BAs in the serum of pig ( $75.96 \pm 4.00\%$ ), but for only very  
176 small portions in those of human ( $4.99 \pm 0.14\%$ ) and mouse ( $3.11 \pm 0.12\%$ ). All 6 HCA  
177 species, i.e., HCA, GHCA, tauro-HCA (THCA), HDCA, GHDCa and tauro-HDCA  
178 (THDCa), were detected in pig serum, but only some of these HCA species were  
179 detected in human and mouse. Meanwhile, the fasting blood glucose level was the  
180 lowest in pig ( $4.4 \pm 0.1$  mmol/L), followed by human ( $5.5 \pm 0.0$  mmol/L) and mouse ( $5.3 \pm$

181 0.2 mmol/L) (Fig 4b), which was in the opposite order of the abundance of serum HCA  
182 species in these species.

183 We further treated the pigs with GW4064, a FXR agonist, via oral gavage at a dose  
184 of 10 mg/kg (twice with a 12 h interval), in an effort to suppress hepatic BA synthesis.  
185 This was done to answer the question whether GW4064 would reduce serum HCA  
186 levels in pigs and furthermore, whether HCA depletion would decrease circulatory GLP-1  
187 concentration and increase blood glucose levels. After GW4064 treatment, the  
188 concentration of HCA species in serum decreased by 60 % (Fig 4c, and Figs S6a-g).  
189 Meantime, the blood glucose levels increased by 25 % (Fig 4d) and that of serum GLP-1  
190 decreased by 72 % (Fig 4e). Blood glucose levels were also measured 15 and 35  
191 minutes after GW4064 treatment, the data and interpretation can be found in Figs S6h-i.

192 To investigate whether HCA species have direct impact on glucose homeostasis, we  
193 treated healthy C57BL/6J mice for 4 weeks with HCA (100 mg/kg/day), HDCA (100  
194 mg/kg/day), metformin (200 mg/kg/day), and 6 % sodium bicarbonate ( $\text{NaHCO}_3$ ) as  
195 vehicle control. Mice in metformin, HCA, and HDCA groups showed improved oral  
196 glucose tolerance at 4 weeks (Fig 4f). The hypoglycemic effect was more rapid with HCA  
197 species intervention (significant at 1 week) compared to metformin (significant at 4  
198 weeks) (Figs S7a-d). Moreover, mice treated with HCA and HDCA showed higher  
199 circulating GLP-1 levels (Fig 4g) and fasting insulin (Fig 4h) than metformin at 4 weeks.

200 We then investigated whether HCA could improve glucose homeostasis under  
201 obese and diabetic conditions in a high-fat diet-streptozotocin (HFD + STZ) induced  
202 diabetic and a db/db mouse model. For the HFD + STZ model, mice were treated with  
203 HCA (100 mg/kg/day), HDCA (100 mg/kg/day), metformin (200 mg/kg/day), and 6 %  
204  $\text{NaHCO}_3$  as vehicle control, respectively. At 4 weeks, mice treated with metformin, HCA,  
205 or HDCA showed significantly lower fasting blood glucose levels than controls (Fig 4i).  
206 Similarly, the hypoglycemic effect was more rapid with HCA or HDCA treatment  
207 compared to metformin (Figs S7e-h). Furthermore, mice treated with HCA or HDCA  
208 showed increased circulating GLP-1 levels (Fig 4j). In a db/db mouse model, mice were  
209 treated with HCA (100 mg/kg/day), metformin (200 mg/kg/day), and vehicle control. At 4  
210 weeks, db/db mice showed significantly lower fasting blood glucose levels in metformin  
211 and HCA treatment groups (Fig 4k, Figs S7j-m), higher circulating GLP-1 levels (Fig 4l)  
212 and higher fasting insulin in HCA group (Fig S7n), compared to controls.

213

214 **HCAs upregulated GLP-1 via TGR5 and FXR signaling**

215 We compared the responses of intestinal enteroendocrine STC-1 and NCI-H716  
216 cells<sup>17,18</sup> to HCA species and other BAs on GLP-1 transcription and protein expression.  
217 The results showed that no apparent GLP-1 upregulation using all BAs at 5  $\mu$ M (Figs  
218 S8a, b). When the concentration increased to 25  $\mu$ M, all of the BAs upregulated GLP-1  
219 transcription and protein expression (Figs S8c-e), among which, HCA species were most  
220 effective. At 50  $\mu$ M, HCA species upregulated GLP-1 transcription and protein  
221 expression significantly more than HCA species at 25  $\mu$ M (Figs 5a, b), while other BAs  
222 did not upregulate GLP-1 expression. These results showed the difference between HCA  
223 species and other BAs on regulating the GLP-1 expression, in that the effect of GLP-1  
224 stimulation was dose dependent with HCA species, while the effect was suppressed with  
225 other BAs at relatively high concentrations.

226 Two BA receptors, TGR5 and FXR, are involved in regulating the GLP-1 expression  
227 in enteroendocrine L-cells. We found that each HCA species significantly increased the  
228 level of GLP-1 secretion as well as CREB phosphorylation (S133) (p-CREB) (a marker  
229 of TGR5 activation) (Fig 5c, left panel of western-blot and bar chart; Figs S9a, b),  
230 compared to other BAs. However, GLP-1 and p-CREB expression levels were  
231 significantly decreased in TGR5 knockdown cells (Fig 5c, right panel of western-blot and  
232 bar chart, Fig S10a,b), suggesting that the upregulation of GLP-1 by HCA species was  
233 TGR5 dependent.

234 Our results showed that two FXR agonists, chenodeoxycholic acid (CDCA) and 5 $\beta$ -  
235 Cholanic acid (5 $\beta$ -CA)<sup>19,20</sup> increased nuclear translocation (a marker of FXR activation)  
236 of FXR, and such effect was inhibited by the co-treatment of HCA species (Fig 5d).  
237 Western-blot analysis of FXR translocation and SHP expression, one of the downstream  
238 proteins of FXR activation, verified the inhibitory effect of HCA species on FXR (Figs S9a,  
239 b). Interestingly, non-HCA BAs, at 25  $\mu$ M, promoted GLP-1 expression via TGR5  
240 activation while their FXR binding and activation was not strong. At higher  
241 concentrations (50  $\mu$ M), there was marked upregulation of FXR by non-HCA BAs (Figs  
242 S9a, b) but the GLP-1 production was suppressed.. Such observation was further  
243 verified in FXR knockdown cells, where GLP-1 transcription and protein expression was  
244 increased significantly with non-HCA BAs intervention in shFXR cells compared to  
245 control due to the loss of FXR. No obvious difference was observed between HCA and  
246 non-HCA treatments (Figs S11b-d). Previous studies have identified 5 $\beta$ -CA as both a  
247 FXR agonist and a TGR5 antagonist, and as expected, the upregulation of GLP-1 by  
248 HCA was abolished by 5 $\beta$ -CA co-treatment as shown by transcription (Fig S12a), ELISA



249 (Fig S12b), western blot (Fig S12c), and 2D and 3D IF staining (Figs S12d,e).

250 We also intended to understand whether the inhibition of FXR by HCA species  
251 directly regulated GLP-1 secretion independent of TGR5 signaling, or such inhibition  
252 also regulated TGR5 expression and subsequently regulated GLP-1 expression. The  
253 control and shFXR cells (Fig S11a) were exposed to HCA species and 4 other  
254 representative BAs, cholic acid (CA), CDCA, LCA, deoxycholic acid (DCA). FXR  
255 knockdown had no apparent effect on TGR5 and p-CREB expression (Fig S13),  
256 suggesting that the effect of TGR5 expression and activation by HCA species was not  
257 regulated by FXR. Taken together, in enteroendocrine L-cells, BAs induce GLP-1  
258 secretion through BA- TGR5 and FXR signaling. . More specifically, BA-TGR5 signaling  
259 promotes GLP-1 expression, whereas BA-FXR signaling inhibits GLP-1 expression. HCA  
260 species promoted GLP-1 expression and secretion through a unique mechanism that  
261 involved both action as an agonist for TGR5 and action as an antagonist for FXR,  
262 simultaneously.

263 To validate whether HCA species induced GLP-1 secretion depended on TGR5  
264 activation as well as FXR inhibition, we conducted *in vivo* studies for 4 weeks using 5 $\beta$ -  
265 CA (100 mg/kg/day, i.g.) to inhibit TGR5 and activate FXR simultaneously, as well as  
266 Fexaramine (FEX; 100 mg/kg/day, i.g.) to activate only intestinal FXR. The results (Fig  
267 6a) showed that 5 $\beta$ -CA intervention significantly inhibited HCA-induced GLP-1 secretion.  
268 Such inhibition was not as strong with FEX treatment as with 5 $\beta$ -CA treatment, because  
269 the presence of HCA-TGR5 signaling was still significant. Meanwhile, HCA induced  
270 insulin secretion and blood glucose reduction was reversed by 5 $\beta$ -CA, but was  
271 attenuated, to some extent, by FEX (Figs 6b, c).

272 We further determined whether HCA induced GLP-1 secretion was an essential  
273 pathway involved in HCA regulated glucose metabolism. We inhibited the GLP-1  
274 receptor in a mouse model using a GLP-1 receptor antagonist, Exendin-3(9-39) amide  
275 (Exendin; 25 nmol/kg/day, i.p.) for 4 weeks, HCA induced insulin secretion and  
276 hypoglycemic effects were abolished (Figs 6d,e).

277

## 278 **DISCUSSION**

279 Among the HCA species, HCA and HDCA were first isolated by Windaus from pig  
280 bile <sup>22,23</sup>. The biosynthetic pathways and physiological levels of HCA and HDCA are  
281 different among mammalian species. Synthesis of HCA and HDCA in humans is not fully  
282 understood. Early *in vitro* data demonstrated that HDCA can be synthesized from TLCA

283 and LCA via 6 $\alpha$ -hydroxylation in human liver microsomes<sup>24,25</sup>. This pathway was later  
284 confirmed<sup>26</sup> and attributed to the function of CYP3A4<sup>27,28</sup>. A study from the same group  
285 reported that HCA can also be synthesized from CDCA through the same CYP3A4-  
286 mediated 6 $\alpha$ -hydroxylation pathway<sup>1,29</sup>. Furthermore, HCA and HDCA can also be  
287 synthesized from CDCA via hepatic CYP3A1 in combination with gut microbial  
288 epimerase enzymes. In rats, HDCA can be synthesized via bacterial biotransformation of  
289  $\beta$ -muricholic acid<sup>30</sup>, or synthesized from LCA by hepatic enzymes that convert LCA to  
290 3 $\alpha$ ,6 $\beta$ -dihydroxy cholanoic acid that can be further oxidized by gut bacteria to 3 $\alpha$ -  
291 hydroxy-6-keto cholanoic acid, and then reduced to HDCA<sup>31</sup>.

292 As a key incretin, GLP-1 is produced and secreted by the intestinal enteroendocrine  
293 cells. Our *in vitro* data showed that HCA species upregulated GLP-1 gene and protein  
294 expression and secretion in intestinal enteroendocrine NCI-H716 and STC-1 cells more  
295 effectively than other BA species tested. This was achieved through the simultaneous  
296 activation of TGR5 and inhibition FXR by unique interactive signaling of HCA species  
297 that has not been observed for other BA species. Our animal studies also showed  
298 simultaneous changes in GLP-1 and glucose levels in the blood following HCA species  
299 treatment. The effect of HCA species on blood glucose regulation was more potent than  
300 the antidiabetic agent, metformin. Therefore, the regulatory effect of HCA species on  
301 glucose homeostasis is mainly mediated through promotion of intestinal secretion of  
302 GLP-1.

303 An interesting finding in our study was that although all of the BAs including HCA  
304 species have the effect on stimulating GLP-1 secretion, the dose effects were different.  
305 At lower concentrations (25  $\mu$ M), all of the BAs promoted GLP-1 secretion. However,  
306 HCA species upregulate GLP-1 secretion in a dose-dependent manner, while other BA  
307 species failed to upregulate GLP-1 secretion at relatively higher concentrations (50  $\mu$ M).  
308 Such a unique feature of HCA species suggested that HCA and derivatives could be  
309 applied with sufficiently high concentrations (pharmacological levels) in maintaining  
310 glucose homeostasis, thus having great potential for therapeutic applications.

311 In clinical studies, T2DM is inherently associated with obesity and aging<sup>32</sup>, so we  
312 tried to eliminate the confounding effects of BMI and age when evaluating the role of  
313 HCA species in T2DM. By matching age and/or BMI between the groups in comparison,  
314 we demonstrated that HCA species had direct correlations with glycemic markers and  
315 future metabolic outcome. These results provide evidence that HCA species play critical

316 roles in regulating glucose homeostasis and are protective against the development of  
317 T2DM in humans.

318 We also showed that, compared with healthy controls, pre-diabetic and diabetic  
319 patients had ~27 % lower serum levels of HCA species, but strikingly ~57 % lower HCA  
320 species in feces, although these patients had similar levels of total BAs in feces as  
321 controls. Notably the pre-diabetic and diabetic patients had higher BMIs than the healthy  
322 controls, which suggest that they may also have altered gut microbiota<sup>33</sup>. Intestinal  
323 microbiota are known to play a critical role in BA metabolism<sup>34-36</sup>. Obesity and/or  
324 diabetes-associated changes in gut microbiota may inhibit the generation or facilitate the  
325 metabolism of HCA species, which, in turn, could lead to their depletion in feces. We  
326 further showed that fecal HCA species had stronger inverse correlations with glycemic  
327 markers than serum HCA species after adjusting for age, sex, and BMI, suggesting that  
328 the intestinal track is a critical site for HCA-mediated glycemic regulation.

329 RYGB surgery is considered a rapid resolution of T2DM. Both HCA and GHCA were  
330 found significantly increased after RYGB<sup>11</sup>. We found that in addition to HCA and GHCA,  
331 HDCA and GHDCa were also increased drastically after RYGB; and among all BAs, the  
332 increases in HCA species were the most pronounced and consistent (Table S12). Our  
333 results further highlighted the critical role of HCA in glucose regulation following bariatric  
334 surgery and their predictive value for the post-operation metabolic outcome.

335

## 336 **CONCLUSION**

337 The composition of the BA profile especially HCA species varies markedly among  
338 mammalian species. We show in this study that obesity and diabetes were closely  
339 associated with significant lower levels of HCA species in serum. Furthermore, the  
340 concentrations of HCA species in both serum and feces were closely correlated with  
341 glycemic markers and were strong predictors of future metabolic outcome in apparently  
342 healthy individuals. HCA species were shown to upregulate the gene transcription,  
343 protein expression and secretion of GLP-1 in both intestinal enteroendocrine NCI-H716  
344 and STC-1 cells to a significantly greater extent than other BA species. This action was  
345 mediated through simultaneous activation of TGR5 and inhibition of FXR. Taken together,  
346 our results provide strong supporting evidence that HCA species are protective against  
347 the development of diabetes in mammals and have the potential to be used as a  
348 treatment for type 2 diabetes. Future research is warranted to further improve our

349 knowledge on the correlations of HCA and gut microbiota in an effort to identify possible  
350 probiotic treatment possibilities.

351

## 352 **METHODS**

### 353 **Human experiments**

#### 354 *Human study 1: cross sectional study 1*

355 A total of 1,107 fasting serum samples obtained from 585 healthy lean (329 men  
356 and 256 women), 419 healthy overweight/obese (229 men and 190 women) and 103  
357 overweight/obese diabetic (52 men and 51 women) participants were selected from the  
358 Shanghai Obesity Study <sup>15</sup>. Individuals were excluded if they had chronic inflammatory  
359 disease, cardiopulmonary, renal or liver disease, active malignancy, or were taking any  
360 medication (including weight loss or psychotropic medication).

361

#### 362 *Human study 2: cross sectional study 2*

363 A group of 106 subjects including 32 healthy controls (12 men and 20 women), 34  
364 pre-diabetic individuals (12 men and 22 women) and 40 diabetic patients (20 men and  
365 20 women) were recruited for this study. The exclusion criteria were the same as in  
366 human study 1. Fasting sera of all the participants and fecal samples of 91 participants  
367 (26 healthy controls, 30 pre-diabetes and 35 diabetic patients) were collected and stored  
368 for later analysis.

369

#### 370 *Human study 3: 10-year longitudinal study*

371 A group of 132 subjects (36 men and 96 women) were selected from the Shanghai  
372 Diabetes Study, which was intended to assess the prevalence of diabetes and diabetes-  
373 associated metabolic disorders in urban Shanghai <sup>16</sup>. All 132 subjects were metabolically  
374 healthy at baseline (year 2000-2001). Ten years later (year 2010-2011), 86 participants  
375 (26 men and 60 women) became metabolically unhealthy (future metabolically unhealthy)  
376 and 46 (10 men and 36 women) remained healthy (future metabolically healthy). Fasting  
377 serum samples of the 132 participants at baseline were collected and stored for future  
378 analysis.

379

#### 380 *Human study 4: Gastric bypass surgery intervention study*

381 A total of 38 obese diabetic patients who received Roux-en-Y gastric bypass surgery  
382 were enrolled in the study <sup>10</sup>. Any patient with a history of open abdominal surgery, a

383 serious disease (such as heart or lung insufficiency) that was incompatible with surgery,  
384 an acute type 2 diabetes complication, severe alcohol or drug dependency, a mental  
385 disorder, type 1 diabetes, secondary diabetes, an unstable psychiatric illness, or who  
386 was at a relatively high surgical risk (such as a patient with an active ulcer) was  
387 excluded. The fasting serum specimens of these subjects were collected and stored for  
388 future analysis before (baseline) and 1, 3, 6, and 12 months after the surgery.

389

#### 390 *Clinical measurements*

391 Fasting and 2 h postprandial plasma glucose and insulin levels, serum lipid profiles  
392 (total cholesterol TC, triglyceride TG, high-density lipoprotein-cholesterol HDL, low-  
393 density lipoprotein-cholesterol LDL), blood pressure (systolic and diastolic blood  
394 pressure SP and DP), waist circumference, BMI, liver and kidney function tests were  
395 determined as previously described<sup>38</sup>.

396

#### 397 *Definitions of lean, overweight/obesity, pre-diabetes, diabetes, metabolically healthy and* 398 *unhealthy*

399 Individuals with BMI < 25 kg/m<sup>2</sup> were considered lean and those with BMI ≥ 25 were  
400 classified as overweight/obese. Individuals with 6.1 mmol/L ≤ fasting blood glucose < 7.0  
401 mmol/L or 7.8 mmol/L ≤ oral glucose tolerance test (OGTT) (2 h) < 11.1 mmol/L were  
402 classified as pre-diabetic. Subjects with fasting blood glucose ≥ 7.0 mmol/L and/or OGTT  
403 (2 h) ≥ 11.1 mmol/L were classified as diabetic. Subjects were considered “metabolically  
404 healthy” if they met all of the following criteria: fasting blood glucose < 6.1 mmol/L,  
405 OGTT (2 h) < 7.8 mmol/L and no previous history of diabetes; SBP/DBP < 140/90 mmHg  
406 and no previous history of high blood pressure; fasting plasma TG < 1.7 mmol/L and  
407 fasting plasma HDL ≥ 0.9 mmol/L (men) or ≥ 1.0 mmol/L (women), and no previous  
408 history of high cholesterol (TC < 5.18 mmol/L); no history of cardiovascular or endocrine  
409 disease<sup>39</sup>. Those who failed to meet all criteria above were classified as “metabolically  
410 unhealthy”.

411

#### 412 *Sample collection*

413 All human samples were collected and stored following the standard operating  
414 protocol of the hospital. Briefly, fasting venous blood samples were obtained before 10  
415 AM and were centrifuged immediately. The serum was removed from the cells, divided  
416 into aliquots and delivered on dry ice to the study laboratory. Wet fecal samples were

417 collected by the participants (single collection), frozen within 30 min in a sterilized tube  
418 and brought to the laboratory immediately. All samples were stored in a -80°C freezer  
419 until analysis.

420

## 421 **Animal experiments**

422 All animal studies were performed following the national legislation and was  
423 approved by the Institutional Animal Care and Use Committee at the Center for  
424 Laboratory Animals, Shanghai Jiao Tong University Affiliated Sixth People's Hospital  
425 (Shanghai, China) and China Agricultural University (Beijing, China).

426 The pig study was conducted in the Metabolism Laboratory of the National Feed  
427 Engineering Technology Research Center (Fengning, Hebei Province, China). Six  
428 crossbred growing pigs (Duroc x Landrace x Yorkshire, weighing around 25 kg) were  
429 used in this experiment. The pigs were housed individually in stainless steel metabolism  
430 cages (1.4 x 0.7 x 0.6 m) equipped with a feeder and a nipple drinker. The crates were  
431 located in three environmentally controlled rooms with the temperature maintained at 22-  
432 24 °C. The pigs were allowed a 10-day period to adapt to the metabolism crates and the  
433 environment of the room, and were fed commercial corn-soybean meal based diets.

434 The C57BL/6J mice (male, 6 weeks old) were purchased from Shanghai Laboratory  
435 Animal Co Ltd. (Shanghai, China), and the db/db mice inbred on BKS background (male,  
436 8 weeks old) were purchased from Model Animal Research Center of Nanjing University  
437 (Nanjing, China). The mouse studies were conducted at the Center for Laboratory  
438 Animals, Shanghai Jiao Tong University Affiliated Sixth People's Hospital (Shanghai,  
439 China) after one week of acclimatization. All experimental mice were housed in specific-  
440 pathogen-free (SPF) environments under a controlled condition of 12 h light/12 h dark  
441 cycle at 20-22 °C and 45 ± 5 % humidity, with free access to purified rodent diet and  
442 ultrapure water. The body weights and the consumption of food and water were  
443 measured weekly for the duration of the experiments. The blood glucose levels were  
444 measured each week, and OGTT was carried out as described in the results. At the end  
445 of each experiment, the retro-orbital blood was collected before sacrifice to measure  
446 serum insulin, and GLP-1 concentrations for all of the mice. All samples were stored in a  
447 -80 °C freezer until analysis.

448

### 449 *Animal experiment 1: GW4064 treatment in pigs*

450 Six pigs including 3 males and 3 females were used in this experiment. All the pigs

451 were orally administered GW4064 (Hanxiang Corp.) at a dose of 10 mg/kg. The  
452 administration was carried out twice with a 12 h interval between doses. Blood samples  
453 were collected through a catheter embedded in the precaval vein 15 min, 35 min, 60 min,  
454 and 24 h after the second GW4064 administration for BA, blood glucose, and GLP-1  
455 measurements. All samples were stored in a -80°C freezer until analysis.

456

457 *Animal experiment 2: HCA species oral administration in C57BL/6J mice*

458 Twenty C57BL/6J wild type mice were divided into four groups and were orally  
459 administrated with the following agents for 28 days: 1) control group: mice (n = 5) were  
460 administered with control vehicle, 6 % NaHCO<sub>3</sub> (S6014, Sigma-Aldrich); 2) metformin  
461 group: mice (n = 5) were administered with metformin (D150959, Sigma-Aldrich) at a  
462 daily dose of 200 mg/kg/day; 3) HCA group: mice (n = 5) were administered with HCA  
463 (700159P, Sigma-Aldrich) at a daily dose of 100 mg/kg/day; 4) HDCA group: mice (n = 5)  
464 were administered with HDCA (H3878, Sigma-Aldrich) at a daily dose of 100 mg/kg/day.

465

466 *Animal experiment 3: HCA species oral administration in HFD+STZ mice*

467 Forty C57BL/6J mice were placed on a high-fat diet (HFD: 60 % kcal from fat;  
468 D12492, Research Diets). After 12 weeks of HFD, mice were fasted for 5 h and then  
469 injected with a single dose of streptozotocin (STZ; V900890, Sigma-Aldrich) (75 mg/kg  
470 i.p.) as a freshly prepared solution in 0.1 mmol/L sodium citrate (S4641, Sigma-Aldrich),  
471 pH 5.5. After 72 h post-injection, only STZ-treated mice exhibiting a fasting glucose level  
472  $\geq 11.1$  mmol/L were used in the study (n = 32). Thirty-two HFD+STZ mice were divided  
473 into four groups and were orally administrated with the following agents for 28 days: 1)  
474 control group: mice (n = 8) were administered with control vehicle, 6 % NaHCO<sub>3</sub>; 2)  
475 metformin group: mice (n = 8) were administered with metformin at a daily dose of 200  
476 mg/kg/day; 3) HCA group: mice (n = 8) were administered with HCA at a daily dose of  
477 100 mg/kg/day; 4) HDCA group: mice (n = 8) were administered with HDCA at a daily  
478 dose of 100 mg/kg/day.

479

480 *Animal experiment 4: HCA oral administration in db/db mice*

481 Twenty-four db/db mice were divided into three groups and were orally  
482 administrated with the following agents for 28 days: 1) control group: mice (n = 8) were  
483 administered with control vehicle, 6 % NaHCO<sub>3</sub>; 2) metformin group: mice (n = 8) were  
484 administered with metformin at a daily dose of 200 mg/kg/day; 3) HCA group: mice (n =

485 8) were administered with HCA at a daily dose of 100 mg/kg/day.

486

487 *Animal experiment 5: TGR5 antagonist, FXR agonist, and GLP-1 receptor antagonist*  
488 *administration in mice*

489 Forty C57BL/6J mice were divided into eight groups and were administrated with the  
490 following agents for 28 days: 1) control group: mice (n = 5) were administered with  
491 control vehicle, 6 % NaHCO<sub>3</sub> (i.g.); 2) HCA group: mice (n = 5) were administered with  
492 HCA (100 mg/kg/day, i.g.); 3) 5 $\beta$ -CA group: mice (n = 5) were administered with control  
493 vehicle, 6 % NaHCO<sub>3</sub> (i.g.), and 5 $\beta$ -CA (C7628, Sigma-Aldrich) in 0.5 % Sodium  
494 Carboxymethyl Cellulose (CMC-Na; 419338, Sigma-Aldrich) (100 mg/kg/day, i.g.); 4)  
495 HCA+5 $\beta$ -CA group: mice (n = 5) were administered with HCA (100 mg/kg/day, i.g.), and  
496 5 $\beta$ -CA in 0.5 % CMC-Na (100 mg/kg/day, i.g.); 5) FEX group: mice (n = 5) were  
497 administered with control vehicle, 6 % NaHCO<sub>3</sub> (i.g.), and FEX (Hanxiang Corp.) in 0.5 %  
498 CMC-Na (100 mg/kg/day, i.g.); 6) HCA+FEX group: mice (n = 5) were administered with  
499 HCA (100 mg/kg/day, i.g.), and FEX in 0.5 % CMC-Na (100 mg/kg/day, i.g.). 7) Exendin  
500 group: mice (n = 5) were administered with control vehicle, 6 % NaHCO<sub>3</sub> (i.g.), and  
501 Exendin in saline (25nmol/kg/day, i.p.); 8) HCA+ Exendin group: mice (n = 5) were  
502 administered with HCA (100 mg/kg/day, i.g.), and Exendin (2081, R&D Systems) in  
503 saline (25nmol/kg/day, i.p.).

504

505 *Fasting blood glucose measurement and OGTT*

506 Fasting blood glucose measurement and OGTT was carried out in mice after  
507 overnight fasting. The glucose levels of tail vein blood samples were analyzed using a  
508 glucose analyzer (OneTouch Ultra, Lifescan, Johnson&Johnson, Milpitas, CA). In OGTT,  
509 a glucose solution (1.5 g/kg) was orally administered to each mouse, and samples were  
510 analyzed for glucose level before (0 min) and at 15min, 30 min, 60 min, and 120 min  
511 after the oral glucose load.

512

513 *Serum GLP-1 and insulin measurement*

514 Blood samples were collected and centrifuged at 3,000 x g, 4 °C, for 10 min for  
515 serum collection. For GLP-1 analysis, dipeptidyl peptidase IV inhibitor (10  $\mu$ L/mL;  
516 Millipore Corp, Missouri) was added to the blood before serum collection. High sensitivity  
517 GLP-1 active chemiluminescent ELISA kit (Millipore Corp, Missouri) and high sensitive  
518 mouse insulin immunoassay ELISA kit (ImmunoDiagnostics Limited, Hong Kong) were



519 used for GLP-1 and insulin measurement, respectively.

520

## 521 **Statistical analysis**

522 The BA profile raw data acquired using UPLC-TQ/MS were processed and  
523 quantified using TargetLynx software (Waters Corp., Milford, MA). Manual checking and  
524 correction were carried out in order to ensure data quality. The HCA species  
525 concentration was calculated by combining the concentrations of HCA, HDCA, GHCA,  
526 GHCA, THDCA, and THCA. Non-parametric Mann Whitney U test and Wilcoxon  
527 signed-rank test were carried out for comparison of unpaired and paired samples in the  
528 human studies. In animal and cell studies, parametric unpaired t-test and paired t test  
529 were applied to compare the unpaired and paired samples, respectively. Spearman's  
530 rank correlation coefficients were calculated to examine the association of BAs and  
531 typical clinical measurements. ROC (Receiver Operation Curve) analysis was used to  
532 test the sensitivity and specificity of total and individual HCA species in group separation.  
533 Logistic regression models were constructed to assess the predictive potentials of  
534 individual and combined HCA species on future metabolic health. For human studies,  
535 the p values were corrected by FDR. For human, animal and cell studies,  $p < 0.05$  were  
536 considered statistically significant (two tailed). SPSS (V19, IBM, USA), GraphPad Prism  
537 (6.0, Graphpad, USA), and MATLAB (2014a, MathWorks, USA) were used for statistical  
538 analyses and graphic generation. Analyte levels in tables and figures were presented as  
539 mean  $\pm$  S.E. or mean  $\pm$  S.D.

540 Materials and methods on cell studies and quantitative analysis of BAs are provided  
541 in supplementary information.

542

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669



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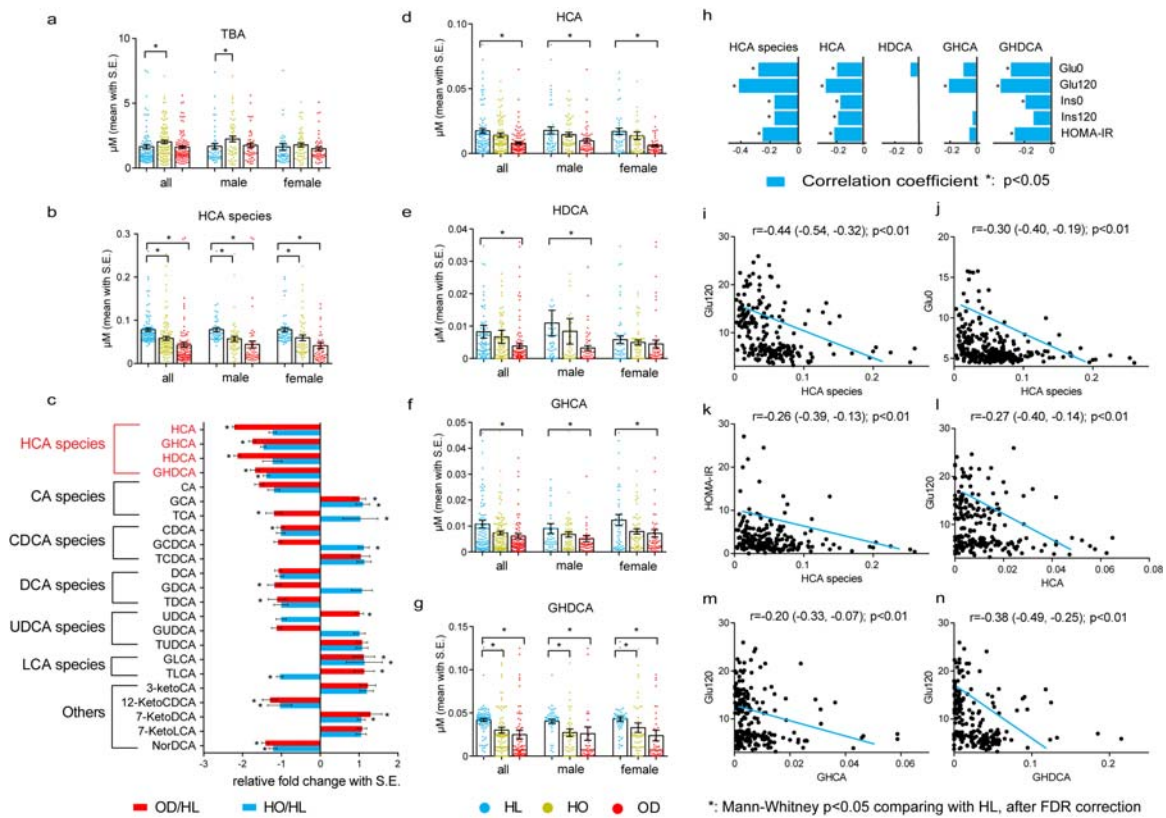
675

676 **Author Contributions** W.J. conceptualized the study and designed the research. X.Z.  
677 and T.C. performed the data preprocessing and statistical analysis. W.P.J was the leader  
678 of the cohort studies and together with Y.B. provided biospecimens from their studies.  
679 W.J., X.Z., T.C., and R.J. drafted the manuscript. W.J., C.R., J.P., X.Z. critically revised  
680 the manuscript. A.Z., C.R., J.T., G.X., A.L. and W.Z. provided valuable suggestions in  
681 data analysis and interpretation. X.M., Y.B., C.W., H.Y., M.J., A.L., and Y.Y. were  
682 responsible for human sample collection and explanation. X.Z., A.H., Y.Z., M.W., M.L.,  
683 D.L., X.H., F.H., Y.Y., J.L., Q.Z., K.G., S.L., S.W., and Y.L. were responsible for animal  
684 sample collection. A.Z., X.Z. and F.H. were responsible for sample preparation and  
685 analysis. R.J., M.W. and Q.Z. were responsible for cell studies.

686

687 **Author Information** The authors declare that they have no conflicts of interest.

688

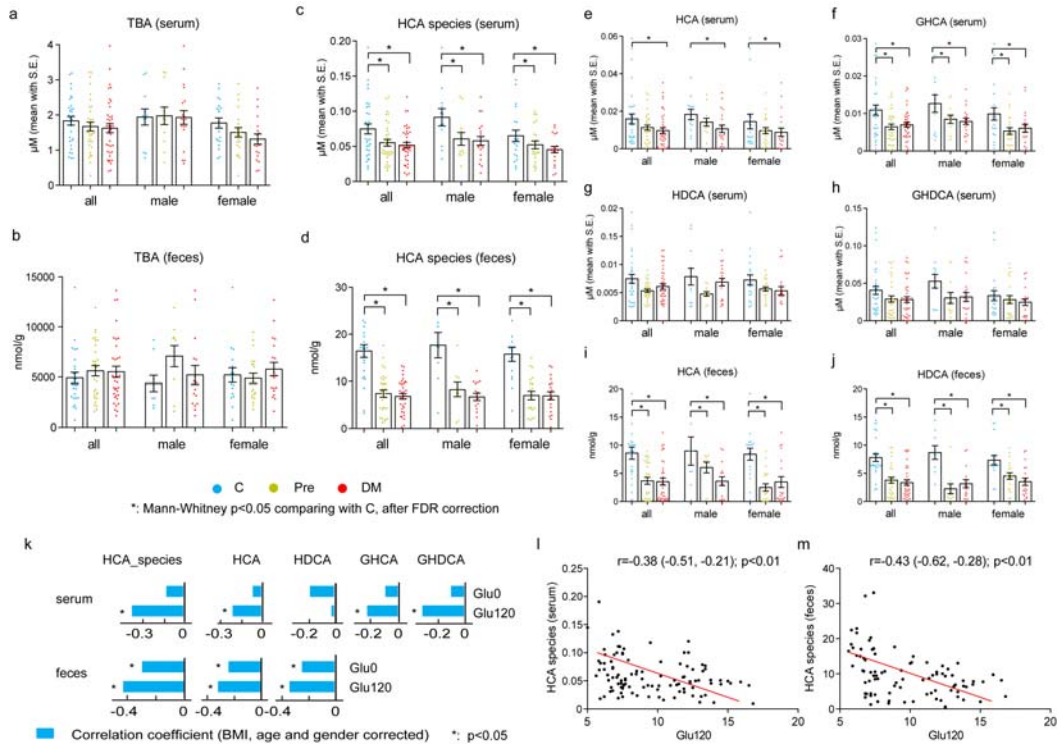


689

690 **Figure 1** Performances of HCA species in the first cross-sectional study.

691 (a) Total bile acid (TBA) and (b) HCA species levels (mean with S.E.) in matched healthy  
 692 lean (HL,  $n=103$  from 585), healthy overweight/obese (HO) ( $n=103$  from 419) and  
 693 overweight/obese with type 2 diabetes (OD) ( $n=103$ ) groups. \* Corrected (FDR=0.05)  
 694 Mann-Whitney  $p < 0.05$  when compared with HL. (c) Fold of change (mean with S.E.) of  
 695 23 BAs in HO and OD groups relative to HL group. \* FDR corrected Mann-Whitney  
 696  $p < 0.05$  when compared with HL. Levels of HCA species (HCA, HDCA, GHCA and  
 697 GHDCA, highlighted in red) were consistently lower in HO and OD groups compared  
 698 with HL group. (d -g) Group differences (mean with S.E.) of individual HCA species  
 699 based on matched all ( $n=309$ ), male ( $n=156$ ) and female ( $n=153$ ) samples. \* FDR  
 700 corrected Mann-Whitney  $p < 0.05$  when compared with HL. (h) Correlation coefficients of  
 701 total and individual HCA species with representative metabolic markers (matched  
 702 samples). \*  $p < 0.05$ . (i -n) Scatter plots of total or individual HCA species versus  
 703 representative metabolic markers.

704



705

706 **Figure 2** Performance of HCA species in the second cross-sectional study.

707 (a -d) Total bile acids (TBA) and total HCA species in serum and feces in healthy control

708 (C,  $n=32$ ), pre-diabetes (Pre,  $n=34$ ) and diabetes (DM,  $n=40$ ) groups. (e -j) Individual

709 HCA species in the 3 groups in all ( $n=106$ ), male ( $n=44$ ) and female ( $n=62$ ) samples.

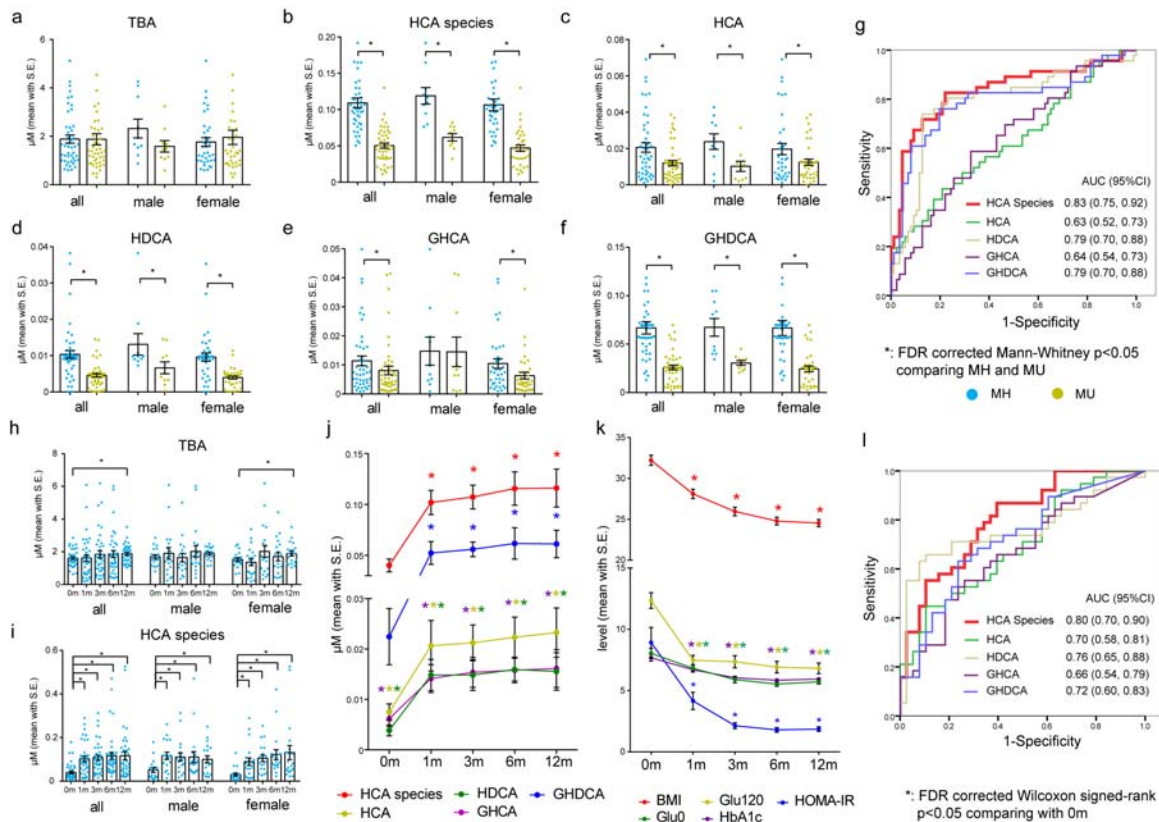
710 Mean with S.E. \* FDR corrected Mann-Whitney  $p < 0.05$  when compared with C. (k)

711 Correlation coefficients of total and individual HCA species in serum and feces with

712 glycemic markers. \*  $p < 0.05$ , adjusted for BMI, age and sex. (l, m) Scatter plots of total

713 HCA species in serum or feces versus a representative glycemic marker.

714

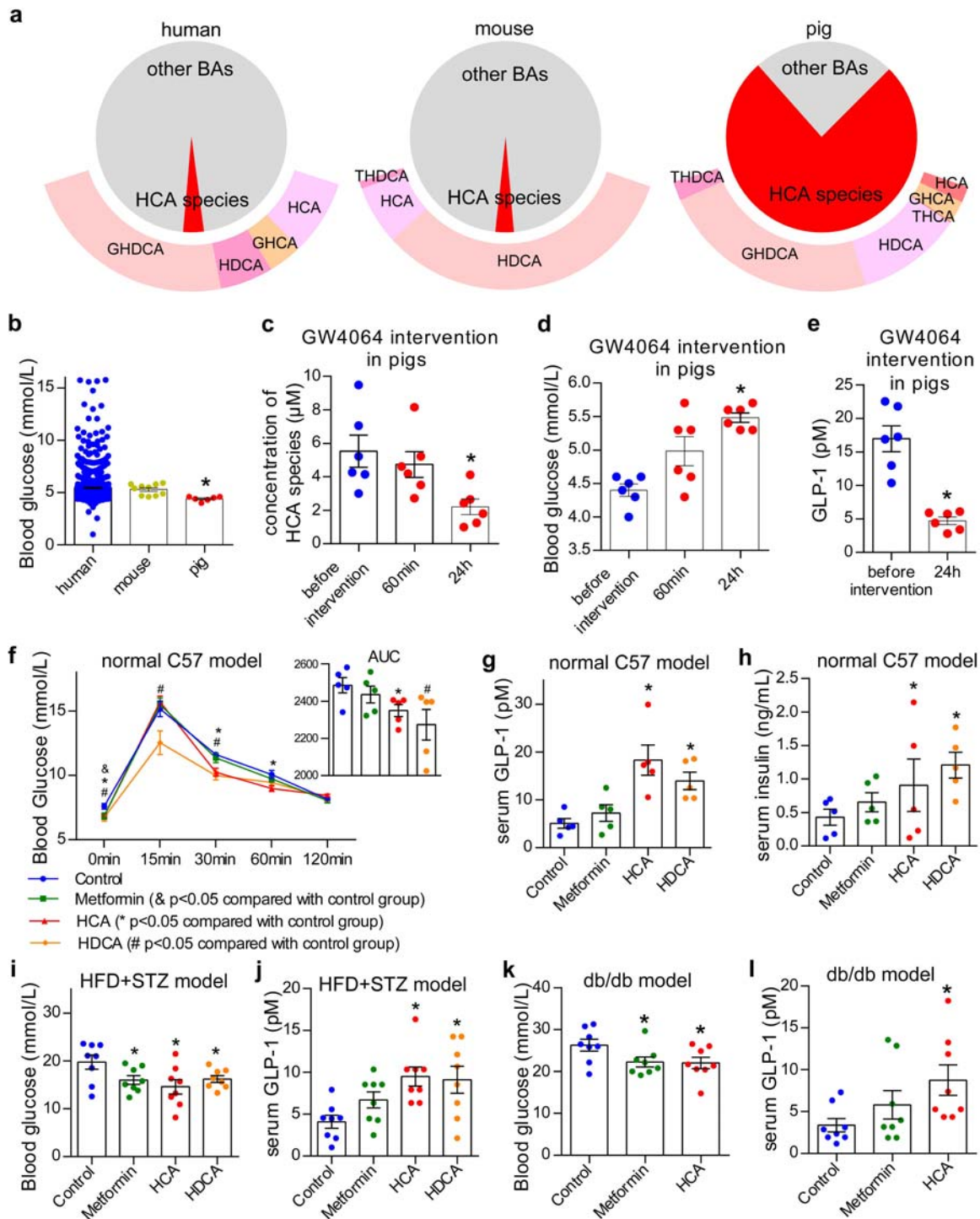


715

716 **Figure 3** Performances of HCA species in the 10-year longitudinal study and surgery-  
 717 induced changes of HCA species in the gastric bypass surgery intervention study.

718 (a -f) Total bile acids (TBA), total and individual HCA species in serum of age and BMI  
 719 matched all (n=92), male (n=20) and female (n=72) individuals in future metabolically  
 720 healthy (MH) and metabolically unhealthy (MU) groups. Mean with S.E., \* FDR corrected  
 721 Mann-Whitney  $p < 0.05$  when comparing MH and MU. (g) Receiver operating  
 722 characteristic (ROC) analyses of total and individual HCA species for the metabolic  
 723 health longitudinal study (all samples). (h, i) TBA and total HCA species serum  
 724 concentrations before and after gastric bypass surgery in 38 obese and diabetic patients  
 725 (j) Serum concentrations of total and individual HCA species before and after surgery (k)  
 726 BMI and glycemic markers before and after surgery. \* FDR corrected Wilcoxon signed-  
 727 rank test  $p < 0.05$  when compared with baseline (0m). (l) ROC analysis of the changes  
 728 (12 months vs. baseline) of total and individual HCA species.

729

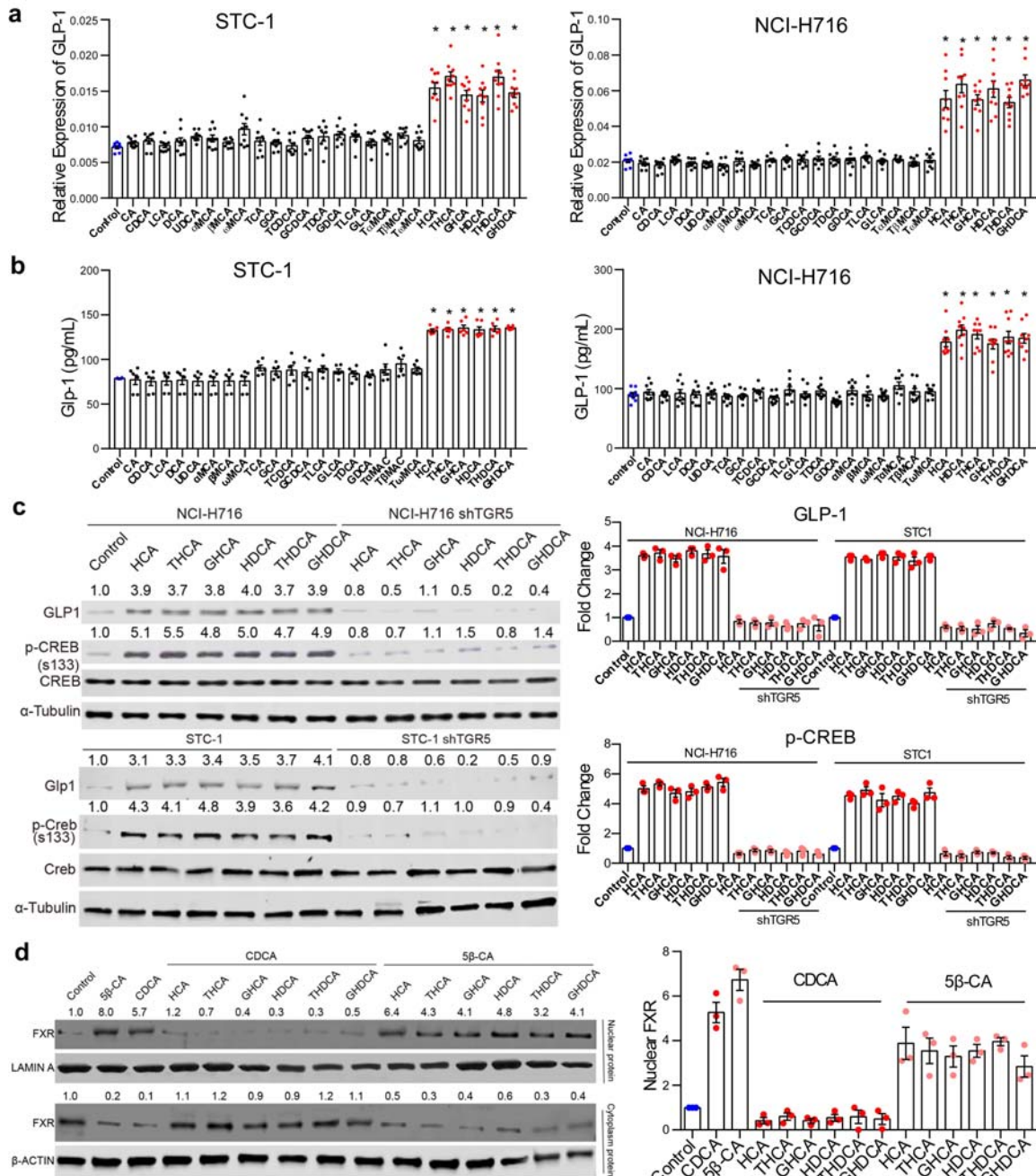


730

731 **Figure 4** Effects of HCA species on the levels of blood glucose, GLP-1 and insulin in  
 732 animal models.

733 (a) BA composition in the serum of humans, C57BL/6J mice, and pigs. The pie charts  
 734 are composed of HCA species (red) and other BAs (grey). The outer rings are composed  
 735 of detected individual HCA species. (b) The fasting blood glucose levels of humans,

736 mice, and pigs. (c) Serum concentrations of total HCA species and (d) blood glucose  
737 before and 60 min and 24h after GW4064 oral administration (10 mg/kg, twice with a 12  
738 h interval) in pigs. (e) Serum GLP-1 level before and 24 h after GW4064 treatment in  
739 pigs. (f) Blood glucose levels and AUC of OGTT, (g) serum GLP-1 levels, and (h) insulin  
740 levels of normal C57BL/6J mouse models treated with metformin (200 mg/kg/day), HCA  
741 (100 mg/kg/day), HDCA (100 mg/kg/day) and vehicle control for four weeks. (i) Blood  
742 glucose levels, and (j) serum GLP-1 levels of HFD+STZ mouse models treated with  
743 metformin (200 mg/kg/day), HCA (100 mg/kg/day), HDCA (100 mg/kg/day) and vehicle  
744 control for four weeks. (k) Blood glucose levels and (l) serum GLP-1 levels (g) of db/db  
745 mouse models treated with metformin (200 mg/kg/day), HCA (100 mg/kg/day) and  
746 vehicle control for four weeks. Mean with S.E. \*  $p < 0.05$  when compared with control  
747 group using unpaired t-test, except compared with before intervention group in pig model  
748 using paired t-test.  
749



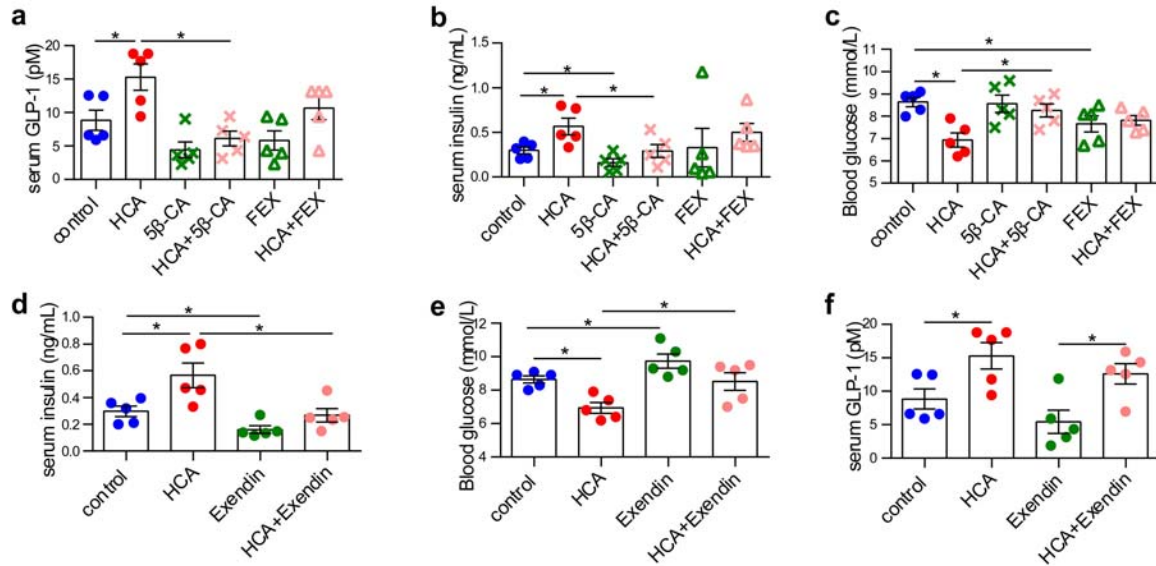
750

751 **Figure 5** HCA species more effectively upregulated GLP-1 protein expression in  
 752 enteroendocrine cell lines than other bile acids through effects of TGR5 and FXR. NCI-  
 753 H716 and STC-1 cells were treated with all 6 HCA species and 19 other BAs, each at 50  
 754  $\mu$ M for 48 h.

755 (a) The GLP-1 transcription was measured using Real-time PCR. (b) The GLP-1  
 756 secretion was measured using ELISA. (c) NCI-H716 and STC-1 as well as their TGR5  
 757 knockdown cells were treated with 6 HCA species for 24h, and intracellular GLP-1, p-

758 CREB and total CREB were determined using western blot. (d) FXR protein  
759 concentration in nuclear and cytosolic fractions of NCI-H716 cells treated with 50  $\mu$ M of  
760 CDCA or 5 $\beta$ -CA for 24 hours, with or without the presence of HCA species, each at 50  
761  $\mu$ M. Representative images are shown, and data were obtained from 3 independent  
762 experiments. Mean with S.E. \*  $p < 0.05$  when compared with control using unpaired t-test.  
763





764

765 **Figure 6** Effect of HCA on the levels of GLP-1, insulin, and blood glucose with TGR5,  
766 FXR and GLP-1 receptor intervention.

767 The levels of (a) serum GLP-1, (b) serum insulin, and (c) blood glucose of normal  
768 C57BL/6J mice in control, HCA (100 mg/kg/day, i.g.), 5 $\beta$ -CA (TGR5 antagonist and FXR  
769 agonist; 100 mg/kg/day, i.g.), HCA+5 $\beta$ -CA, FEX (FXR agonist; 100 mg/kg/day, i.g.), and  
770 HCA+FEX groups at 4 weeks. The serum levels at 4 weeks of (d) insulin, (e) glucose,  
771 and (f) GLP-1 of normal C57BL/6J mice in control, HCA, Exendin-3(9-39) amide  
772 (Exendin, GLP-1 receptor antagonist; 25 nmol/kg/day, i.p.) and HCA+Exendin groups.  
773 Mean with S.E. \* p<0.05 when compared with control group using unpaired t-test.

774