# Targeted metabolomics study of serum bile acid profile in patients with end-stage renal disease undergoing hemodialysis

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**Background.** Bile acids are important metabolites of intestinal microbiota, which have profound effects on host health. However, whether metabolism of bile acids is involved in the metabolic complications of end-stage renal disease (ESRD), and the effects of bile acids on the prognosis of ESRD remain obscure. Therefore, this study investigated the relationship between altered bile acid profile and the prognosis of ESRD patients.
**Methods.** A targeted metabolomics approach based on ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) was used to determine the changes in serum bile acids between ESRD patients (n=77) and healthy controls (n=30). Univariate and multivariate statistical analyses were performed to screen the differential proportions of bile acids between the two groups. **Results.** A total of 6 differentially expressed bile acids were identified as potential biomarkers for differentiating ESRD patients from healthy subjects. The decreased concentrations of chenodeoxycholic acid (CDCA), deoxycholic acid (DCA) and cholic acid (CA) were significantly associated with dyslipidemia in ESRD patients. Subgroup analyses revealed that the significantly increased concentrations of taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), taurohyocholic acid (THCA) and tauro α-muricholic acid (TαMCA) were correlated to the poor prognosis of ESRD patients. **Conclusions.** The serum bile acid profile of ESRD patients differed significantly from that of healthy controls. In addition, the altered serum bile acid profile might contribute to the poor prognosis and metabolic complications of ESRD patients.

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24    **Abstract**

25    **Background.** Bile acids are important metabolites of intestinal microbiota, which have profound

26    effects on host health. However, whether metabolism of bile acids is involved in the metabolic

27    complications of end-stage renal disease (ESRD), and the effects of bile acids on the prognosis of

28    ESRD remain obscure. Therefore, this study investigated the relationship between altered bile acid

29    profile and the prognosis of ESRD patients.

30    **Methods.** A targeted metabolomics approach based on ultra performance liquid chromatography-

31    tandem mass spectrometry (UPLC-MS/MS) was used to determine the changes in serum bile acids

32    between ESRD patients (n=77) and healthy controls (n=30). Univariate and multivariate statistical

33    analyses were performed to screen the differential proportions of bile acids between the two

34    groups.

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36    differentiating ESRD patients from healthy subjects. The decreased concentrations of

37    chenodeoxycholic acid (CDCA), deoxycholic acid (DCA) and cholic acid (CA) were significantly

38    associated with dyslipidemia in ESRD patients. Subgroup analyses revealed that the significantly

39    increased concentrations of taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA),

40    taurohyocholic acid (THCA) and tauro α-muricholic acid (TαMCA) were correlated to the poor

41    prognosis of ESRD patients.

42    **Conclusions.** The serum bile acid profile of ESRD patients differed significantly from that of

43    healthy controls. In addition, the altered serum bile acid profile might contribute to the poor

44    prognosis and metabolic complications of ESRD patients.

45 **1 Introduction**

46 Chronic kidney disease is an emerging health problem in China, with an overall prevalence of

47 10.8%. End-stage renal disease (ESRD) accounts for 0.03% of the Chinese population, and the

48 total number of ESRD patients has reached approximately 3.3 million (Yano et al. 2012). Loss of

49 renal function in ESRD patients can result in the abnormal metabolism of proteins, fatty acids,

50 carbohydrates, vitamins, water, electrolytes and acid-base, leading to dysfunction of various organ

51 systems and poor quality of life. In recent years, the concept of intestinal-renal axis has been

52 increasingly accepted (Evenepoel et al. 2017), which refers to the interaction between chronic

53 kidney disease and endogenous intestinal microbiota as well as their metabolites that may affect

54 the pathophysiological state of ESRD patients. On the one hand, uremia affects the composition

55 and metabolism of intestinal microbiota (Vaziri et al. 2013; Wang et al. 2012; Wong et al. 2014);

56 on the other hand, disordered intestinal microbiota increases the production of enterogenous

57 urotoxin and impairs the barrier function of intestinal mucosa, thereby aggravating renal

58 microinflammation and dyslipidemia, promoting the progression of chronic kidney disease, and

59 elevating the risks of atherosclerosis, cardiovascular and cerebrovascular diseases (Evenepoel et

60 al. 2017; Vaziri et al. 2016). Hence, enterogenous metabolites have become an important research

61 topic for combating the metabolic complications of chronic kidney disease.

62 Bile acids are essential metabolites of intestinal microbiota. Primary bile acids are initially

63 synthesized from cholesterol in the liver (Javitt 1994), and then conjugated with taurine or glycine

64 to form conjugated bile acids (Russell 2003), which are secreted into the intestine via the bile duct.

65 These bile acids are mostly reabsorbed at the end of the ileum, and re-transferred through the portal

66    vein back to the liver, thereby forming a complete enterohepatic circulation of bile acids. The

67    remaining small amount of unreabsorbed bile acids may undergo a series of biotransformation

68    under the action of intestinal microbiota, such as deconjugation, dehydroxylation,

69    dehydrogenation and epimerization, to produce secondary and tertiary bile acids (Midtvedt 1974).

70    Bile acids have been known to play a pivotal role in promoting the absorption of lipids and fat-

71    soluble nutrients in intestinal tract. More recently, they have also been found to affect lipid, glucose

72    and energy homeostasis by acting as endogenous signaling molecules conjugated to bile acid

73    receptors, including farnesoid X receptor (FXR) and G protein-coupled bile acid receptor

74    (GPBAR) (Hylemon et al. 2009).

75    At present, ESRD is commonly diagnosed by glomerular filtration rate test ($\leq$15 ml/min/1.73 m$^2$).

76    Considering the recent advancements in various "omics" fields such as genomics, epigenomics,

77    transcriptomics, proteomics and metabolomics, the introduction of novel omics techniques may

78    allow for the identification of novel biomarkers in ESRD. Several studies have suggested the

79    potential role of bile acids in chronic kidney disease (Marečková et al. 1990; Jimenez et al. 2002;

80    Chu et al. 2015). However, whether bile acid metabolism is involved in the metabolic disorders of

81    ESRD and the impacts of bile acids on the prognosis of ESRD are still obscure. Thus, the purpose

82    of this study was to investigate the relationship between altered bile acid profile and the prognosis

83    of ESRD patients receiving hemodialysis. UPLC-MS/MS, a well-adopted technique in

84    metabolomics research, was used to quantitatively analyze the bile acid profile of ESRD patients

85    on regular hemodialysis.

## 2 Subjects and methods

### 2.1 Patient recruitment and sample collection

A total of 77 ESRD patients (43 males and 34 females) who underwent regular hemodialysis at the Department of Nephrology, the Second Affiliated Hospital of Chongqing Medical University from December 2013 to December 2016 were recruited, and followed up for a long period. Patients were eligible for this study if they were aged between 40 and 70 years, had no previous infections, received no antibiotic treatment within the past three months, had glomerular filtration rate <15 mL/min/1.73 $m^2$ (calculated by the Cockcroft-Gault formula), and underwent regular hemodialysis 2-3 times per week. Exclusion criteria were cholestasis or abnormal markers (serum or imaging) for liver function, history of viral hepatitis, alcoholic liver disease or steatohepatitis that may cause liver damage, history of gallbladder disease or gallbladder removal, and concomitant active malignant tumor.

Meanwhile, 30 age- and gender-matched healthy subjects (17 males and 13 females) were selected as a control group from the physical examination center of the Second Affiliated Hospital of Chongqing Medical University. Fasting blood samples were collected from all subjects, and transported on ice to the laboratory within 2 hours of collection. Subsequently, the blood components were separated, aliquoted and stored at -80°C until further analyses. Patient data such as age, gender, height, and weight were collected, and the body mass index (BMI) was calculated as follows: [(weight (kg) / $height^2$ ($m^2$))]. The present study was approved by the Ethics Committee of the Second Affiliated Hospital of Chongqing Medical University,the approval number was (2018)028. Written informed consent was obtained from all subjects prior to enrollment.

**2.2 Materials and Reagents**

HPLC-MS grade methanol was purchased from Fisher Scientific. Both formic acid (HPLC grade) and ammonium acetate (HPLC-MS grade) were purchased from Sigma-Aldrich. The authentic standards of both unconjugated and conjugated bile acids were purchased from Steraloids (Newport, RI, USA). Four deuterium-labeled bile acids (CA-d4, CDCA-d4, LCA-d4 and GCA-d4) were purchased from Steraloids (Newport, RI, USA) and other six deuterium-labeled bile acids (DCA-d4, GCDCA-d4, GDCA-d4, UDCA-d4, GLCA-d4 and GUDCA-d4) were purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA). The ten deuterium-labeled bile acids were used as isotope-labeled internal standards (ISs) for quantitation and identification of compounds. Other materials were purchased from Anpel Laboratory Technologies (Shanghai, China).

**2.3 Preparation of standard curves**

Aliquots of each BA standard solution were mixed to generate a stock solution at 1000 ng/mL in methanol. The stock solution was serially diluted with methanol to 400, 200, 100, 40, 20, 10, 4 and 2 ng/mL. The stock solutions of the 10 ISs were prepared with methanol, and further diluted to a concentration of 100 ng/mL using methanol. To prepare a standard curve, the above 9 stock solutions were isometrically mixed with 100 ng/mL of IS solution to generate calibration points covering a concentration range of 1-500 ng/mL for all the analytes.

**2.4 Serum samples preparation**

A 50-μL aliquot of serum sample was mixed with 200 μL methanol/acetonitrile solution (5:3,v/v). After centrifugation for 15 min at 14000 g and 4 °C, 200 μL supernatant was dried under a stream

128    of nitrogen. The residues were re-dissolved in 100 μL of 50% aqueous methanol containing 50

129    ng/mL ISs prior to UPLC-MS/MS analysis, with an injection volume of 7.5 μL.

130    **2.5 UPLC-MS/MS analysis**

131    The samples were analyzed by UPLC-MS/MS using an Acquity UPLC system (Waters

132    Corporation, Milford, MA) coupled with a Triple Quad™ 5500 tandem mass spectrometer (AB

133    Sciex, Framingham, MA). All the samples were injected onto a Waters UPLC BEH C18 column

134    (100 mm × 2.1 mm, 1.7 μm) at a flow rate of 0.35 mL/min. The mobile phase consisted of 10 mM

135    ammonium acetate, 0.012% formic acid in water (A) and methanol (B). The chromatographic

136    separation was conducted by a gradient elution program as follows: 0-0.5 min, 35% B; 1 min,

137    60%B; 4 min, 80%B; 5.3-6 min, 100%B; and 6.1-8 min, 35%B. The column temperature was set

138    to 45 ºC.

139    The analytes eluted from column were ionized in a negative ion mode electrospray ionization (ESI-

140    ) mode. The source temperature was set at 550°C. The pressures of curtain gas (CUR), ion source

141    gas 1 (GS1), ion source gas 2 (GS2) and collision gas (CAD) were 30, 50, 50 and 8 psi,

142    respectively. The voltage of ion spray (IS) was -4500V, while -10 V for both entrance potential

143    (EP) and collision cell exit potential (CXP1). Multiple reaction monitoring (MRM) was used to

144    acquire data under optimal conditions of MRM transition (precursor > product), declustering

145    potential (DP) and collision energy (CE), as described in Supplementary Table 1. The optimal

146    dwell time was 8 ms. Both samples and standard curves samples were analyzed simultaneously.

147    Analyst software (version 1.5.2; AB Sciex) was used for instrument control and data acquisition.

148 **2.6 Mass spectrometry data pretreatment**

149     Mass spectrometry data were also analyzed by Analyst version 1.5.2 software (AB Sciex).

150 The default parameters and assisting manual inspection were adopted to ensure the qualitative and

151 quantitative accuracies of each compound. The peak areas of target compounds were integrated

152 and output for quantitative calculation. Considering the extensive individual variations of BA

153 concentrations, the relative proportion of TBA was calculated for comparison. TBA was defined

154 as a sum of all the 26 BAs associated with ESRD risk. The proportion of each BA was calculated

155 as follow: concentrations of each BA / TBA *100% (Yu et al. 2015).

156 **2.7 Statistical analysis**

157     Statistical analyses were performed using SPSS 24.0 software (SPSS, Chicago, IL, USA).

158 Continuous variables were tested for normal distribution via the Shapiro-Wilk test. Mann-Whitney

159 U test or independent sample t test was used to compare the metabolomic data between ESRD and

160 HC groups. One-way analysis of variance (ANOVA) followed by post-hoc testing was used to

161 compare the metabolomic data among ESRD death group, ESRD survival group and HC group.

162 Least significant difference (LSD) and Dunnett's T3 methods were applied to the absence and

163 presence of heterogeneity of variance, respectively. P value of less than 0.05 was considered

164 statistically significant.

165 Multivariate analyses were performed with SIMCA-P+ 14.0 software (Umetrics AB). Principal

166 component analysis (PCA) was used for visual clustering and grouping, as well as initial evaluation

167 of the model's validity. Supervised orthogonal partial least squares-discriminant analysis (OPLS-

168 DA) was used to further compare the differences between the two groups, where the effects of

169     model fitting were evaluated with R2X, R2Y and Q2Y model parameters. R2X and R2Y represent

170     the fractions of the variance in X and Y, respectively, that are explained by OPLS-DA model,

171     while Q2Y indicates the predictive performance of the model. Permutation test was used to verify

172     the model in order to avoid over-fitting. Loading plots and variable importance of projection (VIP)

173     > 1 were adopted as the criteria for screening differentially expressed bile acids. Next, bile acid

174     components contributed most to the intergroup difference were identified using univariate

175     statistical tests (Kondo et al. 2014; Zhu et al. 2015). Receiver operating characteristic (ROC) curve

176     was plotted to calculate the area under the curve (AUC) in order to assess the diagnostic efficacies

177     of the differential metabolites.

178     **3 Results**

179     **3.1 Demographic and clinical characteristics of patients**

180     Demographic and clinical characteristics of 107 subjects are shown in Table 1. There were no

181     significant differences between ESRD group and HC group in terms of age, BMI and sex ratio.

182     With regard to laboratory indices, the levels of serum creatinine (CREA), blood urea nitrogen

183     (BUN), uric acid (UA), phosphorus (P), triglyceride (TG) and total bile acid (TBA) were

184     significantly elevated ($P<0.05$) in ESRD patients compared to HC group, while the levels of blood

185     calcium (Ca), albumin (ALB), total cholesterol (CHOL), high-density lipoprotein cholesterol

186     (HDL-C), low-density lipoprotein cholesterol (LDL-C), hemoglobin (HGB) and platelet count

187     (PLT) were significantly decreased ($P<0.05$). These findings were consistent with the general

188     clinical characteristics of ESRD patients. Nonetheless, there were no significant differences in the

189     levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase

190     (ALP), total bilirubin (TBIL) and direct bilirubin (DBIL) between the two groups. Subgroup

191     analyses revelaed that ESRD death group had increased mean age, reduced serum creatinine level,

192     and elevated levels of aspartate aminotransferase and total bile acid compared to ESRD survival

193     group. However, no significant differences was found in other parameters between the two

194     subgroups (Table 2).

195     **3.2 Univariate analyses of serum bile acid profile**

196     The serum bile acid profiles of 77 ESRD patients and 30 HC were analyzed by HPLC-MS/MS.

197     The differences in bile acid profile between ESRD group and HC group was compared by Mann-

198     Whitney U test, and the results are shown in Table 3. A total of 32 bile acid components in serum

199     were quantified, of which 26 bile acids were significantly correlated with ESRD. The proportions

200     of unconjugated bile acids CA, CDCA, DCA, HDCA, UDCA, α+ωMCA, γMCA, 7KLCA,

201     12KLCA and 6,7-diketoLCA were all decreased, while only the proportion of unconjugated

202     βMCA was significantly increased (P<0.05) in ESRD group compared to HC group. The

203     proportion of LCA showed a downward trend, but the difference was not statistically significant

204     (P=0.062). Altogether, these results indicate that the proportions of most unconjugated bile acids

205     are reduced in ESRD patients. In addition, the proportions of conjugated bile acids GCA, GCDCA,

206     TCA, TCDCA, THCA, TαMCA and TUDCA in ESRD group were significantly higher (all

207     P<0.05) than those in HC group. Nevertheless, the proportions of the remaining conjugated bile

208     acids GDCA, GHCA, GLCA, GUDCA, TDCA and TβMCA were not significantly different

209     between the two groups.

210     To further analyze the changing patterns of bile acids, all the bile acid components were classified

211    according to their chemical properties and subsequently compared. The results showed that ESRD

212    patients exhibited lower proportions of total unconjugated bile acids (UnconBA) and secondary

213    bile acids (SBA), while higher proportions of total conjugated bile acids (ConBA), primary bile

214    acids (PBA), taurine-conjugated bile acids (TaurineBA) and glycine-conjugated bile acids

215    (GlycineBA) compared to HC group (P<0.05).

216    **3.3 Multivariate analyses of serum bile acid profile**

217    The distribution of bile acids in the samples was preliminarily investigated by PCA in order to

218    verify the rationality of experimental design and the homogeneity of biological replicates. The

219    results of PCA are presented as a three-dimensional score plot (Fig. 1A), where each point

220    represents a serum sample. Notably, a high differentiation was observed between ESRD group and

221    HC group (R2X=0.645, Q2=0.308), with a partial overlap and the top four principal components

222    accounted for 64.5% of the total variance.

223    OPLS-DA model exhibited higher ability for intergroup comparisons than PCA model. Thus,

224    OPLS-DA scores were plotted to further analyze the difference between ESRD group and HC

225    group, and to identify the variables contributed most to the difference between the two groups. As

226    shown in Fig. 1B, the serum bile acid profiles of the two groups displayed an obvious separation

227    trend in OPLS-DA model, with the model parameter values of R2Xcum=0.564, R2Ycum=0.508

228    and Q2Ycum=0.303. These data indicate that the bile acid metabolic profiles of the two groups

229    may manifest as different phenotypes.

230    The results of the permutation test used for validating the OPLS-DA model are presented in Fig.

231    1C. R2 denotes the explanation capacity of the model, while Q2 represents the predictive capacity

232  of the model. The low values of intercepts, R2 (0.109) and Q2 (-0.198), revealed that the

233  constructed model was not over-fitting.

234  The most significant variables were visually displayed by a loading plot. The variables with

235  statistical significance in univariate analysis (P<0.05) and VIP>1 were used as the criteria for the

236  identification of differentially expressed bile acids. A total of 6 bile acids (GCDCA, CDCA, DCA,

237  TCDCA, GCA and CA) were identified as potential biomarkers for differentiating ESRD patients

238  from healthy subjects (Table 4).

239  **3.4 ROC curve analysis of differentially expressed bile acids**

240  The 6 differentially expressed bile acids and TBA screened by OPLS-DA model were presented

241  graphically as ROC curves in order to explore their diagnostic abilities. As shown in Fig. 2, the

242  differentiating ability of TBA (AUC=0.701, 95% CI: 0.591, 0.810; P=0.001) was lower than that

243  of the 6 identified bile acids (except for CA). Among the 6 bile acids, CDCA (AUC=0.815, 95%

244  CI: 0.726, 0.904; P<0.001) exhibited the highest ability to differentiate ESRD patients from

245  healthy subjects. In addition, a combination of all 6 differential bile acids demonstrated the most

246  distinguishing ability (AUC=0.890, 95% CI: 0.818, 0.963; P<0.001), hence can be used as a

247  combined biomarker for ESRD diagnosis.

248  **3.5 Analysis of bile acid compositions among the subgroups**

249  To further assess the differences in bile acids among ESRD patients with different prognoses, we

250  retrospectively analyzed the survival rates of 47 patients whose samples were collected during

251  2013, and found that 17 patients died over a period of 3 years. Therefore, we established two

252  subgroups consisted of 17 dead patients and 30 survivors, and then compared with HC group. The

253  results showed that the abundances of TCA, TCDCA, THCA, TαMCA, TaurineBA and TBA in

254  the control group, survival group and death group increased successively (Fig. 3), with a

255  statistically significant difference among the two groups(P<0.1). Altogether, these findings

256  indicate that poor prognosis and mortality are positively correlated to the concentrations of the

257  above-mentioned bile acids.

258  **4 Discussion**

259  In this study, we quantitatively analyzed the serum bile acid profiles in ESRD patients and HC

260  using UPLC-MS/MS in order to determine the roles of bile acids in ESRD. The results showed

261  that ESRD patients and HC displayed significant alterations in the proportions of bile acids.

262  Notably, the levels of TBA were significantly increased in ESRD patients, as consistent with the

263  findings of previous studies (Balestri & Cupisti 1996). Previous studies have shown that the

264  increased TBA level in ESRD patients is probably caused by subclinical liver damage, abnormal

265  enterohepatic circulation (Jimenez et al. 2002), abnormal reabsorption and secretion of bile acids

266  by renal tubule, and diminished excretion of bile acids from kidney due to low glomerular filtration

267  rate (Chu et al. 2015). In addition, Gai et al. (2014) suggest that the elevated plasma BA level

268  represents an early event in the progression of chronic renal failure, and is due to increased efflux

269  across the basolateral membrane of human hepatocytes.

270  The roles of bile acids in the body metabolism and disease onset are difficult to understand by the

271  sheer investigative effort involved in the altering levels of TBA due to their complex bile acids

272  metabolism and diverse chemical structures. Therefore, we classified each bile acid according to

273  its chemical property for the purpose of comparison, and found that ESRD patients exhibited

274  decreased proportions of unconjugated bile acids and secondary bile acids, as well as increased

275  proportions of conjugated bile acids and primary bile acids. Increasing evidence has shown that

276  intestinal microbiota regulates the conversion of primary bile acids to secondary bile acids, which

277  in turn affects the size and composition of bile acid pool (Duboc et al. 2013). After the hepatic

278  primary bile acids enter the intestinal tract, the hydrolysis of the amino bond at the 24th carbon

279  atom of bile acid conjugates is catalyzed by bile salt hydrolase (produced by intestinal microbiota)

280  to form unconjugated bile acids. This reaction is followed by 7α dehydroxylation to form

281  secondary bile acids. However, only the free bile acid salts can carry out 7α dehydroxylation.

282  Hence, the conversion of conjugated bile acids to free bile acids by bile salt hydrolase is the

283  premise of 7α dehydroxylation (Begley et al. 2005). Our results suggest that the decreased

284  proportions of unconjugated bile acids and increased proportions of conjugated bile acids in ESRD

285  patients may be related to the low activity of bile salt hydrolase resulted from the disordered

286  intestinal microbiota and diminished hydrolysis of bile acids in the intestinal tract. This may lead

287  to an increase in the synthesis of primary bile acids and a decrease in the synthesis of secondary

288  bile acids.

289  To further analyze the bile acid compositions that play key roles in the onset and progression of

290  ESRD, 6 typical differential bile acids that differentiate ESRD patients from HCs were screened

291  and identified by OPLS-DA model, including CDCA, DCA, CA, GCDCA, TCDCA and GCA.

292  ROC analysis revealed that the combination of the above 6 bile acids was superior to total or

293  individual bile acids for detecting metabolic complications in ESRD patients. Notably, these 6 bile

294  acids are mainly responsible for the abnormal changes in bile acid metabolism in ESRD patients.

295  Bile acids have been well known to act as emulsifiers to promote the absorption of lipids and fat-

296  soluble vitamins in the small intestine, but the knowledge of bile acids has been constantly

297  evolving over the last 20 years. They were recently found to act as endogenous signaling molecules

298  in mammals for activating FXR, GPBAR and cell signaling pathways *in vivo*. Activation of these

299  receptors and cell signaling pathways is involved in the regulation of many important life

300  processes, including the synthesis, metabolism, and transport of bile acids (Ananthanarayanan et

301  al. 2001; Goodwin et al. 2000; Pircher et al. 2003), blood glucose (Katsuma et al. 2005; Stayrook

302  et al. 2005), and lipoprotein (Hirokane et al. 2004; Watanabe et al. 2004), as well as energy

303  metabolism (Watanabe et al. 2006).

304  FXR is the most important bile acid receptor, not only expressed in the enterohepatic circulation,

305  but also in the heart and kidneys (Forman et al. 1995; Kawamata et al. 2003). Unconjugated bile

306  acids such as CDCA, DCA, LCA and CA are a group of FXR receptor agonists with high affinity,

307  among which CDCA possesses the highest (Jia et al. 2017; Makishima et al. 1999; Song et al.

308  2015; Vaquero et al. 2013). FXR activation was shown to reduce circulating triglyceride levels by

309  lowering the production of very low-density lipoprotein and increasing its clearance (Watanabe et

310  al. 2004). Moreover, the serum levels of triglyceride and high-density lipoprotein cholesterol were

311  increased in FXR-deficient mice (Lefebvre et al. 2009). The enterohepatic circulation of bile acids

312  is disturbed in hyperlipidemia patients receiving bile acid sequestrants or patients undergoing

313  ileectomy, thus leading to an increase in plasma triglyceride level (Angelin et al. 1978; Buchwald

314  et al. 1990). Interestingly, oral administration of CDCA contributes to a significantly greater

315  reduction in triglycerides compared to placebo (Carulli et al. 2013). Likewise, our study found that

316  the levels of CDCA, DCA and CA were decreased in ESRD patients, suggesting that the inhibition

317  of FXR activity may increase plasma triglyceride levels, which represents one of the mechanisms

318  of dyslipidemia in ESRD patients.

319  In addition, our study found that the serum levels of cholesterol and low-density lipoprotein

320  cholesterol decreased remarkably in ESRD patients (Table 1). The serum cholesterol levels in

321  patients with chronic renal failure and patients undergoing hemodialysis may be different from

322  those in healthy population. The lower the serum cholesterol level (50-150 mg/dl), the higher the

323  mortality rate, so-called a "reverse epidemiology" phenomenon (Kalantarzadeh et al. 2003). The

324  activation of FXR and its related signaling pathways has been found to reduce bile acid synthesis

325  by decreasing the biological activity of rate-limiting enzyme CYP7A1, and leading to an increase

326  in serum cholesterol level (Lefebvre et al. 2009). It is speculated that the FXR activity in ESRD

327  patients can be inhibited due to the decreased expression of FXR agonists, which may explain the

328  lower levels of serum cholesterol and low-density lipoprotein cholesterol in ESRD patients

329  compared to HC.

330  Subgroup analysis was performed on ESRD patients with differing prognoses and HC, and the

331  results showed that the abundances of TCA, TCDCA, THCA, TαMCA, TaurineBA and TBA were

332  significantly increased in ESRD death group compared to ESRD survival group (Figure 3).

333  Noticeably, the above bile acids with high abundances were all taurine-conjugated bile acids.

334  These findings demonstrate that the increased taurine-conjugated bile acid levels are positively

335  correlated to the poor prognosis of ESRD patients. Besides, the increased levels of taurine-

336  conjugated bile acids could also promote the absorption of lipids in the small intestine, and increase

337    the risk of hyperlipidemia and subsequent cardiovascular diseases. It has been reported that free

338    taurine maintains the barrier function of intestinal epithelia by promoting NLRP6 inflammasome

339    activation and enhancing intestinal epithelial IL-18 production (Levy et al. 2015). Therefore, it is

340    speculated that the elevated levels of taurine-conjugated bile acids can reduce the concentrations

341    of free taurine in ESRD patients, leading to an impaired intestinal epithelia barrier function and

342    increased enterogenous urotoxin accumulation.

343    Of the 6 bile acids associated with ESRD risk, only TCDCA was differentially expressed between

344    ESRD death group and ESRD survival group. The other metabolites (i.e. CA, CDCA, DCA, GCA

345    and GCDCA) were not significantly different between ESRD death and survival groups. These

346    results indicate that these metabolic biomarkers have no role in the prognosis of ESRD. It is

347    postulated that abnormal bile acid metabolism occurs during the early stage of chronic kidney

348    disease, which does not aggravate ESRD progression and prognosis. The significant roles of

349    TCDCA in the diagnosis and prognosis of ESRD patients remain largely unknown, which warrants

350    further studies.

351    There are still some shortcomings in this study. First, the study subjects were selected from a single

352    center, thus the sample size was relatively small. Second, in the subgroup analyses, the average

353    age of ESRD patients who died during the three-year follow-up was older than that of ESRD

354    survival group, which may influence the findings of bile acid compositions between the two

355    subgroups. Third, the mechanism of bile acid profile change affecting the prognosis of ESRD

356    patients was speculated on the basis of the existing researches. Fourth, the elevated levels of

357    conjugated bile acids in ESRD patients may be a consequence of imbalanced gut microbiota,

358   altered enterohepatic circulation and/or hepatic dysfunction. Thus, further in vitro, in vivo and

359   well-designed clinical studies are warranted to confirm the usefulness of bile acids in ESRD

360   diagnosis and prognosis. Despite these limitations, this is the first study that utilizes metabolomics

361   approach to identify a panel of bile acid biomarkers for the diagnosis and prognosis of ERSD.

362   Given that the currently available diagnostic methods can only examine the symptoms of ESRD,

363   this metabolomics approach is more appropriately used to describe the etiological mechanisms of

364   the disease.

365   **5 Conclusion**

366   In conclusion, this study reveals that the serum bile acid profile of ESRD patients is significantly

367   different from that of healthy subjects. In addition, the altered serum bile acid profile may affect

368   the occurrence of metabolic complications and prognosis of ESRD patients. Therefore, targeting

369   bile acid compositions may provide a novel therapeutic strategy for the treatment of relevant

370   metabolic complications in ESRD patients.

371 **Figure Legends**

372 **Fig. 1** Metabolomic analysis of serum samples.

373 (A) PCA score plots. Ellipse:Hotelling's T2 (95%). (B) OPLS-DA score plots showing a clear

374 discrimination between ESRD (blue sphere) and HC subjects (red octahedron). Ellipse:Hotelling's

375 T2 (95%). (C) Permutation test demonstrating the robustness of OPLS-DA model. R2=0.109 and

376 Q2=-0.198.

377 **Fig. 2** ROC curves for bile acid biomarkers.

378 ROC curves analysis for the predictive power of 6 bile acid biomarkers and TBA for distinguishing

379 ESRD from healthy control.The final logistic model included six bile acid biomarkers. Using a

380 cutoff probability of 50%, we obtained sensitivity of 94.8% and specificity of 73.3% by ROC. The

381 calculated area under the ROC curve was 0.890 (95% confidence intervals, 0.818,0.963).

382 **Fig. 3** Differential serum bile acid profiles among the two cohorts.

383 The fold-changes in bile acid levels between ESRD death and survival groups (relative to the

384 control cohort) are graphically displayed. Normally-distributed data are depicted in a bar graph

385 with means and standard errors of the mean (SEMs).*P<0.1; **P<0.05; ***P<0.01

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Demographic and clinical characteristics of ESRD and control group

1 **Table 1** Demographic and clinical characteristics of ESRD and control group

| Variables | ESRD (N=77) | HC (N=30) | P-value |
|---|---|---|---|
| Age(year) | 59±13.9 | 59.3±6.8 | 0.956 |
| Sex(M/F) | 77(43/34) | 30(17/13) | 0.939 |
| BMI(kg/m²) | 23±3.2 | 22.1±1.5 | 0.091 |
| PTH(pg/ml) | 246.7±47.9 | — | — |
| CREA(umol/L) | 905±268.9[a] | 65.4±14.7 | <0.001 |
| BUN(mmol/L) | 22.6±6.2[a] | 5.2±1.1 | <0.001 |
| UA(umol/L) | 444.9±110.8[a] | 284.3±66.7 | <0.001 |
| Ca(mmol/L) | 2.2±0.2[a] | 2.4±0.2 | <0.001 |
| P(mmol/L) | 1.8±0.5[a] | 1.0±0.1 | <0.001 |
| TG(mmol/L) | 1.9±1.1[a] | 1.1±0.5 | <0.001 |
| CHOL(mmol/L) | 3.5±1.0[a] | 4.7±0.5 | <0.001 |
| HDL-C(mmol/L) | 1.0±0.4[a] | 1.5±0.3 | <0.001 |
| LDL-C(mmol/L) | 1.7±0.7[a] | 2.8±0.5 | <0.001 |
| WBC(10^9/L) | 5.7±1.7 | 5.4±1.3 | 0.416 |
| HGB(g/L) | 106±17.2[a] | 140±19 | <0.001 |
| PLT(10^9/L) | 151±62.6[a] | 193.7±50.6 | 0.003 |
| GLU(mmol/L) | 6.1±2.8 | 5.4±0.9 | 0.061 |
| ALB(g/L) | 38.3±4.0[a] | 44.8±3.0 | <0.001 |
| AST(U/L) | 20±16.3 | 18.1±7.5 | 0.548 |
| ALT(U/L) | 13.4±8.3 | 15.7±6.3 | 0.190 |
| ALP(U/L) | 96.2±47.2 | 92.2±28.8 | 0.675 |
| TBIL(umol/L) | 6.9±2.8 | 7.3±2.3 | 0.459 |
| DBIL(umol/L) | 3.3±1.5 | 3.3±1.9 | 0.920 |
| TBA(ng/ml) | 3151.4±270.0[a] | 1849.2±269.8 | 0.001 |

2 Data represent means±SD or means±SEM.Comparisons performed via t-test (ESRD vs HC), [a] P<0.05.

Demographic and clinical characteristics of ESRD Survival,ESRD death and control group

1  **Table 2** Demographic and clinical characteristics of ESRD Survival,ESRD death and control group.

| Variables | ESRD Survival(N=30) | ESRD death(N=17) | HC (N=30) |
|---|---|---|---|
| Age(year) | 55.9±13.5[b] | 70.7±9.0[a] | 59.3±6.8 |
| Sex(M/F) | 30(17/13) | 17(9/8) | 30(17/13) |
| Duration(months) | 30±21 | 29±22 | — |
| BMI(kg/m$^2$) | 23.8±3.2 | 21.5±3.1 | 22.1±1.5 |
| PTH(pg/ml) | 338.0±104.1 | 240.8±82.7 | — |
| CREA(umol/L) | 939.3±301.5[ab] | 731.1±206.8[a] | 65.4±14.7 |
| BUN(mmol/L) | 22.2±5.4[a] | 21.4±7.5[a] | 5.2±1.1 |
| UA(umol/L) | 444.0±99.6[a] | 442.4±113.6[a] | 284.3±66.7 |
| Ca(mmol/L) | 2.2±0.2[a] | 2.2±0.3[a] | 2.4±0.2 |
| P(mmol/L) | 1.7±0.4[a] | 1.6±0.6[a] | 1.0±0.1 |
| TG(mmol/L) | 2.2±1.4[a] | 2.1±1.1[a] | 1.1±0.5 |
| CHOL(mmol/L) | 3.9±0.8[a] | 3.7±1.0[a] | 4.7±0.5 |
| HDL-C(mmol/L) | 1.0±0.4[a] | 1.0±0.4[a] | 1.5±0.3 |
| LDL-C(mmol/L) | 2.1±0.7[a] | 1.7±0.8[a] | 2.8±0.5 |
| WBC(10^9/L) | 5.6±1.4 | 5.7±1.8 | 5.4±1.3 |
| HGB(g/L) | 112.5±16.0[a] | 103.8±20.8[a] | 140±19 |
| PLT(10^9/L) | 171.6±67.1 | 144.2±72.4[a] | 193.7±50.6 |
| GLU(mmol/L) | 6.1±3.5 | 6.9±2.6 | 5.4±0.9 |
| ALB(g/L) | 38.9±4.2[a] | 38.4±4.3[a] | 44.8±3.0 |
| AST(U/L) | 15.6±6.9[b] | 22.4±11.4 | 18.1±7.5 |
| ALT(U/L) | 12.8±7.1 | 14.9±9.5 | 15.7±6.3 |
| ALP(U/L) | 95.1±49.0 | 113.1±50.5 | 92.2±28.8 |
| TBIL(umol/L) | 6.4±1.8 | 7.2±2.3 | 7.3±2.3 |
| DBIL(umol/L) | 3.2±1.0 | 3.7±1.2 | 3.3±1.9 |
| TBA(ng/ml) | 2888.83±373.4[ab] | 4213.0±785.7[a] | 1849.2±269.8 |

2  Data represent means±SD or means±SEM.Comparisons performed via one-way ANOVA. [a] P<0.05 vs. HC cohort; [b] P<0.05

3  vs.ESRD death cohort.

The percentages of individual bile acid relative to TBA in ESRD and heathy control group(%)

1    **Table 3.** The percentages of individual bile acid relative to TBA in ESRD and heathy control group(%)

| Metabolite | ESRD (N=77) | HC (N=30) | Z-value | P-value |
|---|---|---|---|---|
| CA | 1.32(0.71~2.82) | 2.43(1.06~6.56) | 2.927 | 0.003 |
| CDCA | 3.86(1.88~8.10) | 14.76(6.49~22.74) | 5.049 | <0.001 |
| DCA | 1.37(0.21~4.37) | 6.50(1.39~17.28) | 3.544 | <0.001 |
| HDCA | 0.13(0.05~0.38) | 0.51(0.08~1.72) | 2.592 | 0.010 |
| LCA | 0.10(0.02~0.25) | 0.26(0.00~0.64) | 1.865 | 0.062 |
| UDCA | 0.48(0.17~1.06) | 1.17(0.71~1.89) | 3.024 | 0.002 |
| α+ωMCA | 0.10(0.03~0.29) | 0.39(0.13~0.93) | 2.871 | 0.004 |
| βMCA | 0.05(0.00~0.17) | 0.00(0.00~0.00) | -3.700 | <0.001 |
| γMCA | 0.48(0.15~1.05) | 0.93(0.50~2.23) | 3.329 | 0.001 |
| 7KLCA | 0.22(0.12~0.48) | 0.48(0.28~0.70) | 3.440 | 0.001 |
| 12KLCA | 0.07(0.00~0.22) | 0.36(0.00~1.03) | 2.646 | 0.008 |
| 6,7-diketoLCA | 0.08(0.00~0.22) | 0.23(0.04~0.40) | 2.330 | 0.020 |
| GCA | 11.06(8.22~14.88) | 6.13(4.04~8.76) | -4.383 | <0.001 |
| GCDCA | 41.62(33.90~48.88) | 30.44(25.60~38.81) | -4.016 | <0.001 |
| GDCA | 2.59(0.20~8.16) | 5.53(0.97~11.10) | 1.298 | 0.194 |
| GHCA | 0.52(0.32~0.90) | 0.48(0.35~0.76) | -0.194 | 0.846 |
| GLCA | 0.02(0.00~0.20) | 0.09(0.00~0.26) | 0.813 | 0.416 |
| GUDCA | 8.79(4.93~13.32) | 6.67(2.76~11.00) | -1.345 | 0.178 |
| TCA | 2.52(1.66~3.77) | 1.13(0.61~1.57) | -5.229 | <0.001 |
| TCDCA | 5.33(3.69~9.71) | 2.56(1.79~3.88) | -4.834 | <0.001 |
| TDCA | 0.67(0.09~1.68) | 0.74(0.16~1.44) | 0.097 | 0.922 |
| THCA | 0.13(0.08~0.26) | 0.11(0.06~0.15) | -2.057 | 0.040 |
| TαMCA | 0.10(0.06~0.16) | 0.05(0.00~0.07) | -4.483 | <0.001 |
| TβMCA | 0.45(0.27~0.71) | 0.42(0.24~0.67) | -0.388 | 0.698 |
| TUDCA | 0.63(0.36~1.41) | 0.33(0.24~0.67) | -3.593 | <0.001 |
| UnconBA | 12.22(6.77~20.86) | 35.65(22.65~52.91) | 6.124 | <0.001 |
| TaurineBA | 11.38(8.05~17.91) | 5.78(4.21~8.42) | -4.459 | <0.001 |
| GlycineBA | 71.15(65.22~80.59) | 54.54(42.78~66.70) | -4.862 | <0.001 |
| ConBA | 87.78(79.14~93.23) | 64.35(47.09~77.35) | -6.124 | <0.001 |
| PBA | 74.95(65.37~83.39) | 65.49(54.34~74.97) | -3.274 | 0.001 |
| SBA | 20.43(12.13~29.42) | 26.20(17.70~36.79) | 2.303 | 0.021 |
| PBA/SBA | 3.63(2.15~7.09) | 2.65(1.44~4.40) | -2.538 | 0.011 |
| UnconBA/ConBA | 0.14(0.07~0.26) | 0.55(0.29~1.13) | 6.124 | <0.001 |

2    Data presented as median and inter-quartile range (IQR).

3    Abbreviations: CA, cholic acid;CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; HDCA, hyodeoxycholic acid; LCA,

4    lithocholic acid; UDCA, ursodeoxycholic acid; α+ωMCA, α-muricholic acid and ω-muricholic acid; βMCA, β-muricholic acid;

5    γMCA, γ-murocholic acid; 7KLCA, 7-ketolithocholic acid; 12KLCA, 12-ketolithocholic acid; 6,7-diketoLCA, 6,7-

6    diketolithocholic acid; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid;GDCA, glycodeoxycholic acid; GHCA,

7    glycohyocholic acid; GLCA, glycolithocholic acid; GUDCA, glycoursodeoxycholic acid; TCDCA, taurochenodeoxycholic

8    acid;TDCA, taurodeoxycholic acid; THCA, taurohyocholic acid; TαMCA, tauro α-muricholic acid; TβMCA, tauro β-muricholic

9    acid; TUDCA, tauroursodeoxycholic acid; UnconBA, unconjugated bile acid; ConBA, conjugated bile acid;PBA, primary bile

10    acid; SBA, secondary bile acid. TaurineBA,taurine conjugated bile acid; GlycineBA,glycine conjugated bile acid.

Potential biomarkers discovered by VIP value and ROC analysis

1    **Table 4** Potential biomarkers discovered by VIP value and ROC analysis

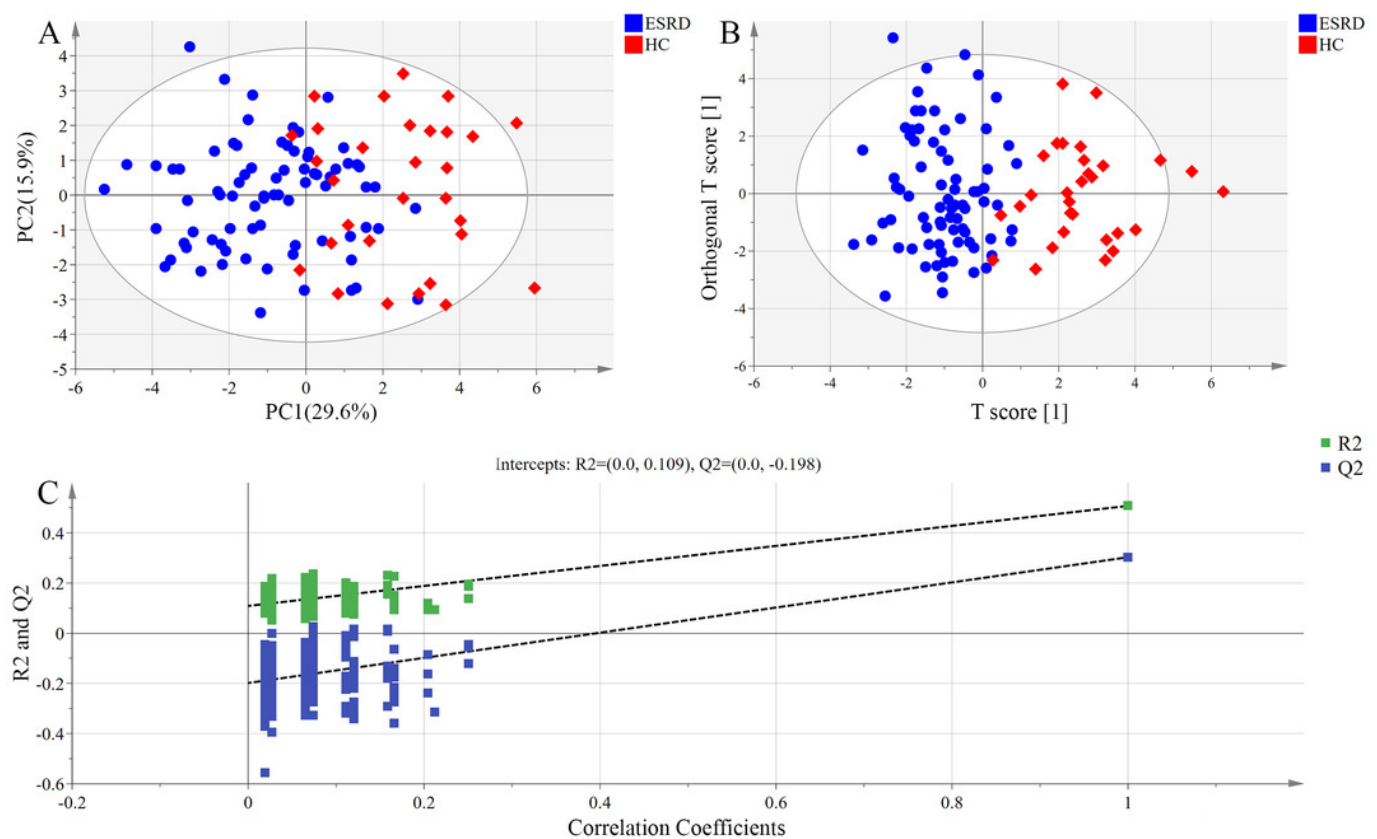
| Biomarkers | VIP | P-value | AUC |
|---|---|---|---|
| GCDCA | 2.48 | <0.001 | 0.751 |
| CDCA | 2.20 | <0.001 | 0.815 |
| DCA | 2.06 | <0.001 | 0.721 |
| TCDCA | 1.41 | <0.001 | 0.802 |
| GCA | 1.38 | <0.001 | 0.774 |
| CA | 1.10 | 0.003 | 0.683 |

2    Variable important in projection (VIP) was obtained from OPLS-DA with a threshold of 1.0; P-value was calculated from Mann-

3    Whitney U test; Area under curve (AUC) was obtained from ROC curves.

# Figure 1

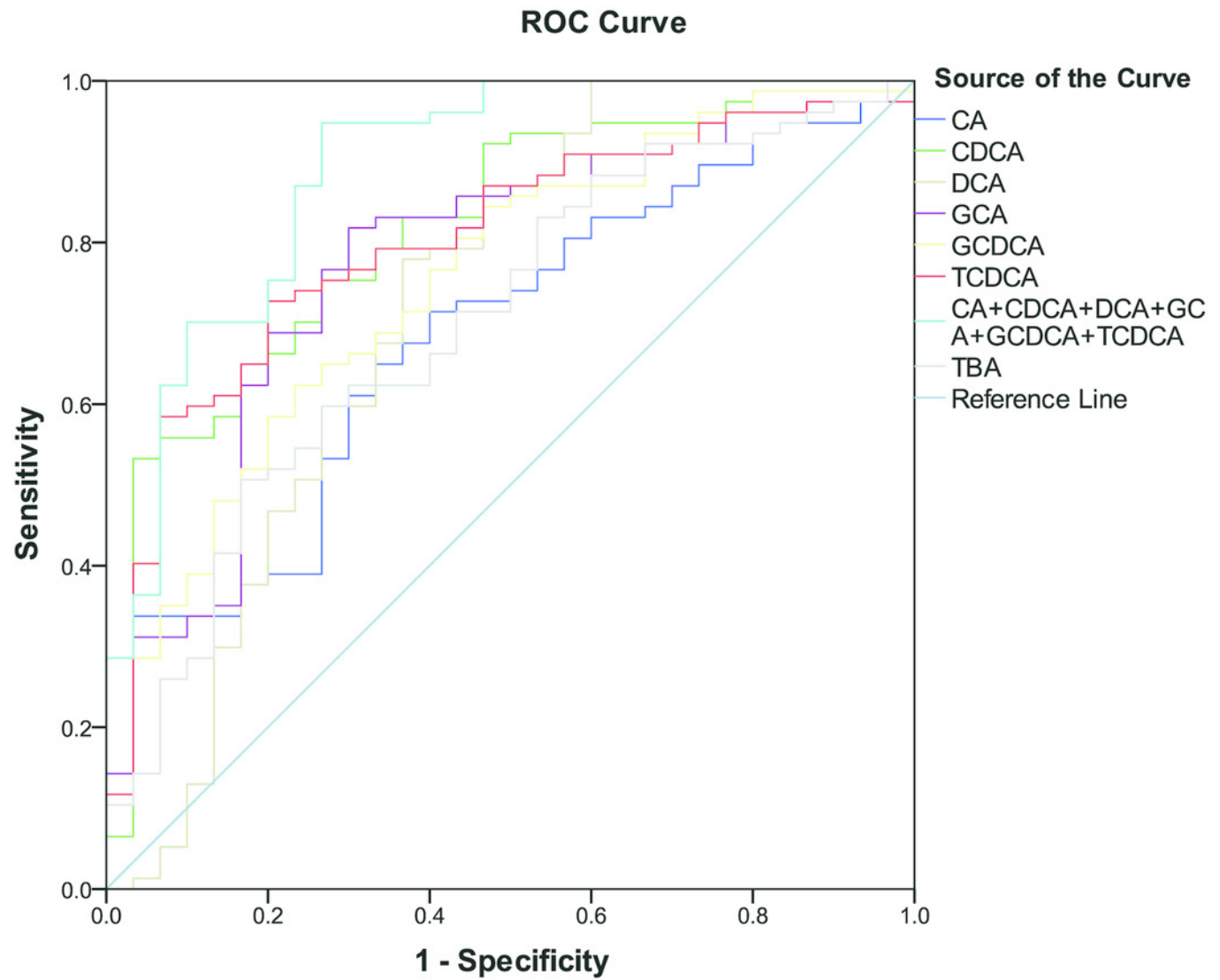
Metabolomic analysis of serum samples.

(A) PCA score plots. Ellipse:Hotelling's T2 (95%). (B) OPLS-DA score plots showing a clear discrimination between ESRD (blue sphere) and HC subjects (red octahedron). Ellipse:Hotelling's T2 (95%). (C) Permutation test demonstrating the robustness of OPLS-DA model. R2=0.109 and Q2=-0.198.

# Figure 2

ROC curves for bile acid biomarkers.

ROC curves analysis for the predictive power of 6 bile acid biomarkers and TBA for distinguishing ESRD from healthy control.The final logistic model included six bile acid biomarkers. Using a cutoff probability of 50%, we obtained sensitivity of 94.8% and specificity of 73.3% by ROC. The calculated area under the ROC curve was 0.890 (95% confidence intervals, 0.818,0.963).

**ROC Curve**

*Source of the Curve*
- CA
- CDCA
- DCA
- GCA
- GCDCA
- TCDCA
- CA+CDCA+DCA+GCA+GCDCA+TCDCA
- TBA
- Reference Line

# Figure 3

Differential serum bile acid profiling among the three cohorts

The fold-changes in bile acid levels between ESRD death and survival groups (relative to the control cohort) are graphically displayed. Normally-distributed data are depicted in a bar graph with means and standard errors of the mean (SEMs).*P<0.1; **P<0.05; ***P<0.01.

*Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.*