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The urinary bile acid profiling analysis of asymptomatic hypercholanemia of pregnancy: A pseudo-targeted metabolomics study

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ABSTRACT

Background: Asymptomatic hypercholanemia of pregnancy (AHP) is a controversial hypercholanemia, which is difficult to distinguish from intrahepatic cholestasis of pregnancy (ICP). Our aim is to elucidate the characteristics of urinary bile acid (BA) profiling of women with AHP and to find potential biomarkers for the diagnosis and differential diagnosis of AHP.

Methods: We developed a pseudo-targeted approach to perform metabolomics analysis of bile acids (BAs) using ultra-high performance liquid chromatography/hybrid quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS). Urinary BAs profiles were compared among AHP women (n = 20), ICP patients (n = 33) and normal controls (n = 35).

Results: The profiling of urinary BAs was significantly different among the AHP, ICP and control groups. Compared to the control group, the AHP group had higher levels of four possible sulfated BAs and trihydroxy BAs, including the species of muricholic acid (MCA), cholic acid (CA) and six possible BAs, whereas, 20 possible sulfated BAs, taurochenodeoxycholic acid (TCDCA), tetrahydrocannabinolic acid (THCA), and seven possible BAs were significantly lower in the AHP group than those in the ICP group. Based on the receiver operating characteristic (ROC) analysis, glycocholic acid (GCA) combined with T-ω-MCA were found to be the potential combination biomarker for the diagnosis (area under the curve was 0.960) of AHP, and mono-S, Gtri-S-2 combined with TLCA-S were found to be the potential combination biomarker for the differential diagnosis (area under the curve was 0.990) of AHP and ICP.

Conclusions: The metabolisms of urinary Bas were altered in the AHP group compared with the ICP group and the control group. Urinary BA profiling analysis can serve as an effective tool for the diagnosis of AHP and the differential diagnosis of AHP and ICP.

Abbreviations: AHP, asymptomatic hypercholanemia of pregnancy; ICP, intrahepatic cholestasis of pregnancy; BAs, bile acids; UPLC-Triple TOF-MS/MS, ultra-performance liquid chromatography-triple quadrupole time-of-flight tandem mass spectrometry; TBA, total bile acids; TCA, taurocholic acid; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; TDCA, taurodeoxycholic acid; GDCA, glycodeoxycholic acid; TUDCA, tauroursodeoxycholic acid; TCDCA, taurochenodeoxycholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; CA, cholic acid; UDCA, ursodeoxycholic acid; LCA, lithocholic acid; HDCA, hyodeoxycholic acid; DHCA, dehydrocholic acid; GUDCA, glycoursodeoxycholic acid; HCA, hycocholic acid; MCA, muricholic acid; GHCA, glycohycocholic acid; GLCA, glycolithocholic acid; GHDCA, glycohyodeoxycholic acid; GDHCA, glycodehydrocholic acid; THCA, taurohycocholic acid; T-, tauro-; THDCA, taurohyodeoxycholic acid; TDHCA, taurodehydrocholic acid; TLCA, taurolithocholic acid; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; USBA, urinary sulfated bile acid; HCV, hepatitis C virus; TBIL, total bilirubin; DBIL, direct bilirubin; ALT, alanine transaminase; AST, aspartate transferase; ALP, alkaline phosphataseand; GGT, gamma-glutamyl transpeptidase; IS, internal standard; ESI, electrospray ionization; MRM, multiple reaction monitoring; DP, declustering potential; CE, collision energy; PLS-DA, partial least squares- discriminant analysis; PCA, principal component analysis; AUC, area under the curve; YI, Youden index; VIP, variable importance in the projection

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1. Introduction

Asymptomatic hypercholanaemia of pregnancy (AHP) is defined as raised serum bile acids (BAs) in pregnancy without symptoms and other raised biochemical characteristics. Maria et al. [1] reported that women with AHP had been proposed as a sub-healthy status that had no differences with healthy pregnant women in clinical and perinatal features. Moreover, hypercholanemia in AHP was supposed to be the result of the existence of a partial impairment in hepatobiliary clearance of BAs. Because of elevated BA levels, AHP is difficult to distinguish from intrahepatic cholestasis of pregnancy (ICP), which is a pregnancy-specific liver disease characterized by maternal pruritus and raised serum total bile acids (TBA). ICP may have serious consequences for the fetus, such as increased proportion of premature deliveries, fetal distress and perinatal mortality [2]. However, Feng et al. [3] reported a case of intrauterine fetal death in an AHP woman. They further demonstrated that women with AHP had a higher risk of adverse perinatal outcomes than ICP patients [4]. They also considered AHP as an unusual form of ICP. Therefore, AHP is controversial in the definition and perinatal features. Currently, the common understanding of the characteristics of AHP, as well as the diagnostic approach and obstetric treatment regimen remain deficient.

BAs are a group of amphipathic molecules with a steroid backbone that are synthesized exclusively from cholesterol in hepatocytes. Different types of BAs have surprising complexity in structure and different physicochemical properties [5]. Actually, the BA metabolisms are different in different types of hepatobiliary diseases. Chen et al. [6] found that women with ICP had significantly elevated levels of taurocholic acid (TCA), glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), taurodeoxycholic acid (TDCA), glycodeoxycholic acid (GDCA), and tauroursodeoxycholic acid (TUDCA) in the serum, compared with normal pregnant women. Zhou et al. [7] detected serum BA profiles in infants with biliary atresia and suggested the ratio of TCDCA/CDCA as a biomarker to distinguish biliary atresia from neonatal hepatitis syndrome. Trottier et al. [8] reported that the levels of taurine and glycine conjugates of primary BAs in serum were elevated in both patients with primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) compared to un-cholestatic donors. This provides further information for the comprehension of different types of hepatobiliary diseases by the analysis of BA profiling rather than only by TBA. The level of serum BAs elevates after food ingestion due to the release of cholecystokinin, which stimulates gallbladder contraction resulting in the flow of bile into the intestine. Herein, feeding status should be controlled before the serum BAs profiling analysis. As renal excretion is a way for the elimination of BAs, the levels of BAs in urine are less affected by food intake than those in serum [9]. Seventy to 80% of BAs in urine are conjugated by sulfation. Sulfation is an important metabolic pathway for the deintoxication and elimination of BAs [9]. Thomassen [10] and Huang [11] et al. reported that urinary sulfated bile acids (USBAs) were elevated distinctly in ICP patients, which indicated that USBA, superior to serum TBA, was suitable for the diagnosis of ICP. USBA was considered as a more useful indicator for hepatic fibrosis than TBA in patients with hepatitis C virus (HCV)-related liver diseases [12]. USBA was also beneficial for the early detection of fibrosis in primary biliary cirrhosis (PBC) [13] and biliary atresia in infants [14]. Therefore, the comprehensive and detailed profiling of urinary BAs, especially for urinary sulfated BA profiling, is critical to maintain the balance between physiological and pathological effects of BAs in humans. However, few studies have reported urinary BA profiling analysis for AHP.

The triple-quadrupole mass analyzer in combination with multiple reaction monitoring (MRM) is a common tool for the quantitative analysis of the target metabolites. However, recent studies have shown that more and more BAs, including known and some unknown species, are involved in human physiological and pathological conditions [15–17]. The ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS) integrates the characteristics, including qualitative exploration, rapid profiling, and high-resolution quantification workflows on a single platform. In previous work, we established a targeted and un-targeted metabolomics analysis for serum BA profiles by UPLC-QTOF-MS [19]. The results showed that 60 BAs (including 27 confirmed and 33 identified BAs) were simultaneously identified in one serum sample. Meanwhile, a potential combination biomarker consisting of TCA, α-MCA and an identified trihydroxy glycine-conjugated BA was screened for the diagnosis of ICP with a high diagnostic efficiency.

In this study, we developed a pseudo-targeted method for the determination of random urinary targeted and un-targeted BA profiles based on the UPLC-QTOF-MS platform. The characteristics of urinary BA profiles of women with AHP could be elucidated by the holistic profile of urinary BAs in women with AHP, ICP, and normal pregnancies. Furthermore, urinary BA profiling analysis can serve as an effective tool for the diagnosis of AHP and the differential diagnosis of AHP from ICP.

2. Materials and methods

2.1. Chemicals and reagents

Cholic acid (CA), chenodexycholic acid (CDCA), dexycholic acid (DCA), ursodeoxycholic acid (UDCA), their glyco-conjugated (G-) and tauro-conjugated (T-) bile acids (GCA, GCDCA, GDCA, GUCDA, TCA, TCDCA, TDCA, TUDCA), hyodesoxycholic acid (HDCA), dehydrocholic acid (DHCA), and taurolithocholic acid 3-sulfate (TLCA-3S)) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hyocholic acid (HCA), α-muricholic acid (α-MCA), β-MCA, ω-MCA, GHCA, glycolithocholic acid (GLCA), GHDCA, THCA, T-α-MCA, T-ω-MCA, TLCA, THDCA, and TDHCA were purchased from Steraloids INC. (Newport, USA). The acetonitrile and methanol of mass pure grade were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Ammonium acetate of high-performance liquid chromatography (HPLC) pure grade was purchased from Sigma (Poole, Dorset, UK). All water used in the experiment was produced by a Millipore Synergy water device (Millipore, Milford, MA, USA).

2.2. Subjects

A case-control study enrolling a total of 119 pregnant women was designed in this experiment. The subjects were recruited from the First Affiliated Hospital of Chongqing Medical University (Chongqing, China) from December 2015 to December 2016. These women were divided randomly into two sets, including a training set (n = 88) and a validation set (n = 31). The training set contained 33 ICP patients, 20 AHP women, and 35 age-matched normal pregnant women in the third trimester (≥28 gestational weeks) of pregnancy as controls. The validation set included 15 ICP patients, eight AHP women, and eight normal pregnant women. This study was approved by the Ethics Committee of Chongqing Medical University. Written informed consents were obtained from all participants. The enrollment criteria for AHP were as follows: with high levels of TBA (≥10 μmol/L) and with normal results for other biochemical tests, but without pruritus. Patients were clinically diagnosed as ICP according to “the guidelines for diagnosis and treatment of intrahepatic cholestasis of pregnancy (2015)” [19]. Abnormal renal function and medication were excluded for the three groups. The random urine samples were collected and kept at −80 °C until analysis. The liver function tests, including serum TBA, total bilirubin (TBIL), direct bilirubin (DBIL), alanine transaminase (ALT), aspartate transferase (AST), alkaline phosphatase (ALP), and gamma-glutamyl transpeptidase (GGT) were performed on a Modular DDP automatic biochemical analyzer system (Roche Molecular Diagnostics, Pleasanton, CA). Creatinine (Cr) concentrations in urinary samples were performed on a Modular DDP automatic biochemical analyzer system (Roche Molecular Diagnostics, Pleasanton, CA).

2.3. Preparation of urinary samples

The sample preparation was performed according to our previous report [20]. Two hundred microliters of thawed urine samples were spiked with 10 μL of TDHCA (internal standard, IS, 100 μmol/L) and 800 μL of H_2O, vortexed for 1 min, and then loaded onto SPE cartridges (Bond Elut, 200 mg/3 mL, Agilent, Santa Clara, CA, USA), which were activated with 1 mL of methanol and followed by 1 mL of H_2O. Loaded cartridges were then washed with 1 mL of H_2O and eluted with 1.5 mL of methanol/H_2O (7:3, *v*/v). The elution was dried in the SpeedVac Vacuum Concentrator (ISS110 P1, Thermo Fisher Scientific) and re-dissolved in 50 μL of methanol/H_2O (1:1, v/v). Then, the solution was sonicated for 2 min and centrifuged at 13,000 ×*g* for 10 min at 4 °C. Five microliters of supernatant was injected into the UPLC-QTOF-MS system.

2.4. UPLC-QTOF-MS analysis

A CBM20Alite UPLC system (Shimadzu, Kyoto, Japan) coupled with a Triple TOF MS 5600 (AB Sciex, Concord, Ontario, Canada) with an electrospray ionization (ESI) system was used for the analysis. The chromatographic separation was performed using a Kinetex XBC18 column (50 mm × 2.1 mm, 1.7 μm; Phenomenex, USA), which was linked to a security guard C18 ultra-cartridge (2 mm × 2.1 mm , Phenomenex, USA). The column temperature was maintained at 30 °C. The mobile phase consisted of 15 mmol/L ammonium acetate (mobile phase A) and acetonitrile (mobile phase B). The flow rate was set at 0.2 mL/min. The gradient was adjusted from 15% to 35% B in 10 min, increased linearly to 60% B from 10 min to 15 min, held at 60% B from 15 min to 17 min, adjusted to 15% B in 0.1 min, and was maintained for 3 min.

The MS system was operated on negative-ion (ESI-) mode, and the ion source-dependent parameters were set as follows: ionspray voltage, − 4500 V; nebulizing gas, 55 mL/min; heater gas, 55 mL/min; curtain gas, 25 mL/min; ion source temperature, 550 °C.

The mass range was firstly set at 70–1000 *m/z* in 250 ms of the accumulation time. The mass-to-charge ratio (*m/z*) of the parent ion for each target BA was fixed. To obtain signals of fragment ions from each target BA, product ion MS/MS scan mode was carried out at the mass ranges of 70–600 *m/z* in 100 ms of the accumulation time. The retention time and the accurate mass of the parent ion and fragments were used to identify the definite target BAs. Meanwhile, the calibration curve method was employed for the quantification of the definite target BAs. The possible BAs were identified in urinary samples based on the accurate *m/z* value of the known BAs (mass error < 10 ppm), characteristic ions of *m/z*, retention time, and interpretation of the MS/MS spectrum (purity score). Moreover, the relative ratios of the peak area between the possible BAs and internal standard (IS) were determined for the relative quantification of possible BAs. The analytical conditions, including quantitative ions of *m/z*, characteristic ions of *m/z* and DP and CE values of BAs are shown in Table 1 in the Supplementary material.

2.5. Data analysis

The PeakView 2.0 (AB Sciex, MA, USA) was used to identify compounds. The MultiQuant software 3.0 (AB Sciex, MA, USA) was adopted to quantify the confirmed compounds. All of the statistical analyses were performed by SPSS Statistics 22.0.0, and $P < .05$ was considered as statistically significant. The Kolmogorov-Smirnov tests were performed to determine the normality of the data distribution. Statistical differences for the three groups were evaluated by the one-way analysis of variance (ANOVA) or the Kruskal-Wallis test according to their distributions. Percentages were compared using chi-squared (χ^2) analysis and Fisher exact test. Principal components analysis (PCA) and partial least squares discriminant analysis (PLS-DA) in the multivariate statistical analysis were performed by SIMCA-P 14.1 software (Umetrics AB, Umea). In the training set, potential biomarkers were selected by combining with the variable importance (VIP) and S-plot of PLS-DA model. The VIP is a computation of the influence of every *x* term in the model on *y* variables. Larger VIP values indicate a greater influence of a term *x* on the *y* variables. Then, these selected metabolites were subjected to the multiple logistic regression analysis using the stepwise variable selection method. Thus, the prediction model was established. Receiver operating characteristic (ROC) curve analysis was utilized to estimate the diagnostic efficiency with the area under the curve (AUC), sensitivity, specificity, and Youden index (YI). The prediction model was re-evaluated using different samples in the validation set, and the specificity and sensitivity of the model were examined using the cut-off value obtained from the training set.

2.6. Validation of quality control and analytical method

The quality control (QC) samples were prepared by mixing an equal aliquot of urinary samples from 15 healthy pregnant women, 15 women with ICP, and 15 women with AHP. QC samples were injected at the beginning/end of the batch analysis every 10 runs.

The calibration samples were prepared by adding 10 μL of a series of mixed-standard solutions of 27 known BAs into 200 μL of pooled urine, and then the sample preparation was performed as described above. The calibration curve was constructed in triplication by plotting the ratio of the peak area of the calibration samples to that of IS, subtracting the ratio of pooled urine peak-area to IS (y) peak-area vs. the concentration of each bile acid (*x*) in the calibration samples.

The precision was studied by the analysis of urine from the ICP group as the high level and the control group as the low level. The intra-day CV was determined by analyzing five replicates of the pooled urinary sample on the same day. The inter-day precision was determined by analyzing the pooled urinary sample on five consecutive days. The recoveries of BAs were determined by adding high, medium, and low concentrations of standard solutions of 27 known BAs to aliquots of urinary samples and measuring the ratio of the increased concentration to the added concentration.

3. Results

3.1. Validation of quality control samples and analytical method

The variance of the first principal component of PCA model in all QC samples was no more than two-fold of the standard deviations, indicating that the analytical system was stable, and all data were under control, as shown in Fig. 1. Twenty-seven target BAs in urine were linear in the range of 0.0030–20.0 μmol/L, with a good correlation ($r > 0.99$), as given in Table 2 in the Supplementary material. The average recoveries for BAs were in the range of 71.6% to 129.4%, as shown in Table 3 in the Supplementary material. The intra-day and inter-day precisions of the method were < 17.8% and 25.9%, respectively, as displayed in Table 4 in the Supplementary material.

3.2. Clinical characteristics of study participants

The clinical characteristics of all study participants are summarized in Table 1. No significant differences were observed among the three groups in terms of age and body mass index (BMI).

Compared with the control group, the ICP and AHP groups showed elevated concentrations of TBA (mean ± SD, 2.1 ± 1.3 for the control group, 25.4 ± 12.6 for the ICP group and 21.8 ± 23.3 for the AHP group, $P < .001$). However, no significant differences in TBA levels were observed between the AHP and ICP groups. Moreover, ICP patients had much higher levels of ALT, AST, TBIL, and DBIL than AHP women and the controls.

Women with ICP delivered earlier than women with AHP and the

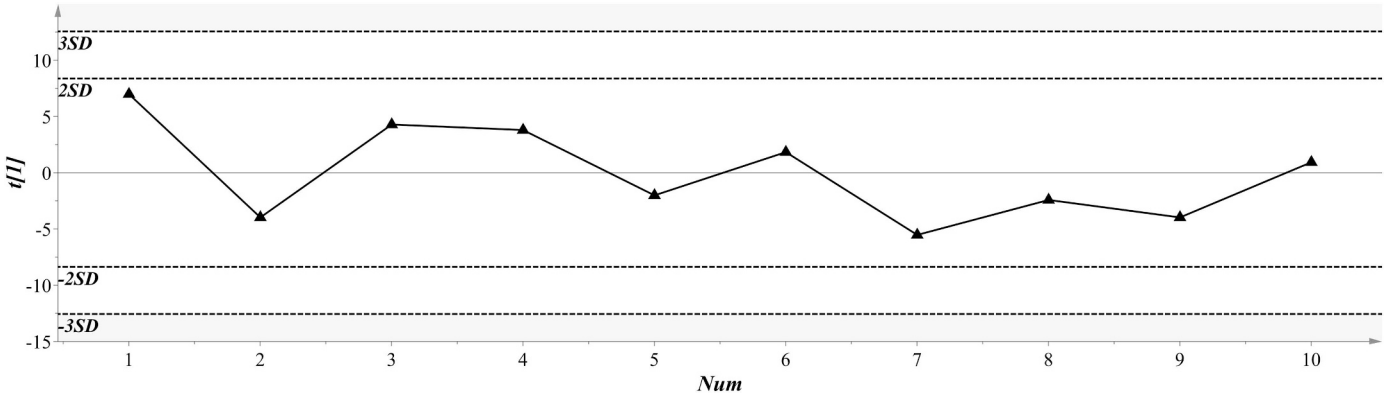

Fig. 1. Quality control charts.

controls ($P < .001$). One case of adverse fetal outcome was recorded when one pregnant woman had one or more adverse fetal outcomes. The rates of total adverse fetal outcomes in women with AHP and ICP were increased significantly compared with that in the control group ($P < .05$). For example, the rate of meconium-stained amniotic fluid, NICU admission, and spontaneous and iatrogenic preterm birth in the ICP population was significantly higher than that in the control group ($P < .05$). In addition, the rate of meconium-stained amniotic fluid, NICU admission, spontaneous and iatrogenic preterm deliveries in AHP group was lower than that in ICP group. Although no statistical significances were observed in the AHP and control groups, the AHP group still had a higher risk of suffering adverse fetal outcomes.

3.3. Multivariate analysis of UPLC-QTOF-MS data

By the UPLC-QTOF-MS method, 14 targeted BAs and 42 possible untargeted BAs were detected in urinary samples. Representative extraction ion chromatograms of the three groups are shown in Fig. 1 in the Supplementary material. The chromatograms presenting the profiles of urinary BAs were significantly different among the AHP, ICP, and control groups. The results of the quantification and semi-quantification of urinary BAs are shown in Table 2. As Cr excretion is stable in urine, urinary concentration of BAs corrected by concentration of Cr could cancel out the dilution of the urine volume. The quantification and semi-quantification of urinary bile acids were expressed as the concentration of BA/concentration of Cr and the rate of the peak area of BA/IS to the concentration of Cr, respectively. The prefixes "mono-", "di-", and "tri-" mean the BAs may have one, two, and three -OHs, respectively.

The level of urinary total BAs in the AHP group was significantly higher than that in the control group, whereas, it was significantly lower than that in the ICP group. The sulfated BAs were the most abundant form in the conjugated forms of BAs, which contained 57.3%, 51.7%, and 79.3% in the AHP, ICP, and the control groups, respectively. Moreover, the levels of total sulfated BAs and most individual sulfated BAs (especially for di-S-4, di-S-8, Tdi-S-5, Gdi-S-1, Ttri-S-1, Ttri-S-2, Gtri-S-2, and TLCA-S) in the AHP and control groups decreased significantly compared to those in the ICP group, and the levels of four sulfated BA species, including di-S-6, Gdi-S-1, Gmono-S-3, and Gmono-S-4 in the AHP group increased significantly compared to those in the control group. For non-sulfated BAs, the levels of trihydroxy Bas, including the species of MCA, CA, and six possible BAs increased in the AHP group compared to the control group, whereas, the levels of TCDCA, THCA, and four possible di-BAs, two possible tri-BAs and one possible mono-BA were significantly lower in the AHP group than those in the ICP group. It is noteworthy that the tri-2 in the AHP group was

Table 1
Clinical, biochemical and obstetrics informations for the recruits with AHP, ICP and normal pregnancies as controls.

	Control (n = 35)	AHP (n = 20)	ICP (n = 33)	P
Maternal age (years)	28.8 ± 3.9	29.7 ± 3.0	28.4 ± 4.6	> 0.05
BMI before pregnancy (kg/m^2)	20.7 ± 2.6	21.6 ± 2.4	21.1 ± 2.8	> 0.05
Gestational age at delivery (wks)	39.0 ± 1.5	37.8 ± 1.8#	37.4 ± 1.6*	< 0.001
Birth weight (g)	3204.0 ± 396.4	3456.0 ± 289.3#	3054.7 ± 448.3*	< 0.05
Apgar score				
At 1 min	9.8 ± 0.4	9.8 ± 0.4#	9.5 ± 0.8*	< 0.05
At 5 min	10 ± 0.0	10 ± 0.0	9.9 ± 0.4	> 0.05
Adverse fetal outcome	1 (2.9%)	4 (20.0%)*	12 (36.4%)*	< 0.05
Meconium-stained amniotic fluid (n)	1 (2.9%)	2 (10.0%)	6 (18.2%)*	< 0.001
NICU admission (n)	0	2(10.0%)	4 (12.1%)*	< 0.001
Fetal distress (n)	0	0	0	> 0.05
Asphyxia neonatorum (n)	0	0	1 (3.0%)	> 0.05
Spontaneous preterm delivery (n)	0	1 (5.0%)	5 (15.2%)*	< 0.05
Iatrogenic preterm delivery (n)	0	1 (5.0%)	6(18.2%)*	< 0.05
Liver function tests				
TBA (μmol/L)	2.1 ± 1.3	25.4 ± 12.6*	21.8 ± 23.3*	< 0.001
TBIL (μmol/L)	7.6 ± 2.1	9.2 ± 4.7#	16.1 ± 13.4*	< 0.001
DBIL (μmol/L)	1.7 ± 0.8	2.9 ± 2.3#	9.0 ± 11.1*	< 0.001
ALT (U/L)	15.1 ± 9.2	19.4 ± 9.5#	162.8 ± 110.1*	< 0.001
AST (U/L)	17.5 ± 4.9	19.8 ± 9.5#	109.8 ± 81.2*	< 0.001

Data were shown as mean ± SD. The normal ranges for TBA, TBIL, DBIL are 0–10.0, 3.4–20.5, 0–6.8 μmol/L, respectively. The normal ranges for ALT and AST are 7–40 and 13–35 U/L, respectively.

means compared to ICP group, $P < .05$.

* means compared to the control group, $P < .05$.

Table 2
Comparisons for concentrations of individual bile acids and semi-quantified levels of identified urinary bile acids in the three groups.

Bile acids	Control (n = 35)	AHP (n = 20)	ICP (n = 33)	P	Bile acids	Control (n = 35)	AHP (n = 20)	ICP (n = 33)	P
Confirmed bile acids (μmol/mmol Cr)					di-S-2	0.096 ± 0.140	0.249 ± 0.643	0.616 ± 1.389	> 0.05
TUDCA	0.005 ± 0.007	0.006 ± 0.006	0.014 ± 0.023*	< 0.001	di-S-3	0.066 ± 0.077	1.152 ± 2.715	2.033 ± 4.947*	< 0.001
TCDCA	0.006 ± 0.008	0.006 ± 0.006#	0.012 ± 0.010*	< 0.001	di-S-4	0.136 ± 0.245	0.239 ± 0.683#	2.054 ± 4.363*	< 0.001
GUDCA	0.004 ± 0.005	0.050 ± 0.071*	0.249 ± 0.406*	< 0.001	di-S-5	0.486 ± 0.723	0.886 ± 1.186	2.187 ± 3.243*	< 0.001
GHDCA	0.000 ± 0.000	0.008 ± 0.019	0.014 ± 0.034*	< 0.001	di-S-6	0.038 ± 0.053	0.286 ± 0.794*	0.189 ± 0.275*	< 0.001
GCDCA	0.006 ± 0.008	0.005 ± 0.006	0.011 ± 0.014	> 0.05	di-S-7	0.485 ± 0.735	0.584 ± 0.637	0.869 ± 0.898*	< 0.001
GDCA	0.005 ± 0.006	0.007 ± 0.007	0.007 ± 0.010	> 0.05	di-S-8	0.008 ± 0.006	0.018 ± 0.019#	0.238 ± 0.435*	< 0.001
α-MCA	0.010 ± 0.014	0.018 ± 0.019*	0.011 ± 0.011	< 0.001	Tdi-S-1	0.017 ± 0.022	0.128 ± 0.212#	1.469 ± 2.018*	< 0.001
T-ω-MCA	0.021 ± 0.021	0.114 ± 0.098*	0.230 ± 0.323*	< 0.001	Tdi-S-2	0.015 ± 0.021	0.022 ± 0.063#	0.273 ± 0.548*	< 0.001
T-α-MCA	0.052 ± 0.056	0.098 ± 0.086	0.209 ± 0.395*	< 0.001	Tdi-S-3	0.013 ± 0.012	0.007 ± 0.017#,*	0.062 ± 0.087*	< 0.001
THCA	0.001 ± 0.002	0.002 ± 0.002#	0.033 ± 0.048*	< 0.001	Tdi-S-4	0.020 ± 0.018	0.009 ± 0.014#	0.087 ± 0.098*	< 0.001
TCA	0.000 ± 0.001	0.016 ± 0.027*	0.058 ± 0.097*	< 0.001	Tdi-S-5	0.037 ± 0.029	0.027 ± 0.024#	0.523 ± 0.480*	< 0.001
GHCA	0.007 ± 0.009	0.003 ± 0.006	0.014 ± 0.026	> 0.05	Tdi-S-6	0.088 ± 0.082	0.060 ± 0.070#	0.569 ± 0.505*	< 0.001
GCA	0.009 ± 0.012	0.057 ± 0.052*	0.097 ± 0.114*	< 0.001	Gdi-S-1	0.100 ± 0.070	1.811 ± 3.075#,*	7.651 ± 8.870*	< 0.001
TLCA-S	0.045 ± 0.067	0.050 ± 0.082#	0.314 ± 0.477*	< 0.001	Gdi-S-2	0.088 ± 0.072	0.126 ± 0.078#	0.251 ± 0.180*	< 0.001
Primary/ Secondary	1.991 ± 0.985	1.618 ± 1.510	1.265 ± 1.204*	< 0.005	Gdi-S-3	0.182 ± 0.118	0.224 ± 0.292#	1.501 ± 1.046*	< 0.001
Total CA/CDCA	1.867 ± 4.239	8.016 ± 8.127*	7.685 ± 6.847*	< 0.001	Gdi-S-4	0.402 ± 0.337	0.449 ± 0.433#	1.545 ± 1.503*	< 0.001
Sum of confirmed BAs	0.172 ± 0.132	0.441 ± 0.261*,#	1.273 ± 1.334*	< 0.001	Ttri-S-1	0.004 ± 0.004	0.001 ± 0.002#,*	0.039 ± 0.039*	< 0.001
Identified bile acids (A(BA)/A(IS)/c(Cr))					Ttri-S-2	0.005 ± 0.005	0.004 ± 0.004#	0.374 ± 0.499*	< 0.001
Tdi-1	0.010 ± 0.013	0.007 ± 0.018#	0.107 ± 0.255*	< 0.001	Gtri-S-1	0.059 ± 0.076	0.034 ± 0.059#	0.111 ± 0.167*	< 0.001
Tdi-2	0.004 ± 0.004	0.001 ± 0.002#	0.140 ± 0.343*	< 0.001	Gtri-S-2	0.045 ± 0.036	0.041 ± 0.047#	0.921 ± 0.749*	< 0.001
Tdi-3	0.001 ± 0.002	0.007 ± 0.013#	0.280 ± 0.517*	< 0.001	mono-S	0.052 ± 0.051	0.058 ± 0.057#	0.198 ± 0.383*	< 0.001
Gdi-1	0.001 ± 0.001	0.006 ± 0.016#	0.125 ± 0.485*	< 0.001	Gmono-S-1	0.004 ± 0.003	0.006 ± 0.004#	0.014 ± 0.019*	< 0.001
Gdi-2	0.057 ± 0.088	0.171 ± 0.155*	0.167 ± 0.524	< 0.01	Gmono-S-2	0.025 ± 0.034	0.013 ± 0.026#	0.067 ± 0.118*	< 0.001
Ttri-1	0.003 ± 0.005	0.008 ± 0.012#	0.092 ± 0.132*	< 0.001	Gmono-S-3	0.020 ± 0.024	0.050 ± 0.035*	0.086 ± 0.082*	< 0.001
Ttri-2	0.006 ± 0.010	0.026 ± 0.020*	0.078 ± 0.144*	< 0.001	Gmono-S-4	0.005 ± 0.005	0.020 ± 0.024*	0.023 ± 0.024*	< 0.001
Ttri-3	0.001 ± 0.001	0.005 ± 0.007*	0.052 ± 0.011*	< 0.001	Non-sulfated BAs	2.448 ± 2.936	4.764 ± 3.006*	6.085 ± 6.274*	< 0.001
Ttri-4	0.011 ± 0.016	0.015 ± 0.014#	0.062 ± 0.081*	< 0.001	mono- BAs	0.001 ± 0.002	0.001 ± 0.002	0.011 ± 0.022	> 0.05
Ttri-5	0.062 ± 0.077	0.115 ± 0.089*	0.393 ± 0.909*	< 0.001	di- BAs	0.104 ± 0.099	0.382 ± 0.283*	1.701 ± 2.364*	< 0.001
Gtri-1	0.373 ± 0.392	1.015 ± 0.832*	0.879 ± 1.092	< 0.01	tri- BAs	2.342 ± 2.953	4.381 ± 2.832*	4.374 ± 4.600*	< 0.001
Gtri-2	0.168 ± 0.152	0.389 ± 0.210*	0.414 ± 0.396*	< 0.001	Sulfated BAs	2.682 ± 1.860	6.498 ± 5.419#	24.061 ± 18.706*	< 0.001
tri-1	0.502 ± 1.375	0.293 ± 0.536	0.446 ± 1.272	> 0.05	mono- BAs	0.055 ± 0.047	0.075 ± 0.048#	0.191 ± 0.202*	< 0.001
tri-2	0.935 ± 1.599	2.346 ± 2.411*,#	1.274 ± 2.359	< 0.001	di- BAs	2.510 ± 1.829	6.342 ± 5.354#	22.384 ± 24.085*	< 0.001
Tmono-1	0.001 ± 0.002	0.001 ± 0.002#	0.009 ± 0.019	< 0.001	tri- BAs	0.117 ± 0.101	0.081 ± 0.091#	1.486 ± 1.348*	< 0.001
di-S-1	0.055 ± 0.073	0.077 ± 0.070	0.080 ± 0.093	> 0.05	Sum of BAs	5.130 ± 3.486	11.262 ± 7.365#,*	30.147 ± 22.315*	< 0.001

Data were shown as mean ± SD.
"A(BA)/A(IS)/c(Cr)" means the rate of the peak area of BA/IS to the concentration of Cr.
means compared to ICP group, $P < .05$.
* means compared to the control group, $P < .05$.

significantly higher than that in both the ICP and control groups. The corresponding heat map of the concentration and relative abundance of these metabolites is presented in Fig. 2. On the other hand, the levels of G- and T-conjugated BAs increased markedly in the AHP and ICP groups compared to the control group. In addition, the level of T-conjugated BAs was obviously lower in the AHP group than that in the ICP group, as shown in Fig. 2 in the Supplementary material.

In the study, the PLS-DA model was applied to elucidate the metabolic characteristics of 56 BAs in the AHP, ICP, and control groups. The PLS-DA score plot (R^2Y cum = 0.429, Q^2 cum = 0.301) was constructed as shown in Fig. 3A. It revealed that ICP patients could be separated from the controls and AHP women. The distinct separations were observed based on PLS-DA models for the three groups of pairwise comparisons as shown in Fig. 3B, C, and D (R^2Y cum = 0.704, Q^2 cum = 0.469; R^2Y cum = 0.814, Q^2 cum = 0.501; R^2Y cum = 0.681, Q^2 cum = 0.432), respectively. It indicated that the metabolite profiles of urinary BAs showed significant differences among the three groups.

3.4. Discovery of candidate biomarkers

Compared with the parameters in the control group, the variables with VIP values of the PLS-DA model > 1.0 and AUC > 0.7 in the AHP group were selected as candidate biomarkers (Table 3). Then, all variables with VIP values of the PLS-DA model > 1.0 were enrolled in the multiple logistic regression analysis using the stepwise variable selection method. Thus, a prediction model for the diagnosis of AHP was established based on the combination of GCA and T-ω-MCA. The AUC, sensitivity, and specificity of this model were 0.960 (95% confidence interval (CI): from 0.915 to 1.000), 95.0%, and 85.3%, respectively , as shown in Fig. 3A. Moreover, the sensitivity and specificity of this model in the validation set were 87.5% and 100.0%, respectively. As these variables were found informative for the differentiation of the AHP group from the ICP group, a prediction model with the combination of mono-S, Gtri-S-2, and TLCA-S was established for the differential diagnosis of AHP from ICP, as shown in Table 3. The AUC, sensitivity, and specificity for the differential diagnosis model were 0.990 (95% CI: from 0.972 to 1.000), 91.2% and 100.0%, respectively, as shown in Fig. 3B. Moreover, the sensitivity and specificity of this model in the validation set were 88.9% and 93.8%, respectively. It indicated that the combined biomarker was feasible for the diagnosis and differential diagnosis of AHP with an acceptable sensitivity and specificity (Fig. 4).

4. Discussion

BAs are synthesized in the liver and secreted into duodenum, and then they are absorbed efficiently in the intestine. After that, they returned to the liver, and secreted into the bile. This process is called the enterohepatic circulation of BAs. Under normal conditions, BAs are sustained in the enterohepatic system, with minimal spilling into the

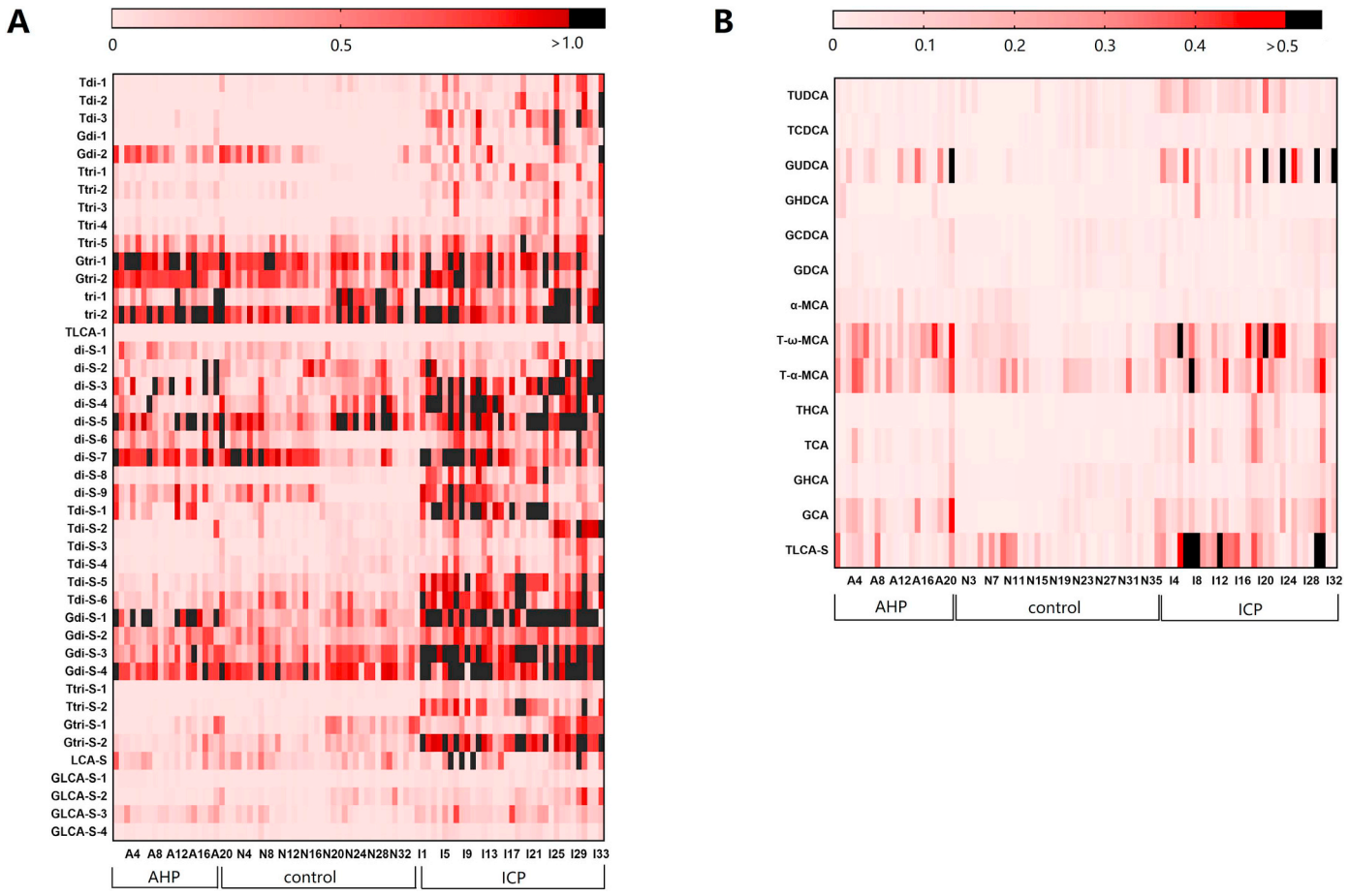

Fig. 2. Heat map of different metabolites. A. The relative abundance of possible identified bile acids. **B.** The concentration of commercial bile acids in the three groups.

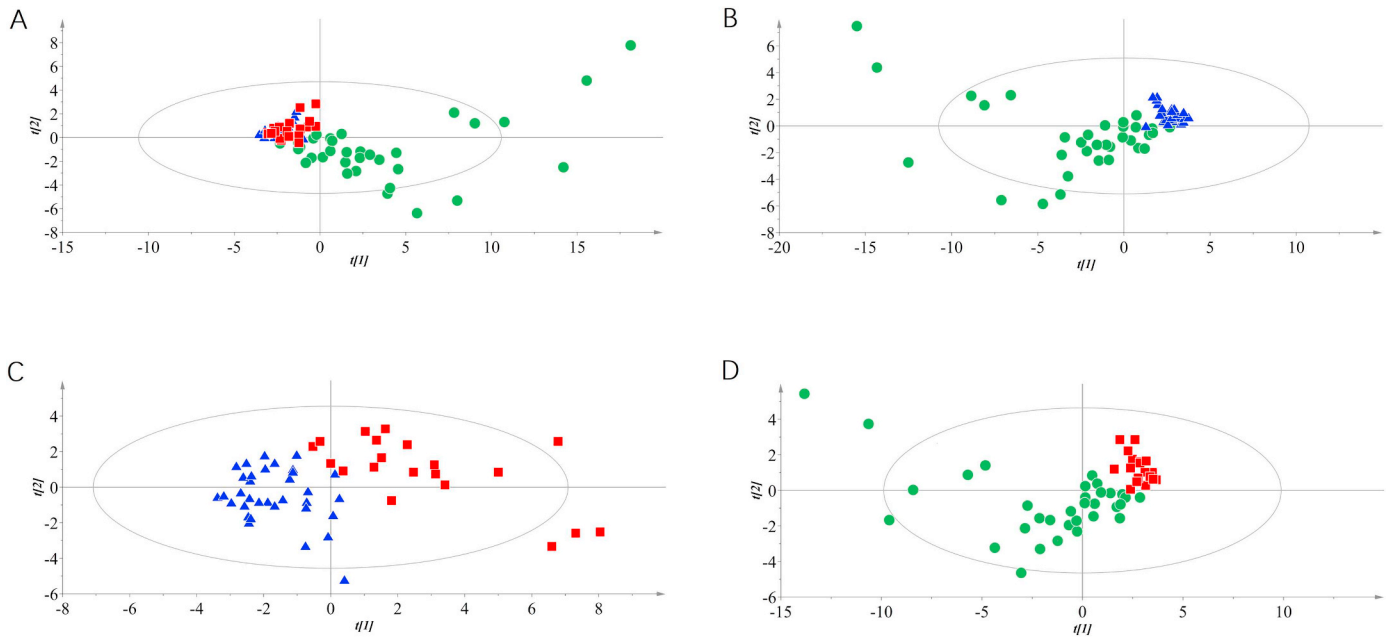

Fig. 3. The metabolic profiles of urinary samples. A. Score plots of PLS-DA model for the comparison of urinary bile acids profilings among AHP (■) women, ICP (●) patients and controls (▲); **B.** Score plots of PLS-DA model for the comparison of urinary bile acids profilings between ICP (●) patients and controls (▲); **C.** Score plots of PLS-DA model for the comparison of urinary bile acids profilings between AHP (■) women and controls (▲); **D.** Score plots of PLS-DA model for the comparison of urinary bile acids profilings between AHP (■) women and ICP (●) patients.

Table 3
Receiver operating characteristic (ROC) analysis to investigate the cut-off value of individual bile acids for the diagnosis of hypercholanemia in pregnancy.

Bile acid	Study group (n = 88)									Validation group (n = 31)	
	VIP	AUC	SEM	95%Cl	Cut-off	YI	Sensitivity	Specificity	P	Sensitivity	Specificity
AHP - Control											
GCA	1.783	0.862	0.055	0.755–0.969	0.018	0.664	75.0%	91.4%	< 0.001	87.5%	87.5%
Ttri-3	1.523	0.886	0.045	0.798–0.973	0.005	0.693	95.0%	74.3%	< 0.001	62.5%	62.5%
Gtri-1	1.607	0.766	0.069	0.631–0.901	0.402	0.464	75.0%	71.4%	< 0.001	75.0%	100.0%
T-ω-MCA	1.912	0.886	0.046	0.796–0.976	0.069	0.700	70.0%	97.1%	< 0.001	50.0%	100.0%
Gtri-2	1.504	0.816	0.060	0.698–0.934	0.222	0.643	90.0%	74.3%	< 0.001	62.5%	100.0%
GCA + T-ω-MCA	–	0.960	0.023	0.915–1.000	0.197	0.807	95.0%	85.3%	< 0.001	87.5%	100.0%
AHP - ICP											
Gdi-S-3	1.651	0.929	0.036	0.859–1.000	0.423	0.832	88.2%	95.0%	< 0.001	88.9%	93.8%
Tdi-S-6	1.475	0.856	0.052	0.754–0.958	0.113	0.715	76.5%	95.0%	< 0.001	88.9%	100.0%
Gtri-S-2	1.417	0.966	0.024	0.919–1.000	0.229	0.882	88.2%	100.0%	< 0.001	88.9%	100.0%
Tdi-S-5	1.367	0.947	0.030	0.888–1.000	0.067	0.832	88.2%	95.0%	< 0.001	88.9%	100.0%
Mono-S + Gtri-S-2 + TLCA-S	–	0.990	0.009	0.972–1.000	0.189	0.912	91.2%	100.0%	< 0.001	88.9%	93.8%

For quantitative bile acids, the cut-off value was expressed as concentration (μmol/mmol Cr); for semi-quantified bile acids, the cut-off value was expressed as the relative ratios of the peak area between bile acids and internal standards, and then each subject was corrected by creatinine (Cr) concentration.

blood, and negligible urinary excretion. Due to the impairment of the biliary excretion route under cholestatic conditions, BAs are transported out of the liver into systemic blood [9]. The elevated serum levels of CA and CDCA as well as a remarkably increased ratio of CA/CDCA were observed in AHP and ICP women [1,6]. Urinary excretion becomes a primary route for BA elimination. Compared with the controls, AHP women had significantly higher urinary levels of trihydroxy BAs, including α-MCA, T-ω-MCA, TCA, GCA and six kinds of possible BAs. The hydrophilicity of BAs is positively related to the number of hydroxyl groups. Therefore, trihydroxy BAs were highly hydrophilic and easily excreted [21], which increased distinctly in urine of AHP women as an adaptive compensated response to eliminate the marked accumulation of BAs in serum. We selected GCA and T-ω-MCA as a combination biomarker for the diagnosis of AHP. Compared with the control group, these two conjugated BAs in the AHP group increased approximately 6.33 and 5.43 times, respectively. In addition, the levels of total G- and T-conjugated BAs increased markedly in the AHP group compared with the control group. It indicated that amidation of BAs with glycine and taurine also played a major role in increasing the solubility, enhancing the urinary excretion, and decreasing the toxicity of BAs in the AHP group.

However, sulfation was a major detoxification pathway of BAs for ICP patients, and sulfated BAs accounted for 79.3% of total BAs. We demonstrated that ICP patients showed elevated urinary total sulfated BAs compared to AHP women and the controls (approximately 4-fold and 9-fold, respectively). As the solubility was increased by the sulfation of BAs, the intestinal absorption decreased and urinary excretion enhanced. BA-sulfates are also less toxic than their un-sulfated counterparts. Sulfotransferase-2A1 (SULT2A1) catalyzes the formation of sulfated BAs. The increase of sulfated BAs in urine might be related to a compensatory increase in sulfated BAs as a result of the upregulation of the hepatic SULT2A activity/expression or the increase in the substrate (BAs) availability for the sulfation during cholestatic conditions [9]. These results showed that urinary BA profiling might be used as a potential biomarker for the differential diagnosis of AHP. Based on the PLS-DA model and ROC curve analysis, we selected mono-S, Gtri-S-2 and TLCA-S as a combination biomarker for the differential diagnosis of AHP and ICP with acceptable selectivity and specificity. We found that TLCA-S, Gtri-S-2, and mono-S increased approximately 6.28, 3.41, and 22.46 times in ICP women compared to AHP women, respectively. This indicated that some BAs might be related to the metabolic characteristics for some diseases. In addition, urinary sulfated BAs may be used as the grade diagnosis of ICP. We reported previously that the combination of GCA-S and Gdi-S-1 were employed to distinguish severe ICP from mild ICP, with a sensitivity of 94.4% and specificity of 100% [20].

AHP women suffered a significantly lower rate of fetal

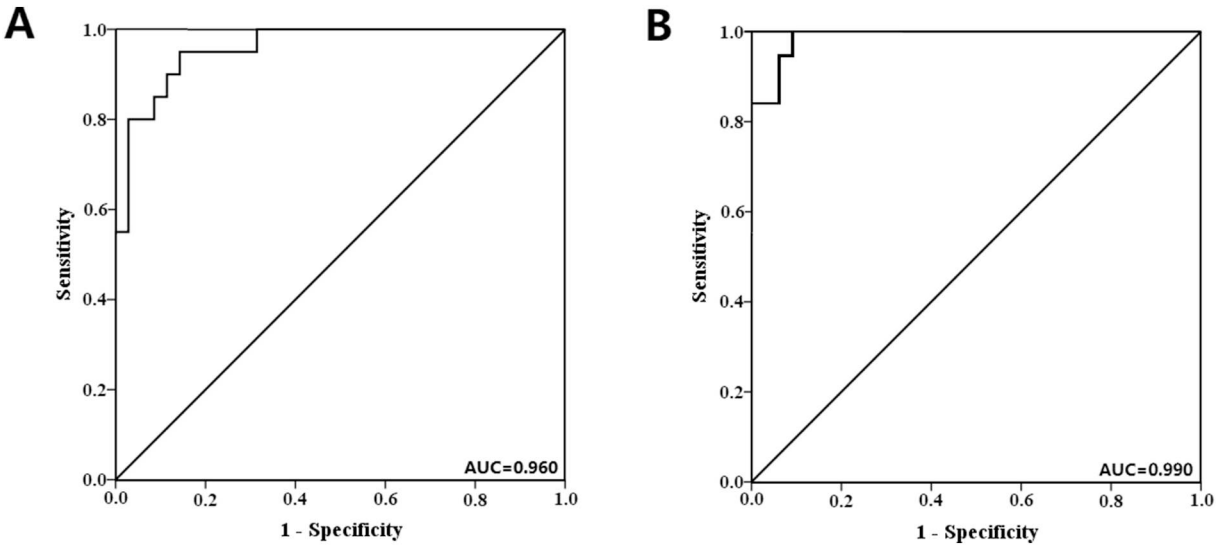

Fig. 4. The ROC curve analysis for the differential diagnosis. **A.** Combination biomarkers for AHP vs. normal controls. **B.** Combination biomarkers for AHP vs. ICP.

complications compared to ICP patients. Some studies confirmed that the fetal complications were related to the toxicity of BAs. BAs have a vasoconstrictive effect on the placental chorionic veins, which leads to fetal distress. This may also be explained by a negative effect of high BA levels on cardiomyocytes, which causes fetal arrhythmias and distress [22,23]. The pathologic effect of BAs is considered to be caused by their detergent properties, which enables them to bind to and solubilize the membrane of lipids. Their detergent properties are inversely proportional to the number of hydroxyl groups on the steroidal backbone, with mono-hydroxyl BAs (such as LCA species) as the most toxic BAs. Mono-hydroxyl BAs are extremely low under normal conditions, which do not produce any toxicity in the enterohepatic system. In this study, urinary sulfated mono-hydroxyl BAs in the AHP group and the normal group were found to be lower than those in the ICP group, which was probably due to the decrease in the substrate (mono-hydroxyl BAs species) availability for the sulfation. This suggests that these women may show a less toxic BA pool than ICP patients. In addition, T-conjugated BAs had superior acidity to the G-conjugated BAs in solution, and they increased slightly in potency as TGR5 agonists in cultured cells [24]. T-conjugated BAs had more negative impact on neonatal rat cardiomyocytes than G-conjugated BAs and could cause dysrhythmic contraction. As a result, the increased proportion of T-conjugated BAs in ICP may contribute to the etiology of intrauterine death [25]. In the study, the level of G-conjugated BAs in the AHP group was 8 times as high as that of the T-conjugated form. By contrast, the ratio of G−/T-conjugated BAs in ICP patients was 4:1. We demonstrated previously that the ICP group had increased levels of T-conjugated BAs in serum compared with the AHP group. These results indicated a decreased risk of adverse pregnancy outcomes in AHP women rather than ICP patients.

Currently, few studies have investigated the pathomechanism of AHP. Shadi et al. [26] reported that pregnancy increased levels of allopregnanolone-sulfate (PM4-S) and epiallopregnanolone-sulfate (PM5-S), which can inhibit Na^+-dependent and -independent influx of taurocholate in primary human hepatocytes and cause competitive inhibition of sodium taurocholate *co*-transporting polypeptide (NTCP)-mediated uptake of taurocholate in Xenopus oocytes. These metabolites influence BA metabolism and transportation, which may contribute to AHP. Frederi et al. [27] described the first case of NTCP deficiency because of homozygous mutations in the SLC10A1 (NTCP) gene in a child with chronically elevated BAs, without symptoms and other raised biochemical parameters. Then, a pregnancy case with NTCP deficiency causing asymptomatic hypercholanemia was reported [28]. The pregnancy developed spontaneous rupture of membranes with meconium-stained fluids at 38 weeks of pregnancy (first pregnancy) and lost the fetus. She was negative in the genetic testing associated with ICP, including ABCB4 (MDR3), ATP8B1 (FIC1), and ABCB11 (BSEP) and maintained elevated serum BA levels post-partum for more than one year. NTCP deficiency was highly suspected to be associated with AHP, which may have been due to genetic changes that differed from ICP. In any case, detailed genetic study requires further investigations.

5. Conclusion

Based on the pseudo-targeted UPLC-Triple TOF/MS method, the profiling of urinary BAs were found to be significantly different among the AHP, ICP, and control groups. Potential combination biomarkers were screened out for the diagnosis of AHP and the differential diagnosis of AHP and ICP. However, the sample size was limited in this study. Therefore, a large-cohort, long-term study enrolling AHP women needs to be performed. Our next work is to validate the application value of the potential biomarkers for the diagnosis and differential diagnosis of AHP.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2019.07.002.

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