Altered Serum Bile Acid Profile Associated with Chronic Allograft Dysfunction in Kidney Transplant Recipients

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Background: Chronic allograft dysfunction (CAD) is the leading cause of graft loss among kidney transplant recipients (KTRs). Bile acids (BAs) play an important role in regulating inflammatory process, which is the major contributor to the development of CAD. The aim of this study was to evaluate the association between BAs metabolic dysregulation and CAD in KTRs.

Material/Methods: Fifteen serum BA species were determined in 43 healthy controls (HCs) and 131 KTRs by UPLC-MS/MS. KTRs were grouped into stable renal function (STA) and CAD1 and CAD2 groups based on eGFR levels. Circulating CYP7A1, CYP7B1, CYP27A1, and SLCO2B1 mRNA levels were determined by RT-PCR.

Results: Total BA concentrations were comparable among the 4 groups. However, KTRs showed significantly different BAs profiling compared to HCs. KTRs with severe CAD (CAD2) had significantly lower unconjugated BAs and secondary BAs (SBAs) compared to the other 3 groups. KTRs had significantly lower SBAs/primary BAs (PBAs) ratios than HCs, which were comparable among the 3 KTR groups. Conjugated/unconjugated BAs ratios increased significantly with the deterioration of allograft function, which was further confirmed by correlation analysis. Differential correlation network analysis revealed that perturbations in intraclass and interclass BA coregulation existed during CAD progression. Moreover, relative gene expressions of CYP7B1 and CYP27A1 were positively correlated with eGFR.

Conclusions: BA species profiling, but not total BA concentrations, was significantly altered in KTRs with CAD. The shifts from unconjugated BAs toward conjugated BAs, SBAs toward PBAs, and distinct pairwise BAs coregulation patterns were the main characteristics of KTRs with CAD.

Keywords: Bile Acids and Salts • Kidney Transplantation

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Background

Kidney transplantation (KT) is the most effective treatment for patients with end-stage renal disease (ESRD). Although the short-term survival rate has been remarkably improved over the past decades, chronic progressive graft loss remains a difficult problem in the field of KT. A wide spectrum of etiologies, including cellular- and antibody-mediated rejection, immunosuppressive drugs toxicity, and recurrence of native kidney disease, are involved in kidney allograft injury and graft function decline [1], which further lead to the imbalance of water, electrolytes, acid base, and metabolites. Recently, a relatively new concept called “gut-kidney axis”, which refers to the pathogenic interconnection between gut microbiota (including their metabolites) and the kidney organ, has been implicated in various kidney diseases such as acute kidney injury (AKI), chronic kidney disease (CKD), and immunoglobulin A (IgA) nephropathy [2,3]. Also, as essential metabolites of gut microbiota, the alteration of bile acids (BAs) concentration and composition were revealed to be associated with host signaling in kidney diseases [4,5], which suggests the potential pathogenic role of BAs in kidney damage. However, whether BAs metabolism dysregulation is presented in kidney transplant recipients (KTRs) with chronic allograft dysfunction (CAD), who are facing the challenges of alloimmune response and lifelong immunosuppressive drugs, remains to be clarified.

There are 2 major categories of BAs – primary BAs (PBAs) and secondary BAs (SBAs) – which are synthesized in the liver and intestine, respectively. Unconjugated PBAs are lipid-soluble molecules initially synthesized in the liver from cholesterol through primary and alternative pathways [6]. The PBAs are then amidated with either glycine or taurine to form water-soluble conjugated PBAs. These conjugated PBAs travel to and are stored in the gallbladder temporarily. During digestion, the majority of BAs are delivered to the duodenum via the biliary tract and are recycled through enterohepatic circulation. The remaining 5% of BAs are not reabsorbed and undergo biotransformation by microbiota in the colon to form SBAs, which will either be transferred back to the liver via the portal vein or excreted in the feces [7]. With the in-depth investigation of their function, BAs are not only considered as an emulsifier to promote fat utilization and activate lipase to lipid synthesis via the interaction with its nuclear farnesoid X receptor, FXR, and cell-surface (Takeda G protein-coupled receptor 5, TGR5) receptors [8].

BA assay kit was purchased from Qlife Lab (Nanjing, China). BA components were extracted from the serum samples strictly according to the manufacturer’s instructions. Serum BAs were analyzed using a Waters ACQUITY ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) system. The UPLC-MS/MS system was coupled to a Waters ACQUITY UPLC and a Waters Xevo TQD triple quadrupole mass spectrometer. The chromatographic separation was performed on a Waters ACQUITY BEH C18 column (1.7 μm, 100 mm × 2.1 mm). The mobile phase consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in 85% acetonitrile (solvent B). The gradient elution program was as follows: 0-0.1 min, 95% solvent A; 0.1-1.5 min, 80% solvent A; 1.5-10 min, 15% solvent A. The flow rate was 0.4 ml/min. The samples were directly injected without dilution. The concentration of BAs in serum was calculated based on the peak area and external standard curve. The detection limit of most BAs was 5 μg/L, and the linearity range was from 5 μg/L to 500 μg/L.

Sample Collection and Pretreatment

Serum samples were collected after centrifugation and stored in aliquots at -80°C until the determination of BAs profile. Fresh EDTA-anticoagulated peripheral blood samples were collected from KTRs (before the morning dose of immunosuppressants) and HCs. White blood cells (WBC) were isolated after erythrolysis and then stabilized in Trizol reagent at -80°C until mRNA quantification was required.
liquid chromatography (UPLC) system coupled with a Waters XEVO TQ-S mass spectrometer with an ESI source controlled by MassLynx 4.1 software. An ACQUITY BEH C18 column (1.7 μm, 100×2.1 mm internal dimensions) (Waters) was used to perform chromatographic separations. Standard curves and internal standards were used for the purpose of quantifying BA metabolites concentrations. Corresponding high and low controls were employed to ensure the accuracy of detection. Fifteen BAs were determined in this study and the precursor and product ion pairs were as follows: cholic acid (CA) (407.3>407.3), chenodeoxycholic acid (CDCA) (391.3>391.3), glycocholic acid (GCA) (464.3>73.95), taurocholic acid (TCA) (514.35>80.01), glycochenodeoxycholic acid (GCDCA) (448.3>73.95), taurochenodeoxycholic acid (TCDCA) (498.3>80.1), deoxycholic acid (DCA) (391.3>391.3), lithocholic acid (LCA) (375.3>375.3), ursochenodeoxycholic acid (UDCA) (391.3>391.3), glycochenodeoxycholic acid (GUDCA) (448.3>73.95), glycolithocholic acid (GLCA) (432.3>73.95), ursodeoxycholic acid (UDCA) (498.3>80.1), tauroursodeoxycholic acid (TUDCA) (498.3>80.1), glycodeoxycholic acid (GDCA) (448.3>73.95), and taurochenocholeic acid (TLCA) (482.3>80.01).

BAs were classified into 4 subgroups based on the conjugation degree:
(1) unconjugated PBAs: CA and CDCA;
(2) conjugated PBAs: GCA, TCA, GCDCA and TCDCA;
(3) unconjugated SBAs: DCA, LCA and UDCA;
(4) conjugated SBAs: GDCA, GLCA, TDCA, TUDCA, GUDCA, and TLCA.

Ratios of selected BA metabolites were calculated to reflect the enzymatic activities involved in BA metabolism referred to the published studies [17,18].

(1) Ratio of CA to CDCA was used to determine the shifts in the BA synthesis from the classical pathway to the alternative pathway.
(2) Ratios of SBAs to PBAs (DCA/CA and LCA/CDCA) were used to reflect changes in gut microbiome enzymatic activity toward the shifted production of SBAs.
(3) Ratios of conjugated PBAs to unconjugated PBAs (TCA/CA, GCA/CA, TCDCA/CDCA, and GCDCA/CDCA) were used to reflect whether the dysregulation of PBAs synthesis was correlated with taurine or glycine conjugation.
(4) Ratios of conjugated SBAs to unconjugated SBAs (GDCA/DCA, TDCA/DCA, GLCA/LCA, TLCA/LCA) were used to reflect whether the dysregulation of SBAs synthesis was correlated with taurine or glycine conjugation.

mRNA Quantification

RNA was extracted from WBC using Trizol reagent according to the manufacturer’s instructions. The optical density value was measured to calculate the concentration and purity of RNA. Reverse transcription was performed with PrimeScriptTM RT reagent kit with gDNA eraser (TAKARA). Quantitative real-time polymerase chain reaction (RT-PCR) analysis was performed on TB Green (TAKARA). The primers used in this study were as follows: CYP7A1 (forward: 5’-CAAGGCAACACATTCCAGCAGC-3’, reverse: 5’-ATAGGATGCTCTCCAAGCTGAC-3’), CYP7B1 (forward: 5’-CACCGAGAACATGGAGACGCC-3’, reverse: 5’-GCTACAAAGCTCCCCTGGCA-3’), CYP27A1 (forward: 5’-GTGCTGCTTCTTGTAGGAGCAT-3’, reverse: 5’-TAGCCAGACACCTTGATGCG-3’), SLC2O2B1 (forward: 5’-TGGGCACAGAAAAACACACCT-3’, reverse: 5’-CGCTGCTGCAAAAATGCTCAC-3’), GAPDH (forward: 5’-GGACCGAGATCCCTCCAAAAT-3’, reverse: 5’-GGCTGTGTGTACATCTTCTCGG-3’).

Amplification was performed with a 2-step PCR protocol: 95°C for 5 minutes, followed by 40 cycles of 95°C for 10 seconds and 60°C for 30 seconds. A melting-curve analysis was applied to ensure the specificity of PCR products. The expressions of CYP7A1, CYP7B1, CYP27A1, and SLC2O2B1 were normalized to GAPDH and determined using the relative quantification method 2^{ΔΔCT}.

Statistical Analyses

Data were presented as the absolute number, mean±standard deviation or median (interquartile range) according to the data type. Chi-square or Fisher exact tests was utilized to compare categorical variables between groups. The t test or Mann-Whitney U test was utilized to compare continuous variables with normal distribution and skewed distribution, respectively. All statistical analyses and graphs were done with SPSS (version 23.0, SPSS Inc., Chicago, IL, USA), R (version 4.2.0, https://cran.r-project.org), and GraphPad Prism (Version 9.0.1, GraphPad, La Jolla, CA, USA). Principle component analysis was completed with MetaboAnalyst (version 5.0, https://www.metaboanalyst.ca). The Spearman correlation matrix was built with the “corplot” package. Differential correlations between BA pairs were calculated with the “DGCA” package. Only BA pairs with differential correlation (P<0.05) were imported into Cytoscape software (Version 3.7.2) [19] to achieve the visualization of correlation networks. Due to the exploratory study design property, we conducted the Bonferroni correction for multiple testing to avoid type I error. P<0.05/n (n refers to the number of comparisons) was considered statistically significant.

Results

Baseline Characteristics of Study Participants

The clinical and laboratory characteristics of study populations are presented in Table 1. Age, sex, trough concentration of...
Table 1. Characteristics of study participants.

|                     | HC      | STA     | CAD1    | CAD2    | P       |
|---------------------|---------|---------|---------|---------|---------|
| N                   | 43      | 56      | 51      | 24      | /       |
| Age, years          | 39.0±7.6| 41.2±11.2| 39.3±8.9| 36.9±9.5| 0.290   |
| Gender (M/F)        | 39/4    | 43/13   | 38/13   | 16/8    | 0.099   |
| Time after Tx, years| NA      | 5.0 (4.0-7.0) | 3.0 (1.0-5.0)* | 5.5 (3.0-8.5)** | 0.001   |
| TAC-C0, ng/ml       | NA      | 5.1 (4.3-6.7) | 5.8 (4.7-6.5) | 4.6 (3.4-6.7) | 0.137   |
| MMF/EC-MMF (N)      | NA      | 50/6    | 45/6    | 20/4    | 0.752   |
| MPA-AUC, mg·h/L     | NA      | 60.0±7.0| 68.7±28.5| 49.7±19.7| 0.535   |
| **Lipid profile**   |         |         |         |         |         |
| CHOL, mmol/L        | 4.6±0.7 | 4.8±0.9 | 5.2±1.2* | 4.7±1.5 | 0.046   |
| TBA, μmol/L         | 2.5 (1.7-3.4) | 2.8 (2.0-4.5) | 3.9 (2.5-6.4) | 2.7 (1.9-6.3) | 0.062   |
| TG, mmol/L          | 1.5 (1.1-1.7) | 1.4 (1.0-1.8) | 1.8 (1.4-2.5)** | 1.5 (1.0-2.1) | 0.011   |
| LDL-C, mmol/L       | 12.2 (10.0-15.1) | 13.1 (11.1-15.1) | 1.4 (1.1-1.6) | 1.1 (1.0-1.8) | 0.513   |
| HDL-C, mmol/L       | 2.8±0.6 | 2.8±0.7 | 3.0±1.1 | 2.8±1.1 | 0.208   |
| Statin (No/Yes)     | 43/0    | 53/3    | 41/10*  | 23/1    | 0.003   |
| **Liver function**  |         |         |         |         |         |
| ALT, IU/L           | 18 (16-27) | 14 (10-22) | 13 (11-19)* | 15 (11-23) | 0.027   |
| AST, IU/L           | 19 (17-23) | 17 (15-21) | 17 (15-19) | 19 (13-24) | 0.094   |
| ALP, IU/L           | 69 (60-82) | 75 (67-96) | 68 (59-83) | 73 (55-103) | 0.083   |
| GGT, IU/L           | 22 (17-30) | 21 (15-32) | 21 (16-27) | 25 (16-52) | 0.488   |
| **Kidney function** |         |         |         |         |         |
| SCR, μmol/L         | 85 (77-93) | 100 (84-110)* | 160 (146-187)** | 270 (255-298)** | <0.0001 |
| eGFR,ml/min/1.73m2  | 99.0 (89.2-109.5) | 76.1 (69.8-85.6)* | 41.5 (35.9-49.6)** | 23.5 (18.2-26.0)** | <0.0001 |
| Urea, mmol/L        | 4.7 (4.0-5.6) | 5.9 (5.0-7.2)* | 9.7 (8.2-11.6)** | 15.9 (12.8-22.9)** | <0.0001 |
| Cystatin C, mg/L    | 0.88 (0.81-0.93) | 1.15 (1.03-1.33)* | 1.88 (1.64-2.19)* | 3.28 (2.76-4.02)** | <0.0001 |
| **Urine protein**   |         |         |         |         |         |
| Negative            | 36      | 29      | 21      | 3       | <0.001  |
| +/-                 | 5       | 12      | 11      | 4       |         |
| +                  | 1       | 11      | 11      | 5       |         |
| ++/+++             | 1       | 3       | 8       | 11      |         |
| **Inflammatory index** |          |          |          |          |         |
| Neutrophil, 10⁹/L   | 3.2 (2.8-4.0) | 4.5 (3.5-5.8)* | 5.0 (4.0-6.7)* | 5.0 (3.5-7.0)* | <0.0001 |
| Lymphocyte, 10⁹/L   | 1.7 (1.5-2.1) | 2.0 (1.6-2.6) | 1.7 (1.3-2.4) | 1.1 (0.8-1.5)** | <0.0001 |
| NLR                 | 1.7 (1.3-2.2) | 2.1 (1.8-2.7)* | 2.7 (2.1-3.9)* | 4.2 (2.4-6.4)* | <0.0001 |

NA – not applicable; TAC-C0 – trough concentration of tacrolimus; MMF – mycophenolate mofetil; EC-MPS – enteric coating mycophenolate mofetil; MPA-AUC – mycophenolic acid area under the concentration-time curve; CHOL – total cholesterol; TBA – total bile acid; TG – triacylglycerol; LDL-C – low density lipoprotein cholesterol; HDL-C – high density lipoprotein cholesterol; ALT – alanine transaminase; AST – aspartate aminotransferase; ALP – alkaline phosphatase; GGT – γ-glutamyl transpeptidase; SCR – serum creatinine; eGFR – estimated glomerular filtration rate; NLR – neutrophil to lymphocyte ratio. * P<0.008 based on Bonferroni correction vs HC; ** P<0.017 or 0.008 based on Bonferroni correction vs STA; *** P<0.017 or 0.008 based on Bonferroni correction vs CAD1.
We found that the total BA levels, whether they were detected by enzyme cycling method or 15 BAs that were detected by LC-MS/MS, were comparable among groups. KTRs in the CAD2 group showed a much lower level of total SBAs than the other groups, but no difference was observed among HC, STA, and CAD1 groups. In addition, we observed that KTRs in the CAD2 group showed significantly lower levels of unconjugated BAs, especially unconjugated SBAs, than the other groups, while no obvious differences were found regarding total conjugated BAs, or glycine/taurine-conjugated BAs among the 3 KTR groups. To determine enzymatic activity changes in the liver and the gut, 4 types of metabolite ratios were evaluated to reflect the potential mechanisms, leading to the noted altered BA profile in KTRs. The ratios of SBAs to PBAs (total SBAs/PBAs and DCA/CA) significantly decreased in KTRs when compared to HCs, but they were not different among KTR groups. The ratio of LCA/CDCA was significantly lower in the CAD1 group compared to the HC and STA groups, but not different from the CAD2 group. However, the ratios of conjugated to unconjugated BAs (total conj./unconj. BAs, TCA/CA, GCA/CA, TCDCA/CDCA, and GCDC/CDCDCA) increased with the decline of renal function, and the CAD2 group had higher values than the other 3 groups. In addition, KTRs showed much higher values of TDCA/DCA ratio than HCs, and KTRs in the CAD2 group had the highest value.

**Associations Between BAs and Clinical Parameters**

Spearman correlation analyses were completed on all study participants. As shown in Figure 2, the results demonstrated that the sum of all 15 measured serum BAs, total PBAs, all individual PBAs, total SBAs, and some of the individual SBAs (UDCA, GUDCA, and TUDCA) were positively correlated with total bile acids (TBA), which was determined by total BA enzyme circulation method. The concentrations of taurine-conjugated BAs (including TCA, TCDCA, and TUDCA) and the ratios of Conj.BAs/Unconj.BAs, TCA/CA, TCDCA/CDCA, TDCA/DCA increased with the decline of renal function, while SBAs (DCA, LCA, GDC, GLCA, TDCA, and TLA), the ratios of SBAs/PBAs, DCA/CA, LCA/CDCDCA, and GLCA/LCA were inversely correlated with renal function. Additionally, SBAs (DCA, LCA, GDC, GLCA, TDCA, TDLC, and TLA) and the ratios of DCA/CA, GLCA/LCA were negatively correlated with the systemic inflammatory index NLR.

**Differential Correlation Network Analysis of BA Coregulation Between HCs and KTR Groups**

To comprehensively evaluate the perturbed BA coregulation underlying CAD development, we generated network plots to visualize differential correlations between various BAs pairs in the tested groups. First, the 3 KTR groups were separately compared to the HCs. The results demonstrated that there was a slight difference in BA coregulation between the HC and STA groups (Figure 3A). However, a total of 21 and 30 differential
**Figure 1.** Comparison of BA profiling, composition, and absolute concentrations of individual BAs among HC, STA, CAD1, and CAD2 groups. (A) Supervised partial least squares-discriminant (PLS-DA) score plot based on 15 BAs. (B) Differences in the composition of BAs in 4 groups. * P<0.05 for the comparison among 4 groups. * P<0.05 for the comparison among the 3 KTR groups. (C) Serum concentrations of individual BAs in 4 groups. ** P<0.008, *** P<0.001, **** P<0.0001 vs HC group. Figure 1A was generated in MetaboAnalyst (version 5.0, https://www.metaboanalyst.ca). Figure 1B and 1C were generated using GraphPad Prism (Version 9.0.1, GraphPad, La Jolla).

**Table 2.** Serum BAs subgroup concentrations and ratios in HCs and KTRs with different allograft function.

|                  | HC             | STA            | CAD1           | CAD2           | P    |
|------------------|----------------|----------------|----------------|----------------|------|
| 15 BAs (ng/mL)   | 1311.9 (864.5-1715.6) | 1370.4 (950.5-2200.2) | 1505.4 (1072.2-2706.1) | 1044.5 (673.0-1866.3) | 0.126 |
| Unconj.BAs (ng/mL) | 465.9 (257.9-777.3) | 333.6 (164.0-581.0) | 360.3 (182.5-778.9) | 127.1 (75.6-324.2)** | 0.002 |
| Conj.BAs (ng/mL)  | 748.2 (448.9-1006.2) | 960.1 (612.3-1691.4) | 1137.6 (650.4-2008.5)* | 855.5 (571.5-1465.0) | 0.026 |
| G-conj.BAs (ng/mL)| 670.6 (392.6-940.5) | 860.7 (574.0-1514.8) | 918.4 (568.0-1743.1) | 653.3 (444.8-1210.3) | 0.031 |
| T-conj.BAs (ng/mL)| 57.0 (36.0-88.7) | 80.0 (43.6-180.1) | 139.2 (52.8-207.1)* | 144.8 (94.5-286.2)* | 0.001 |
Table 2 continued. Serum BAs subgroup concentrations and ratios in HCs and KTRs with different allograft function.

|                | HC                    | STA                   | CAD1                  | CAD2                  | P        |
|----------------|-----------------------|-----------------------|-----------------------|-----------------------|----------|
| **PBAs (ng/mL)** | 845.2 (449.4-1228.4)  | 1012.9 (652.8-1584.5) | 1298.3 (778.7-2122.5)* | 877.1 (488.9-1623.1) | 0.034    |
| Unconj.PBAs (ng/mL) | 238.2 (95.3-443.2)    | 188.7 (97.5-408.3)    | 238.1 (96.2-653.7)     | 87.8 (41.9-266.3)*    | 0.031    |
| Conj.PBAs (ng/mL)  | 538.0 (322.9-784.8)   | 829.7 (454.8-1381.4)* | 897.0 (536.3-1622.9)* | 762.7 (421.7-1211.4) | 0.006    |
| G-conj.PBAs (ng/mL) | 483.9 (287.0-716.2)   | 737.9 (416.9-1198.5)* | 755.7 (334.6-1436.1)* | 326.3 (952.2)         | 0.009    |
| T-conj.PBAs (ng/mL) | 44.1 (29.9-72.3)      | 73.0 (39.0-163.4)*    | 130.2 (47.7-177.8)*    | 137.6 (82.9-248.0)*   | <0.001   |
| **SBAs (ng/mL)**  | 403.6 (252.8-536.7)   | 312.2 (193.9-484.2)   | 300.9 (120.6-675.7)    | 334.6 (73.8-359.5)*   | 0.008    |
| Unconj.SBAs (ng/mL) | 173.9 (124.0-312.8)   | 121.6 (61.4-205.0)*   | 110.8 (49.5-207.2)     | 59.3 (20.4-92.9)*     | <0.001   |
| Conj.SBAs (ng/mL)  | 165.3 (107.9-232.3)   | 178.5 (104.6-286.1)   | 134.2 (73.8-284.4)     | 104.1 (45.6-253.6)    | 0.324    |
| G-conj.SBAs (ng/mL) | 157.9 (94.5-219.9)    | 171.3 (93.5-277.5)    | 128.1 (70.8-278.3)     | 93.1 (42.2-238.4)     | 0.298    |
| T-conj.SBAs (ng/mL) | 8.7 (5.1-16.2)        | 7.8 (3.6-14.4)        | 6.5 (3.1-12.8)         | 6.5 (2.9-19.6)        | 0.675    |
| **SBAs/PBAs**     | 0.48 (0.35-0.66)      | 0.30 (0.14-0.47)*     | 0.22 (0.10-0.40)*      | 0.20 (0.11-0.28)*     | <0.001   |
| Conj./Unconj.BAs   | 1.85 (0.84-2.65)      | 3.07 (1.79-5.57)*     | 2.70 (1.12-6.12)       | 5.64 (2.97-10.93)*    | <0.001   |
| **CA/CDCCA**      | 0.34 (0.24-0.63)      | 0.25 (0.14-0.37)      | 0.44 (0.18-0.76)       | 0.45 (0.24-0.85)*     | 0.017    |
| DCA/CA            | 2.00 (0.42-4.70)      | 0.20 (0.04-2.75)*     | 0.06 (0.01-0.61)*      | 0.06 (0.20-0.58)*     | <0.001   |
| LCA/CDCCA         | 0.03 (0.01-0.10)      | 0.03 (0.008-0.08)     | 0.01 (0.004-0.03)*     | 0.02 (0.006-0.03)     | 0.013    |
| TCA/CA            | 0.15 (0.06-0.45)      | 0.45 (0.16-0.85)*     | 0.31 (0.08-1.26)       | 1.07 (0.73-3.26)**    | <0.001   |
| GCA/CA            | 1.74 (0.85-4.30)      | 4.20 (1.95-7.92)*     | 2.29 (0.92-7.92)       | 4.70 (3.56-15.92)**   | 0.002    |
| TCDCA/CDCCA       | 0.23 (0.11-0.66)      | 0.43 (0.18-0.86)      | 0.44 (0.17-1.20)       | 1.75 (0.91-3.34)**    | <0.001   |
| GCDCA/CDCCA       | 2.76 (1.14-5.12)      | 3.69 (1.82-7.83)      | 3.30 (1.38-6.57)       | 7.51 (3.44-14.04)**   | 0.011    |
| GDCDA/DCA         | 0.58 (0.37-0.99)      | 1.01 (0.48-1.85)*     | 0.69 (0.37-1.59)       | 0.96 (0.47-1.77)      | 0.045    |
| TDCA/DCA          | 0.07 (0.03-0.15)      | 0.15 (0.07-0.36)*     | 0.19 (0.06-0.38)*      | 0.36 (0.15-0.52)*     | <0.001   |
| GLCA/LCA          | 0.71 (0.39-1.14)      | 0.37 (0.23-0.90)*     | 0.38 (0.15-0.70)*      | 0.28 (0.00-0.43)*     | <0.001   |
| TLCA/LCA          | 0.04 (0.02-0.08)      | 0.03 (0.01-0.05)      | 0.03 (0.01-0.07)       | 0.03 (0.02-0.08)      | 0.010    |

G-unconj. – glycine unconjugated; T-conj. – taurine-conjugated; * P<0.008 based on Boferroni correction vs HC; † P<0.008 based on Boferroni correction vs STA; & P<0.008 based on Boferroni correction vs CAD1.
correlations of BA pairs were found between HCs versus CAD1 and HCs versus CAD2, respectively. This suggested that the worse the allograft function was, the larger the alteration of BA coregulation patterns was in KTRs with CAD when compared to HCs (Figure 3B, 3C). Then, we compared the BA coregulation patterns between the 3 KTR groups. The data showed that TUDCA and GUDCA were positively correlated with all conjugated PBAs in the STA group (blue line, ++/0 in Figure 4A), but not in KTRs from the CAD1 group, and the positive correlation between DCA and LCA (pink line, ++/+ in Figure 4A) was stronger in the STA group. In addition, 7 BA pairs (DCA and CDCA, CA, UDCA, LCA; TUDCA and UDCA, TDCA, GDCA) showed no significant correlations in the STA group, but were positively correlated with each other in the CAD1 group (Figure 4A). Similarly, 6 BA pairs had positive correlations in the STA group, but the correlation disappeared in the CAD2 group. By contrast, 18 BA pairs (brown and orange lines in Figure 4B) showed no correlations in the STA group but became positively correlated to each other in the CAD2 group. A total of 26 BA pairs were significantly differentially presented in the 2 CAD groups (Figure 4C).

Comparison of CYP7A1, CYP7B1, CYP27A1, and SLCO2B1 mRNA Levels in Peripheral Blood Among Groups

The total RNA was extracted from peripheral WBC. Relative mRNA expression levels of CYP7A1 and SLCO2B1 in peripheral blood were extremely low, while the abundance of CYP7B1 and CYP27A1 mRNA was higher. As shown in Figure 5, KTRs in the CAD2 group presented a significantly lower relative expression of CYP7B1 and CYP27A1 mRNA levels when compared to HCs and KTRs with stable allograft function, while no significant difference was observed among groups regarding the relative gene expressions of CYP7A1 and SLCO2B1. Correlation analysis demonstrated that CYP7B1 and CYP27A1 mRNA levels were positively but weakly correlated with eGFR levels with correlation coefficients of 0.215 (P=0.034) and 0.290 (P=0.0037), respectively (Figure 6). Moreover, CYP7B1 mRNA levels showed positive correlations with total, conjugated, unconjugated, and individual SBAs species (Figure 6).

Discussion

In this study, we utilized UPLC-MS/MS assay to determine the concentration of 15 serum BAs in a cohort of healthy humans and KTRs with different renal function to systematically evaluate their potential association with CAD courses. Notably, BAs composition was obviously shifted between HCs and KTRs. Unconjugated BAs (including PBAs and SBAs) were dramatically decreased, and skewed compositional profiles of unconjugated BAs toward conjugated forms were observed in KTRs with severely impaired renal function. Moreover, differential
Figure 3. Differential correlation analyses of various BAs between HC and KTR groups. Network analysis illustrates the differential correlation of BAs between study groups (A-C). Only BA pairs with significant differential correlations ($P<0.05$) were included. The colored lines connecting BAs indicates the direction and strength of the correlation groups, and the number of lines that follows indicates the number of BA pairs in the global networks exhibiting this pattern of change. For instance, the red line $+/0$ 1 in Figure 3A indicates that the correlation between GCA and GDCA was positive ($+$) in the HC group, but the correlation disappeared (0) in the STA group. Only 1 BA pair connected by red line in the network displayed this pattern of change ($+/0$). +$P<0.05$, ++$P<0.01$, are positively correlated; -$P<0.05$, is negatively correlated; 0, represents unrelated. Differential correlation coefficients between BA pairs were calculated with the “DGCA” package and the visualization of correlation networks were achieved using Cytoscape software (Version 3.7.2).
Figure 4. Differential correlation analyses of various BAs between KTR groups. Network analysis illustrates the differential correlation of BAs between the 3 KTR groups (A-C).
correlation network analysis revealed that distinct perturbations in intraclass (ie, PBA and SBA) and interclass (ie, unconjugated and conjugated) BA coregulation are involved in the progression of CAD in KTRs. In addition, CYP7B1 and CYP27A1 mRNA levels from peripheral blood significantly decreased with the decline of renal allograft function and were positively correlated with eGFR.

It has been well established that metabolic disorders such as post-transplant diabetes, hyperparathyroidism, and obesity are highly prevalent in KTRs and can adversely influence post-transplant graft outcomes [20,21]. Considering the diverse functions of BAs signaling in glucose and lipid homeostasis, energy balance, fibrosis, and immune homeostasis [7], it is worth exploring whether BAs are involved in allograft diseases of KTRs. Although liver and intestine are the main sites for BA synthesis and biotransformation, circulating BAs are believed to fluctuate almost synchronously with the enterohepatic circulation and may reflect the overall BAs metabolic status of the body [22]. The changes of serum BAs concentration and composition actually occur in various metabolic and inflammatory diseases [23]. Thus, in this study we focused on BAs species from serum. Intriguingly, there was no significant difference among groups in TBA measured by the enzyme cyclizing method, nor in total 15 BAs determined by UPLC-MS/MS. However, the BAs profiles were skewed with the deterioration of allograft function and KTRs in the CAD2 group showed the most distinct spectrum when compared to other groups. In general, the changes in BAs composition and concentrations showed a similar trend among all 4 groups. The BAs profile of KTRs was significantly different from HCs. Further comparison of individual BA species among KTR groups revealed that all unconjugated BAs (CDCA, UDCA, CA, DCA, and LCA) were dramatically reduced, and excessive transition from unconjugated BAs to conjugated form (evaluated from Conj./unconj.BAs, TDCA/DCA, and GLCA/LCA values) was observed in KTRs with severe impaired allograft function, which suggested that the BAs’ amidation process may play important roles in the progression of CAD in KTRs.

Figure 5. Relative gene expressions of CYP7A1, CYP7B1, CYP27A1, and SLCO2B1 in the peripheral blood of HC, STA, CAD1, and CKaD2 groups (A-D). Plots were generated by using GraphPad Prism (Version 9.0.1, GraphPad, La Jolla).
Correlation analysis between BAs and clinical parameters revealed that both total SBAs and individual SBAs (TDCA, GDCA, DCA, TLCA, GLCA, and LCA) were positively correlated with renal function and were negatively correlated with the inflammatory marker NLR. In addition, the ratios of SBAs to PBAs (DCA/CA and LCA/CDCA) that reflect the enzymatic activity toward shifted production of SBAs in the intestines demonstrated similar trends. SBAs are some of the most concentrated microbiota-derived gut metabolites, which are reported to be the key regulators of anti-inflammatory process through activation of the TGR5 BA receptor. Sinha et al found that externally treated colitis mice with DCA and LCA, but not CDCA, had significantly inhibited gene and protein generation of multiple inflammation-associated chemokines and cytokines (such as CCL5, CCL11, IL17A, IFNγ, and IL-1β), which was partly mediated through TGR5 expression on immune cells [24]. Given that inflammation is an essential process contributing to CAD, it is reasonable to speculate that the lower level of SBAs in the CAD2 group directly weakened the anti-inflammatory effect of SBAs and thus participates in the pathogenesis of CAD in KTRs. In addition, a series of studies have demonstrated that BAs are potent antimicrobials that control microbial diversity in host intestines, dysbiosis of which triggered signals regulating inflammation and were significantly associated with kidney graft outcomes [12,25-27]. Therefore, the downregulation of SBAs observed in the current study may have promoted the progression of CAD through indirect regulation of gut microbiota in KTRs. Further functional and basic experiments are required to elucidate whether there is an association between BAs metabolism and microbiota and their roles and interactions in the development of CAD in KTRs.

In addition to the absolute concentration and composition of BAs, we also conducted differential correlation network analyses to investigate the global interconnection of pairwise BAs, which identified significant correlation variations between groups. Overall, our data showed that larger gaps of disease stages between groups were associated with more BA pairs with significant differential correlations. KTRs in advanced CAD stage showed more differentially correlated BA pairs to HCs than those from STA versus HC groups. Moreover, considerable altered BAs co-regulation was found in pairwise comparison among the 3 KTRs.

Figure 6. Correlation between relative gene expression of CYP7B1 and CYP27A1 and eGFR levels (A, B). The correlation matrix displays the Spearman correlations between gene expression and BAs species (C). Figure 6A and 6B were generated by using GraphPad Prism (Version 9.0.1, GraphPad, La Jolla). Figure 6C was generated by using R (version 4.2.0, https://cran.r-project.org) and the “corrplot” package was used.
groups. The distinct BAs coregulation among groups therefore suggest that some BAs metabolism processes are related to the development of CAD in KTRs, which may account for the subsequent shifts of the compositional profiles of serum unconjugated BAs toward conjugated BAs, and SBAs toward PBAs. However, the exact bidirectionally regulatory mechanisms between CAD progression and BA metabolic pathway need further investigation.

Previously, due to the extremely low expression of some BA rate-limiting biosynthesis and transporter genes such as CYP7A1, CYP7B1, CYP27A1, and SLC20A1 existing in the extra-hepatic sites, BA synthesis was believed to exclusively occur in hepatocytes. Recently, this view was challenged by a study revealing that BAs biosynthesis also occurred in human extra-hepatic monocyte cells when induced by viral infection [28]. Therefore, considering the unavailability of liver tissue from participates, we only quantified the gene expression levels of peripheral blood CYP7A1, CYP7B1, CYP27A1, and SLC20A1 to further explore whether the development of CAD would influence the expression BA rate-limiting biosynthesis and transporter genes. Our data demonstrated that very little CYP7A1 and SLC20A1 mRNA was detected in peripheral blood, while CYP7B1 and CYP27A1 mRNA presented higher concentrations and showed a significant positive correlation with eGFR level. In addition, the circulating gene expression of enzymes showed no correlations with serum PBAs but displayed weak positive correlations with SBAs. These data indicated that gene expressions of some BA synthesis-related enzymes were limited during CAD progression in KTRs. However, whether this association was related to BA metabolism remains unknown based on existing data. Also, whether alloantigen would stimulate the extra-hepatic biosynthesis of BAs and then contribute to deterioration of allograft function requires further mechanistic elucidation.

There are several limitations to this study. First, as the kinetics and composition of BAs pool are affected by food intake and lifestyle, the lack of this information in the current study may have resulted in confounding biases affecting our results. Second, due to the inaccessibility of liver tissue from patients, we were unable to directly analyze the effect of CAD on rate-limiting enzyme expression or activity, and only ratios of BA pairs and circulating enzyme gene expression were determined to indirectly reflect the enzyme activity in the liver. Third, our data cannot address the relationship between BA profile and different pathologies because the majority of KTRs in the CAD groups did not undergo an allograft biopsy to confirm the pathological cause. Finally, gut microbiota was not assessed in the current study, which prevented us from further integrating and explaining the data on the dramatic changes of SBAs and, PBAs in the progression of CAD.

Conclusions

Our findings indicate that BA species profiling rather than total BA concentrations was significantly altered with the development and progression of CAD in KTRs. The shifts from unconjugated BAs toward conjugated BAs and SBAs toward PBAs, as well as the distinct pairwise BAs coregulation patterns, were the main characteristic changes in KTRs with CAD. Moreover, positive correlations were found between circulating BA synthesis-related enzyme CYP7B1 and CYP27A1 mRNA levels and eGFR. These results suggest that BAs metabolism plays a role in KT and may provide phenotypic clues for further elucidating the biological mechanisms of BAs in the occurrence and progression of CAD in KTRs.

Declaration of Figures’ Authenticity

All figures submitted have been created by the authors, who confirm that the images are original with no duplication and have not been previously published in whole or in part.
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