Different Gastrointestinal Microbiota Related to Bile-Acid Metabolism in Hypertensive Patients With or Without Nephropathy

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Research

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Keywords

Microbiota, Bile-Acids Metabolism, Hypertension, Nephropathy, Sequencing.

Introduction

Chronic kidney disease (CKD) is an important contributor to morbidity and mortality in the world. It has been recognized as a risk factor for cardiovascular disease and a risk multiplier in patients with hypertension and diabetes[1,2]. While impaired fasting plasma glucose, high blood pressure (HBP), high body-mass index, and a diet high in sodium were risk factors for CKD quantified in the Global Burden of Disease (GBD). This means early detection and treatment of diabetes, hypertension and CKD are readily available, inexpensive treatments to defer or prevent progression to the end-stage of kidney disease and cardiovascular events[3]. Despite major advances in the identification of key pathophysiological mechanisms and in treatment, hypertension remains one of the most important causes of acute and chronic cardiovascular disease[4,5] It is believed that the etiology of hypertension depends on the complex interplay of both genetic and environmental factors[6-8] [6-8]. With the sequencing technology development, more and more researchers switched their attention from dietary components to gastrointestinal microbiota (GM) [9-11].

The potential role of the GM in the altering health status of the hosts has drawn considerable attention. A number of microbial biomarkers specific to various diseases, such as colorectal cancer, liver cirrhosis, arthritis, type 2 diabetes, and atherosclerosis, have been discovered [12-15]. In addition, some studies found that changes in the structure of the intestinal flora are directly related to hypertension, and the richness and diversity of the intestinal flora in hypertensive patients have decreased significantly, specifically as *Prevotella* and *Klebsiella*

significant increase, while the content of beneficial bacteria decreased[16]. Intestinal flora induced hypertension can also affect host gene expression and basic metabolic processes[17]. Some scientists believed that changes in intestinal microflora cause mutation of hepatic flavin monooxygenase-related genes and increase the genes expression ability as well as flavin monooxygenase synthesis, which indirectly leads to an increase in the incidence of hypertension by affecting the metabolism of trimethylamine oxide[18] . It is generally believed that the intestinal flora can also produce metabolites, such as Farnesoid X Receptor (FXR) signal antagonists, TGR5 signal agonists, β-taurocholic acid and other primary bile acids [19,20], thereby promoting atherosclerosis and calcification of blood vessel walls by affecting cholesterol metabolism to indirectly affecting the occurrence and development of hypertension. In addition to multiple factors, such as genes, inflammation, and neuro-endocrine system, changes in the intestinal microbiota attract our attention as a new target for diagnosis and treatment of hypertension.

The previous study found total bile acids (TBA) level of hypertensive patients is significantly higher than that of non-hypertensive patients, and the TBA level is related to the grade of hypertension, and has a certain correlation with the structural damage of the kidney and heart. TBA, a metabolite of cholesterol in the liver, provides a signal from the host to maintain the balance of the intestinal microecology through hepato-intestinal circulation[21,22]. Researchers are currently focusing on Bile Salt Hydrolase (BSH) which can enzymolyze the C-24-N-acyl bond of bile salt bound by glycine or taurine into free bile acid[22]. Many bacteria, such as *Bacteroides*, *Clostridium*, *Lactobacillus*, and *Bifidobacteria*, have BSH activity, which means the imbalance of intestinal flora may influence the metabolism of bile acids[23] .

Considering TBA and GM are related to hypertension and kidney damage, and gut bacteria are involved in the metabolism of bile acid, we explored the relationship between GM and bile acid metabolism in patients with or without nephropathy.

Results

- **GM Diversity in Hypertension and Hypertension-related CKD (H-CKD)**

To determine whether changes in gut microbes are related to H-CKD, we studied the fecal microbiota in a well-characterized cohort that included 30 newly diagnosed hypertensive subjects without nephropathy and 11 patients with hypertensive nephropathy and 10 age, gender and BMI-matched normal controls. All selected patients provided stool samples under their approvals for 16S rRNA gene sequencing and analysis. There are no significant differences in other characteristics, except for a family history of early-onset cardiovascular and cerebrovascular disease: Hypertension vs Normal ($p=0.00136$); H-CKD vs Hypertension ($p=0.00106$), by Kruskal-Wallis test. The detailed demographic, clinical and hypertensive features of the cohort are in Table1.

In this cohort study, rarefaction was performed that the total number of operational taxonomic units (OTUs) of these samples were estimated, the sequencing data were abundant enough that few new OTUs were found. Based on the genera profile, the Shannon index was calculated to estimate the within-sample (α) diversity. The rate of acquisition in the Normal group exceeded the one of new OTUs acquisition in Hypertensive groups, which indicates that the bacterial enrichment level of the Hypertensive groups is lower (Figure 1a). However, compared with the control group by the Kruskal-Wallis test, α diversity of the H-CKD and the Hypertension groups did not significantly decrease at the genus level (Figure 1b). In order to

evaluate the overall diversity of microbial composition, we performed a principal coordinate analysis based on unweighted UniFrac distance and partial least square discriminant analysis (PLS-DA). The multivariate permutation test (MPT) showed that there were no significant differences in the composition of the intestinal taxonomy among the three groups (Figure 1c). However, PLS-DA showed that the difference and the distinction were significant among the groups when ignoring the random error (Figure 1d).

- **Bacteria Differential Abundance in Hypertension and H-CKD versus Controls**

To identify differential abundant taxa, we performed a linear discriminant analysis of effect (LEfSe) analysis on the fecal microbiota composition of the three groups. *Bacteroidetes*, *Firmicutes* and *Proteobacteria* were the most dominant phylum in all three groups (figure 2a). Meanwhile, *Firmicutes*/*Bacteroidetes* (F/B) in both H-CKD and Hypertensive groups is significantly lower than it in Normal groups. No significant difference observed between Hypertension and H-CKD groups: H-CKD F/B=2.30; Hypertension F/B=2.60; Normal F/B=0.90. H-CKD vs Normal p=0.035; Hypertension vs Normal， p=0.035; analyzed by Kruskal-Wallis test (Figure 2b). There were 19 bacterial taxa showing distinct relative abundances among the three groups; Linear Discriminant Analysis (LDA) score >2.0, $p<0.05$ (Figure. 2c; 2d). The contents of *Coprococcus eutactus* (p=0.006, LDA score=3.65) and *Ruminococcus torques* (p=0.042, LDA score=3.63) in the Hypertension with no nephropathy group were significantly increased, while the abundances of *Veillonella parvula* (p=0.017, LDA score=3.28) and *Oxalobacter formigenes* (p=0.019, LDA score=3.73) were higher in the Hypertension with nephropathy group. Compared with the healthy controls, the abundances of *Bacteroides ovatus* (p=0.047, LDA score=4.52), *Clostridium ramsum* (p=0.015, LDA

score=4.06), *Prevotella copri* (p=0.018, LDA score=4.68) and *Erysipelotrichia* (p=0.029, LDA score=4.01) in disease groups were significantly reduced.

- **Correlations between the Gut Microbiota and Bile Acid Metabolism**

In order to further study the correlation between clinical variables and disease-associated bacteria, we found that the mean levels of different types of bile acid were different in the H-CKD, Hypertension and the Normal groups (Figure 3a). Although the level of total bile acid did not change significantly, the content of Chenodeoxycholic acid (CDCA) was significantly higher in the both H-CKD and Hypertension groups than in the Normal group. Meanwhile, the content of CDCA in Hypertension group was higher than that in the H-CKD group: Normal vs Hypertension p=0.031; Hpertension vs H-CKD p=0.018, by Mann–Whitney U test (Figure 3b). The content of taurocholic acid of the H-CKD group was significantly higher than that of Hypertension group. However, there was no significance difference between Hypertension and Normal groups: Normal vs Hypertension p>0.051; Hypertension vs H-CKD p=0.029, by Mann–Whitney U test (Figure 3c).

In addition, we used Partial Spearman's rank-based correlation test to investigate whether the difference of bile acid profile is related to intestinal bacteria and found that deoxycholic acid was positive correlated with *Anaerostipes* (R = 0.46, P <0.01), *Firmicutes* (R = 0.37, P <0.05) and *Clostridium* (R = 0.38, P <0.05) ; it was negatively correlated with *Escherichia* (R = -0.43, P <0.01), *Enterobacteriaceae* (R = 0.48, P<0.05), and *Proteobacteria* (R = 0.47, P <0.05). Glycodeoxycholic acid was negatively correlated with *Escherichia* (R = -0.41, P <0.05) and was positively correlated with *Oscillospira* (R = 0.37, P <0.05). While *Oscillospira* was positively related to taurolithocholic acid (R = 0.38, P <0.05) and was negatively related to

CDCA (R = -0.37, P <0.05). The results showed that a negative correlation between *Prevotella* and taurochenodeoxycholic acid (R=-0.37, P<0.01) and a positive correlation between *Coprococcus* and glycolithocholic acid (R = 0.32, P <0.05) (Figure 3d)

We inferred the metagenomes from the 16s rRNA gene data and analyzed the functional potential of the gut microbiome by PIRUST to investigate the functional changes of the microbial among the H-CKD, Hypertension, and Normal groups. Metabolism, genetic and environmental information processing are the main functions of the human gastrointestinal microbiota. However, no differences of these main functions were observed among H-CKD, Hypertension and Normal groups (Figure 4a). Through principal coordinate analysis, we found that the dominance functions of membrane transport, cell motility, signal transduction, cellular processes and signaling in H-CKD and Hypertension groups were stronger than those in the Normal group (Figure 4b). We also observed D-Glutamine (p=0.014, Anova test) and D-glutamate metabolism, Glycosphingolipid biosynthesis - lacto and neolacto series (p=0.014, Anova test) were significantly different between Hypertension and Normal groups.

Discussion

There is increasing evidence that changes in the intestinal microbiome are related to hypertension. In this research, we studied the structure of the fecal microbiome of hypertension with or without nephropathy by 16S rRNA gene sequencing and analysis. The data showed that although there was no significant difference between healthy participants and hypertension with or without nephropathy, the microbial dysbiosis in the disease groups was characterized by changes in the relative abundance of the 19 bacterial genera. Then, we found that the CDCA level of hypertensive patients without nephropathy was higher than that

of the Normal group and the Hypertension with nephropathy group. It may be related to the bile salt hydrolysis of *Bacteroides* and *Clostridium*.

All patients in the Hypertension and H-CKD groups were newly diagnosed hypertension or hypertension-related kidney disease. Although several studies [9,16,24] discovered that the species richness and evenness of the hypertensive groups were low, the results of this study show that there is no statistical difference of the GM diversity in hypertensive patients with or without nephropathy groups and healthy controls. It may be related to the grade of hypertension[25] . Since only 51 subjects participated in our study, including 10 healthy controls, 30 hypertension patients, and 11 hypertensive nephropathy patients, we did not match them according to the grade of hypertension. We found that the F/B of the disease groups was significantly higher than that of the healthy control group, and *Bacteroides* is the dominant phylum of the healthy control group, consistent with previous animal studies [26]. This suggests that the composition of the intestinal microbiome is different in patients with simple hypertension or hypertensive nephropathy.

The most notable finding is that the characteristic flora of hypertension are *Coprococcus eutactus* and *Ruminococcus torques*, and the characteristic flora of hypertension-related nephropathy are *Veillonella parvula* (*V. parvula*) and *Oxalobacter formigenes* (*O. formigenes*), while the abundances of *Bacteroides ovatus*, *Clostridium Ramsum*, *Prevotella copri* (*P.copri*)， and *Erysipelothrix spp.* of hypertension and H-CKD groups are lower than healthy controls. However, to figure out whether the characteristic flora of these three groups can accurately distinguish between hypertension and hypertensive nephropathy, it requires further research.

The previous studies[27] have proved that *Coprococcus spp.* has a high affinity for carbohydrates and can decompose carbohydrates to produce large amounts of butyric acid and acetic acid, also known as Short-Chain Fatty Acids (SCFAs). SCFAs are ligands for many G protein-coupled receptors (such as Gpr41, Gpr43, Gpr109a) and Olfactory receptor 78 (Olfr78), mainly found in the renal afferent arteriole and smooth muscle cells of the peripheral vascular system[28-30] . Renal afferent arterioles are the main place for storage and secretion of renin, which can increase blood pressure through the renin-angiotensin-aldosterone pathway. The studies[31] have shown that the combination of SCFA and Gpr41 can decrease blood pressure, while the combination of Olfr78 can increase the secretion of renin to raise blood pressure.

This physiological opposition may be related to the different ligand affinities of these two receptors. High concentrations of SCFAs may activate Olfr78 to increase blood pressure. Therefore, it is speculated that *Coprococcus eutactus* can produce more SCFAs from carbohydrates, exceeding the upper limitation to promote the development of hypertension.

We also found that the abundance of *P. copri* is significantly lower in Hypertension and H-CKD groups. It is affected by diet and lifestyle. The previous research[32] suggested that *P. copri* in the intestine of people often taking in a large amount of high-fiber diet has a stronger ability to decompose carbohydrates, while *P. copri* in the intestine of people who have an omnivorous diet contains more leuB, a gene associated branched-chain amino acid synthesis related glucose tolerance and the risk of type-2 diabetes. However, Mushtaq-Nosheenand et al.[25] found that the abundance of *P. copri* in the hypertensive group was higher than that in the healthy controls. There is controversy regarding the beneficial or

harmful effects of *P. copri* strain due to different diet patterns screened different *P. copri* strains, so the relationship between *P. copri* and hypertension still need further research. In addition, in the diabetic mouse model, it was found that *P. copri* was the predominant intestinal type, which means *P. copri* is related to diabetes[33]. Our results are different from the above studies, because we have ruled diabetes and other comorbidities out of the research subjects.

In the hypertensive nephropathy group, *O. formigenes* was the characteristic bacteria that is able to decompose oxalic acid to produce formic acid [34]. Oxalate is one of the uremic toxins in End-Stage Renal Disease (ESRD) patients. The increase of *O. formigenes* content is a compensatory process for the increase in oxalate [35]. In addition, our study also found *V. parvula* is the dominant bacteria in the patients with hypertensive nephropathy. *V. parvula*, a gram-positive bacteria with Lipopolysaccharide (LPS), is an opportunistic pathogen that usually parasitizes the human gastrointestinal tract and oral cavity. In the patients with hypertensive nephropathy, the excretion of various metabolites, such as secondary bile acid and trimethylamine oxide disorders, can destroy the intestinal mucosal barrier and cause LPS to enter the blood, thereby driving the immune response to release inflammatory factors and promote blood vessel walls oxidation, then leading to aggravating kidney damage[36]. However, the specificity of *V. parvula* is insufficient because it is also found in autoimmune hepatitis[37] . The characteristic bacteria in hypertensive nephropathy we found might be different from those in other renal disease. A study on IgA nephropathy showed that the significantly increased genera in the IgAN group were *Escherichia-Shigella*, *Hungatella* and *Eggerthella*, all of which possess pathogenic potential[38,39]. Researchers identified

Ruminococcus (*Firmicutes phylum*) that was associated negatively with renal function and positively with an indoxyl sulfate metabolite as well as 2 genera in the Lachnospiraceae family that were associated positively with renal function and negatively with phenylacetylglutamine metabolite[40].Based on the correlation analysis of the influence of bile acid as an environmental factor on the intestinal flora, it was found that TCDCA was negatively correlated with *Prevotella spp.* Considering the hepatic and intestinal circulation mechanisms of bile acids, it is speculated that the increase in venous plasma TCDCA may inhibit the growth of *Prevotella spp.*, therefore increasing blood pressure. However, there was no difference in TCDCA levels between the Hypertension group and the Healthy group. The content of CDCA in the Hypertension group was significantly higher than that in the healthy control. An animal experiment found that rats fed CDCA continuously had blood pressure increased from the 7th day and remained at a higher level after 14 days of continuous feeding[41,42] . By correlation analysis, we found that CDCA levels are negatively correlated with *Oscillatoria spp.,* a gram-positive anaerobic bacterium. Studies showed that the level of *Oscillatoria spp.* was related to vegetarian diet patterns [43]. Hence, we speculated that a vegetarian diet may be beneficial to blood pressure control[44] . However, we didn't find the difference in *Oscillatoria spp.* among these three groups by LEfSe analysis.

Under the action of cholesterol 7-α dehydrogenase in the liver, cholesterol is decomposed to primary bile acids (CDCA and CA). Then Bile Acid-CoA Synthetase and Bile Acid-CoA (Amino Acid N-Acyltransferase) respectively combine with glycine and taurine to form GCDCA, GCA, TCA and TCDCA. They are then transported to the gallbladder for storage by a bile salt export pump. Under the action of bile salt hydrolase (BSH) in the intestine, the

conjugated bile acids are decomposed into free bile acids. CA and CDCA are dehydroxylated to form secondary bile acids DCA and LCA respectively, which can re-enter the liver through the relevant transport proteins of hepatic intestinal circulation[45]. The previous research[46,47] have found that *Bacteroides spp.* and *Clostridia spp.* have the effect of BSH to generate CA and CDCA. This is consistent with the increased levels of CDCA, *Bacteroides spp.* and *Clostridia spp.*in hypertensive participants in our study.

There was no statistical correlation found among Hypertension, H-CKD, and Normal groups in terms of DNA replication, repair, and certain amino acid metabolism. The PIRUST analysis, however, showed D-Glutamine and D-glutamate metabolism, Glycosphingolipid biosynthesis-lacto and neolacto series were significantly different (p=0.014). Mels et al.[48] found that the metabolism of D-Glutamine and D-glutamate is one of the metabolic pathways of hypertension. Oxidative inflammatory damage of the vascular endothelium is one of the pathogenesis of hypertension. Glutamate is an important substrate of glutathione which is an effective antioxidant in the human body. Therefore, it is speculated that the level of glutamate affects the level of glutathione that mediates the oxidative inflammation of the vessel wall and further participates in the occurrence of hypertension. Other researchers [49] found that the synthesis of neutral glycosphingolipids in the brain of stress-induced hypertension rats was significantly inhibited, and the content of glycosphingolipids was significantly negatively correlated with the prognosis of acute stroke. This suggested Glycosphingolipid biosynthesis-lacto and neolacto series may be related to the compilations of hypertension. To find out the relationship between glutamate and glycosphingolipid metabolism pathways and hypertension, it needs further research with more samples.

The main advantages of our research include collecting hypertensive nephropathy samples before renal protective interventions, using a cohort that includes any other diseases, discovering characteristic intestinal bacteria and preliminary discussion about the correlation between bile acid metabolism and GM in hypertension patients with or without nephropathy. Nevertheless, several limitations need to be noted. Firstly, all the samples were collected at the local hospital. The number of the samples in this cohort is not large enough, so our research is not universal but representative. Secondly, 16S rRNA gene sequencing has some disadvantages: 1.The number of copies of each genome can vary; 2. Although they tend to be specific taxa, there may be variation among strains; 3. The relative abundance measurement is unreliable due to amplification bias; 4.Genetic diversity tends to exaggerate estimates of diversity[50]. Thirdly, this study provides evidence of association rather than causation. Nonetheless, it provides new insights for preventing hypertension with or without nephropathy and also increases the possibility of using bacterial bio-markers to diagnose hypertensive nephropathy.

Materials and Methods

- **Study Design and Sample Collection**

All patient samples were newly diagnosed as primary hypertension or hypertension-related nephropathy in Renji Hospital, School of Medicine, Shanghai Jiaotong University, from September 2019 to December 2019. The diagnosis of hypertension was that systolic blood pressure (SBP) is ≥140 mmHg and/or the diastolic blood pressure (DBP) is ≥90 mmHg after repeated examination in hospital[51] .Patients who meet the following two conditions can be diagnosed with hypertension-related nephropathy: (1) Essential hypertension; (2)

30≤eGFR≤60ml/min and/or urine albumin-creatinine ratio (ACR)≥30mg/g. Finally, we collected a total of 51 individual stool samples for this research, including 10 healthy controls, 30 hypertensive patients with no nephropathy, and 11 patients with hypertensive nephropathy. Those healthy controls were selected from the volunteers to match the disease groups of patients with age, gender and body mass index (BMI). The exclusion criteria for the three groups were: (1) Patients with a history of diabetes or fasting blood glucose higher than 110 mg/dL(6.11 mmol/L) or urinary albumin /creatinine >2.5; (2) Patients with primary or secondary increasing urinary protein or kidney damage such as rheumatic connective tissue disease, acute and chronic glomerulonephritis, multiple myeloma et al; (3) Patients with secondary hypertension such as protoaldehyde/adrenaline adenoma/renal artery stenosis/pituitary disease, etc.; (4) Cerebrovascular diseases occur within 2 months, such as cerebral hemorrhage, cerebral infarction, transient ischemic attack, subarachnoid hemorrhage, angina or myocardial infarction, PTCA, CABG, persistent atrial fibrillation and atrial flutter, combined with stenotic arteriosclerosis etc.; (5) Patients with a history of congenital and rheumatic heart disease, and cardiac insufficiency (NYHA cardiac function class III or higher); (6) Patients with a history of acute infection, acute bleeding or blood transfusion within 2 months, and taking any antibiotic and microbial preparation within 1 month; (7) Patients with severe liver and kidney dysfunction (eGFR less than 30), malignant tumors, inflammatory bowel disease, acute and chronic pancreatitis. This study was approved by the Ethics Committee of Renji Hospital, School of Medicine, Shanghai Jiaotong University, and all the subjects included in the study signed an informed consent. Stool samples from the individuals were all freshly collected in the hospital and frozen at −80°C

immediately after sampling. Meanwhile, we tested the fasting blood bile acids metabolism curve by high-performance liquid chromatography-mass spectrometry.

- **DNA Extraction and 16S rRNA Gene Sequencing**

We performed 16S rRNA gene sequencing. Briefly, after extracting bacterial genomic DNA by the E.Z.N.ATM Mag-Bind Soil DNA Kit(OMEGA, Switzerland)，the 16S rRNA gene V3-V4 region was amplified by PCR and then sequenced by MiSeq platform (Illumina, San Diego, California, USA)

- **Bioinformatic Analysis of 16s rRNA Gene Sequencing**

The 16S rRNA gene sequencing data were analyzed by Quantitative Insights into Microbial Ecology (QIIME)[52]. Then, we used vsearch plugin to cluster sequences into operational taxonomic units (OTUs) at 97% identity and the taxonomy was assigned against the Greengenes database (V.13.8). After filtering, each sample gets an average of 72473 reads of alpha value, and uses q2 diversity to perform β diversity analysis at 2500 rare sampling depths. The metagenomes of gut microbiome were imputed from 16S rRNA gene sequences by PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States)[53] .

- **Statistical Analysis**

We applied LEfSe analysis to determine the abundance of taxa or pathway differences between cases and controls[54]. This method firstly uses the non-parametric factorial Kruskal-Wallis sum-rank test to detect features with significant differential abundance and then uses linear discriminant analysis (LDA) to calculate the effect size of each feature.

The relationship between the microbiota and bile acid profile was analyzed by canonical correspondence analysis/redundancy analysis (CCA/RDA)[55]. The R pheatmap package was used to analyze whether the microbiome was significantly correlated with bile acid, and to calculate the Spearman coefficient[56]. The function of the microbiome was analyzed by the Kyoto Encyclopedia of Genes and Genomes （KEEG） [57], the predictive function was analyzed by ANOVA and Dunnett's test to see if there were significant differences among the groups.

Statistical analyses were performed with R (V 3.51). We used multivariate stepwise logistic regression analysis and the caret package to identify the genera that best distinguished hypertensive patients with or without nephropathy from controls. Other statistical analysis tools included Fisher's exact test, Kruskal-Wallis test and partial Spearman's rank correlation (PResiduals package).

Conclusion

The overall composition of the intestinal microbes has no significant difference in diversity, compared with hypertension and healthy controls. F/B increases significantly in hypertensive patients.

The intestinal characteristic flora of hypertension with no nephropathy is *Coprococcus eutactus* and *Ruminococcus torques*; this flora of hypertension with nephropathy is *Veillonella parvula* and *Oxalobacter formigenes*; and it of the healthy population is *Bacteroides ovatus*, *Clostridium ramsum*，*Prevotella copri* and *Erysipelothrix spp.*

The characteristics of the intestinal flora may be used as a diagnostic biomarker for hypertension-related nephropathy. The use of probiotics may help prevent nephropathy and

cardiovascular disease.

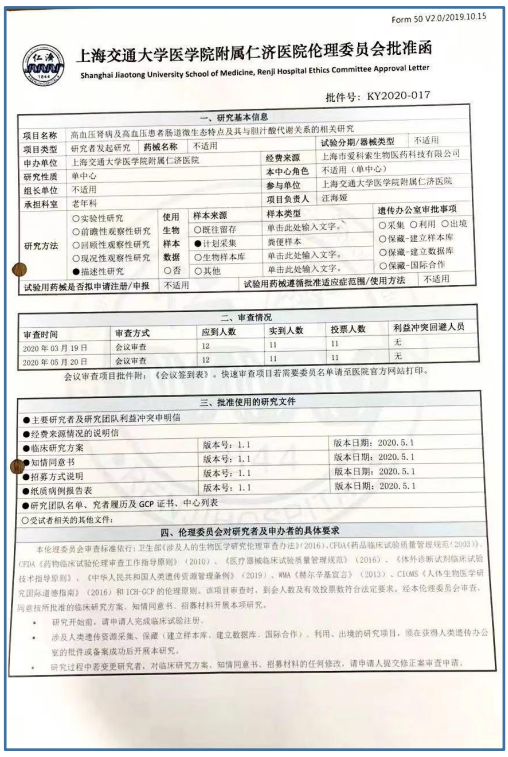
The CDCA level of hypertensive patients without nephropathy is higher than that of healthy controls and hypertensive nephropathy patients. It may be related to the bile-salt hydrolysis of *Bacteroides* and *Clostridium*.

Declarations

- **Ethics approval and consent to participate**

  This study was approved by the Ethics Committee of Renji Hospital, School of Medicine, Shanghai Jiaotong University, and all the subjects signed the consent form to participate in the study.

  Following are the pictures of “Shanghai Jiaotong University School of Medicine, Renji Hospital Ethics Committee Approval Letter”. The “Approval Document No.” is KY2020-017.



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上海交通大学医学院附属仁济医院伦理委员会批准函

Shanghai Jiaotong University School of Medicine, Renji Hospital Ethics Committee Approval Letter

批件号：KY2020-017

一、研究基本信息

项目名称	高血压肾病及高血压患者肠道微生态特点及其与胆汁酸代谢关系的相关研究				
项目类型	研究者发起研究	药械名称	不适用	试验分期/器械类型	不适用
申办单位	上海交通大学医学院附属仁济医院			经费来源	上海市爱科索生物医药科技有限公司
研究性质	单中心			本中心角色	不适用（单中心）
组长单位	不适用			参与单位	上海交通大学医学院附属仁济医院
承担科室	老年科			项目负责人	汪海娅
研究方法	○实验性研究	使用生物样本数据	样本来源	样本类型	遗传办公室审批事项
	○前瞻性观察性研究		○既往留存	单击此处输入文字。	○采集 ○利用 ○出境
	○回顾性观察性研究		●计划采集	粪便样本	○保藏-建立样本库
	○现况性观察性研究		○生物样本库	单击此处输入文字。	○保藏-建立数据库
	●描述性研究		○否 ○其他	单击此处输入文字。	○保藏-国际合作
试验用药械是否拟申请注册/申报		不适用	试验用药械遵循批准适应症范围/使用方法		不适用

二、审查情况

审查时间	审查方式	应到人数	实到人数	投票人数	利益冲突回避人员
2020 年 03 月 19 日	会议审查	12	11	11	无
2020 年 05 月 20 日	会议审查	12	11	11	无

会议审查项目批件附：《会议签到表》。快速审查项目若需要委员名单请至医院官方网站打印。

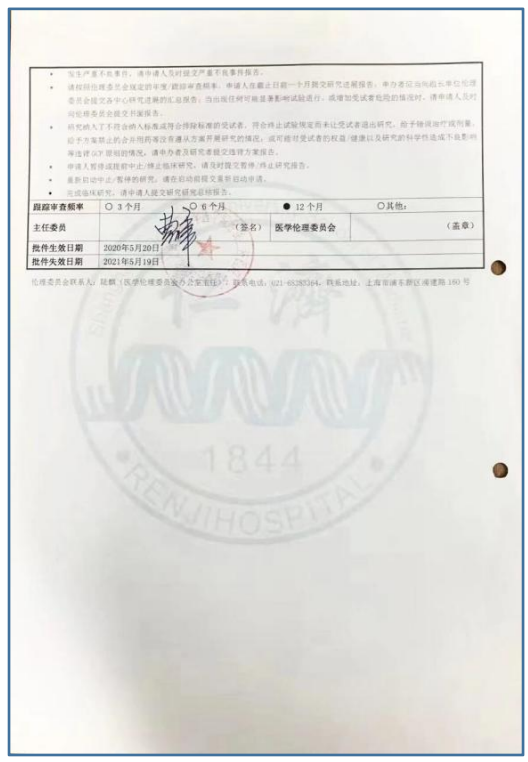
三、批准使用的研究文件

●主要研究者及研究团队利益冲突申明信		
●经费来源情况的说明信		
●临床研究方案	版本号：1.1	版本日期：2020.5.1
●知情同意书	版本号：1.1	版本日期：2020.5.1
●招募方式说明	版本号：1.1	版本日期：2020.5.1
●纸质病例报告表	版本号：1.1	版本日期：2020.5.1
●研究团队名单、完者履历及 GCP 证书，中心列表		
○受试者相关的其他文件：		

四、伦理委员会对研究者及申办者的具体要求

本伦理委员会审查标准依行：卫生部《涉及人的生物医学研究伦理审查办法》（2016）、CFDA《药品临床试验质量管理规范》（2003）、CFDA《药物临床试验伦理审查工作指导原则》（2010）、《医疗器械临床试验质量管理规范》（2016）、《体外诊断试剂临床试验技术指导原则》、《中华人民共和国人类遗传资源管理条例》（2019）、WMA《赫尔辛基宣言》（2013）、CIOMS《人体生物医学研究国际道德指南》（2016）和 ICH-GCP 的伦理原则。该项目审查时，到会人数及有效投票数符合法定要求。经本伦理委员会审查，同意按所批准的临床研究方案、知情同意书、招募材料开展本项研究。

- 研究开始前，请申请人完成临床试验注册。
- 涉及人类遗传资源采集、保藏（建立样本库、建立数据库、国际合作）、利用、出境的研究项目，须在获得人类遗传办公室的批件或备案成功后开展本研究。
- 研究过程中若变更研究者、对临床研究方案、知情同意书、招募材料的任何修改，请申请人提交修正案申查申请。



跟踪审查频率	○ 3 个月	○ 6 个月	● 12 个月 ○其他：
主任委员	（签名）	医学伦理委员会	（盖章）
批件生效日期	2020年5月20日		
批件失效日期	2021年5月19日		

- **Consent for publication**

There manuscript contains no individual person's data in any form.

- **Availability of data and materials statement**

The data that support the findings of this study are available from the first author and the corresponding author upon reasonable request.

- **Competing interests**

The authors declare that they have no competing interests.

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None

- **Authors' contributions**

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1	Xin Li	Signed first for joint first author.	Brewing and designing experiments; Research implementation; Data collection; Data analysis/interpretation; Statistical analysis; Article drafting.
2	Li Wang	Signed second for joint first author.	Brewing and designing experiments; Research implementation; Data collection; Experiment; Statistical analysis; Article draft revision.
3	Shaojun Ma	The second author.	Data collection; Samples collection and management.
4	Shaohui Lin	The third author.	Sample management; Supporting contribution.
5	Chunyan Wang	Signed second co-corresponding author	Brewing and designing experiments; Research implementation; Genome sequencing and analysis/interpretation; Research funding; Critically review the intellectual content of the article.
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Figures and Tables

- **Figure 1**

Figure 1a

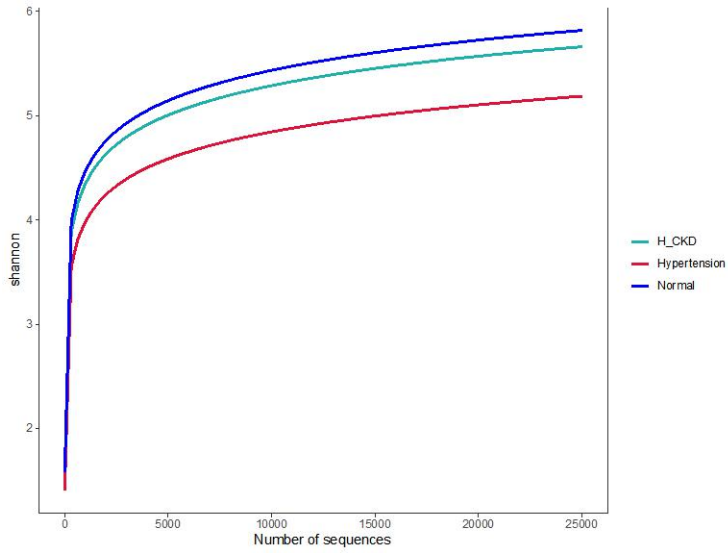


Figure 1b

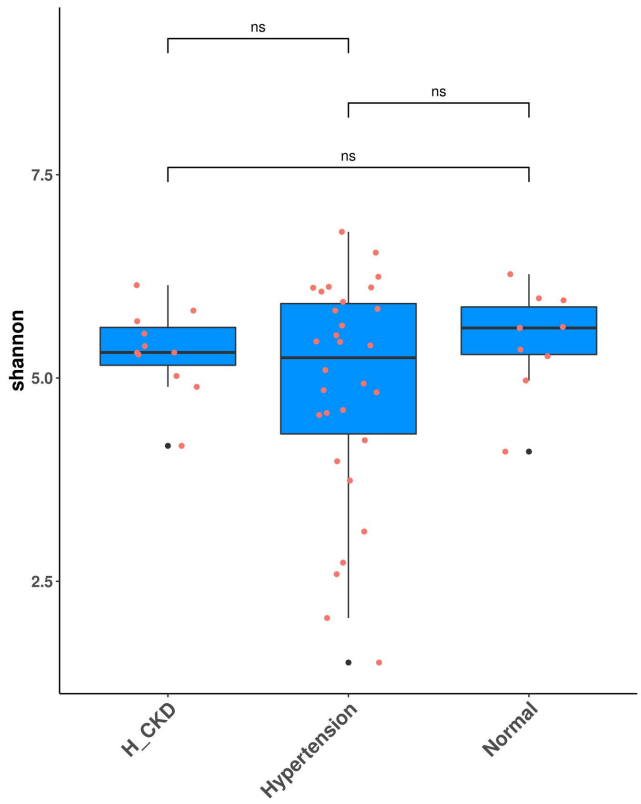


Figure 1c

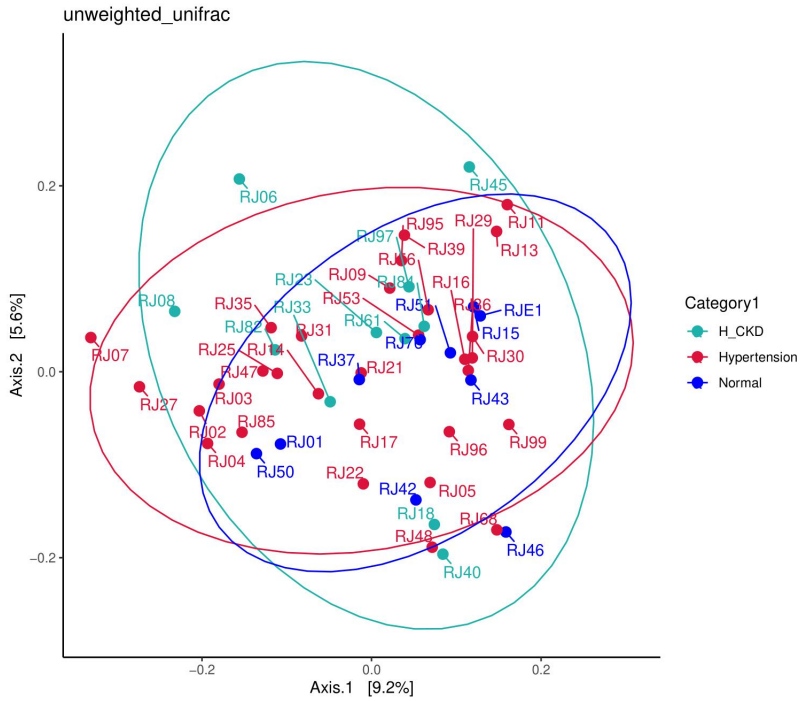


Figure 1d

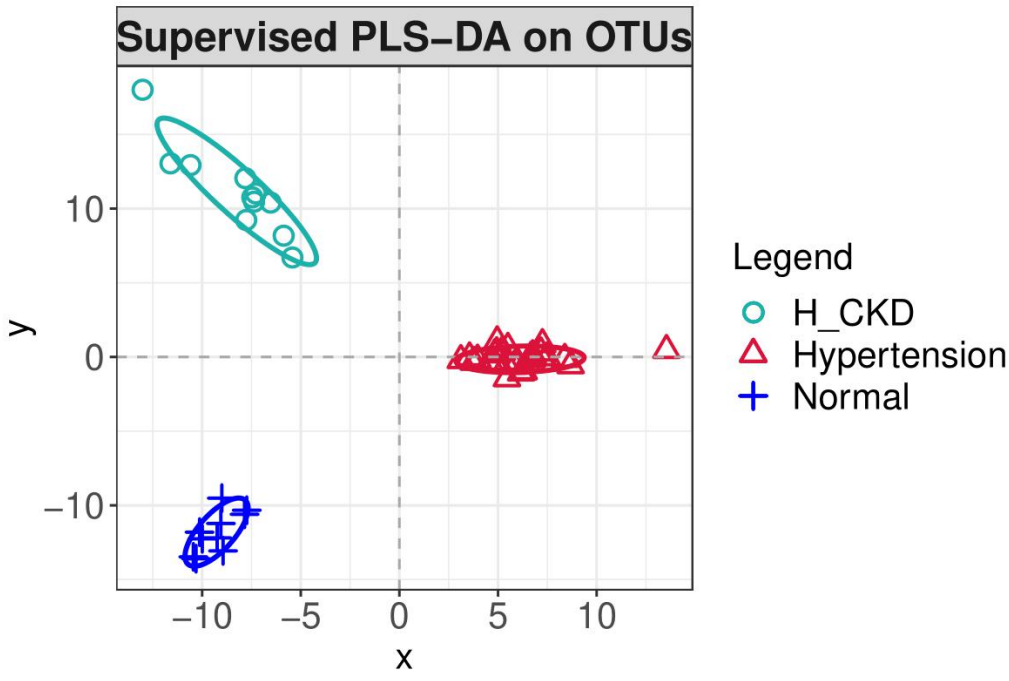


Figure 1: Comparisons of Alpha-diversity and Beta-diversity among the Groups of H-CKD, Hypertension and Normal

(a) Rarefaction curves for the mean of Shannon index in the three groups. The curve in each group is near smooth when the sequencing data is abundant enough with few new OTUs undetected; (b) Comparison of diversity in the three groups accessed by Shannon index. Compared with the control group tested by Kruskal-Wallis, the α diversity of H-CKD group and hypertensive groups did not significantly decrease at the genus level; (c) Based on the unweighted UniFrac matrix, PCoA showed that the overall fecal microbiota composition of the three groups was similar; (d) PL

(b) S-DA shows the significant distinction among the three groups.

OTU, operational taxonomic units; PCoA, principal coordinate analysis; PLS-DA, Partial Least Squares Discrimination Analysis.

- **Figure 2**

Figure 2a

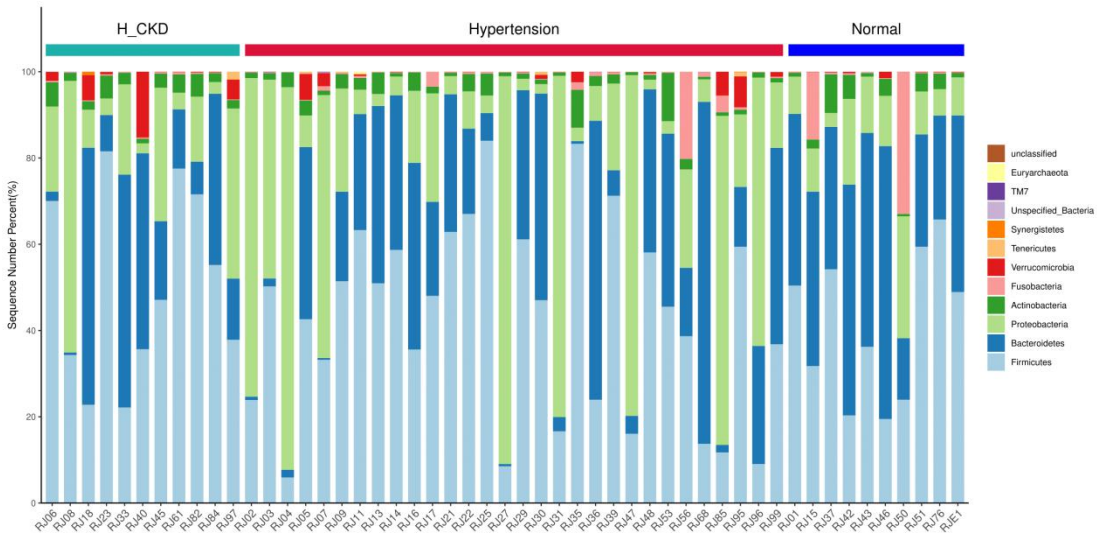
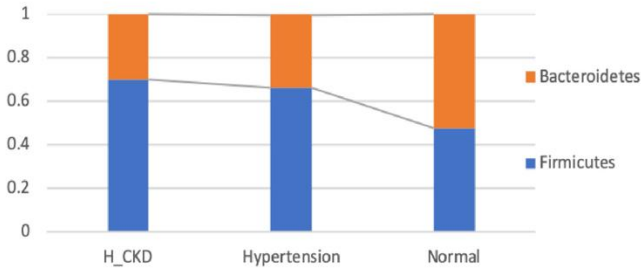


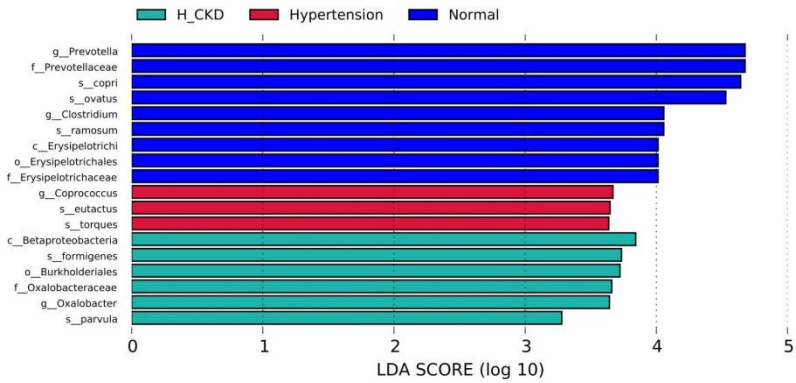
Figure 2b



F/B: H-CKD vs Normal p=0.035

Hypertension vs Normal p=0.041

Figure 2c



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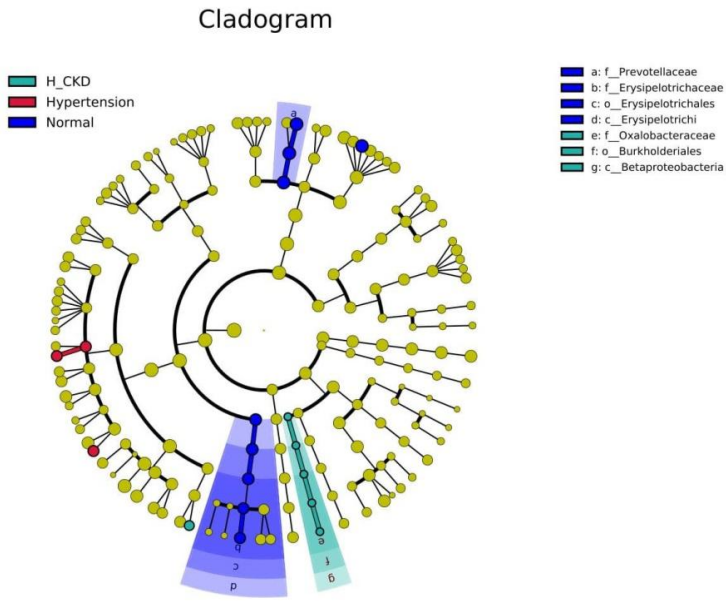
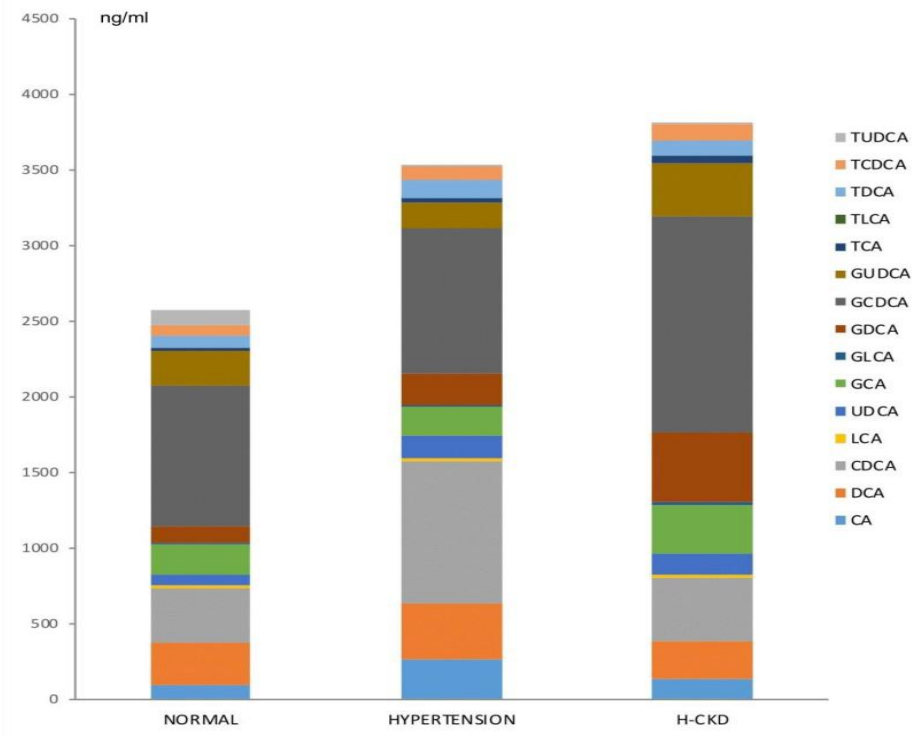


Figure 2：Variations of Fecal Microbiota in H-CKD, Hypertension and Normal Groups

(a) Relative proportions of bacterial phyla in H-CKD (n=11), hypertension (n=30) and normal (n=10); (b) Rate of relative proportions of *Firmicutes* and *Bacteroidetes* (F/B). F/B in both H-CKD and hypertensive groups is significantly lower than it in healthy controls; (c)(d) Linear discriminant analysis (LDA) effect size analysis revealed that the relative abundance of 19 bacteria were significantly different among H-CKD, hypertension and normal groups.

- **Figure 3**

Figure 3a



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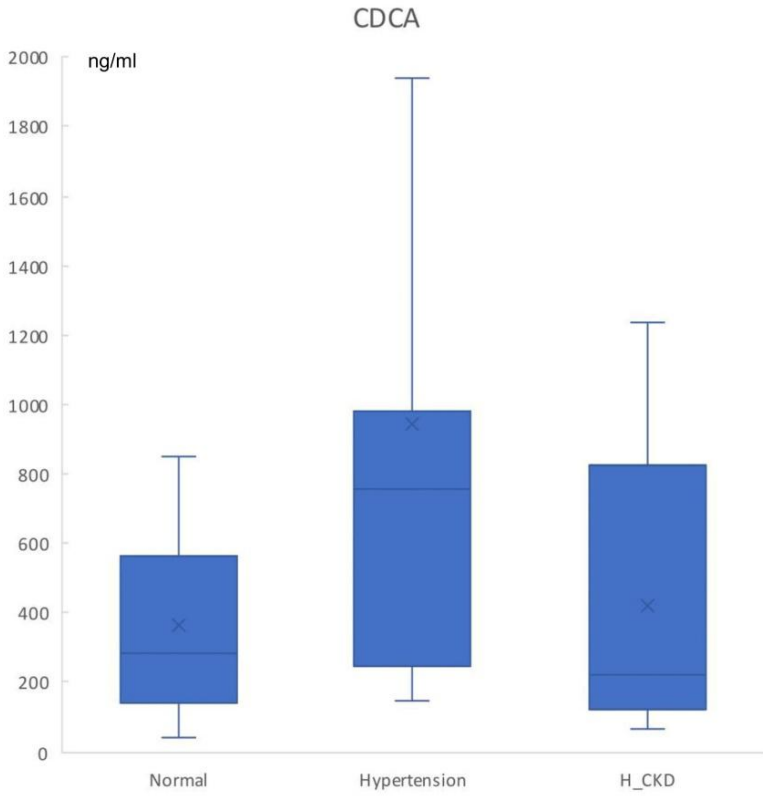
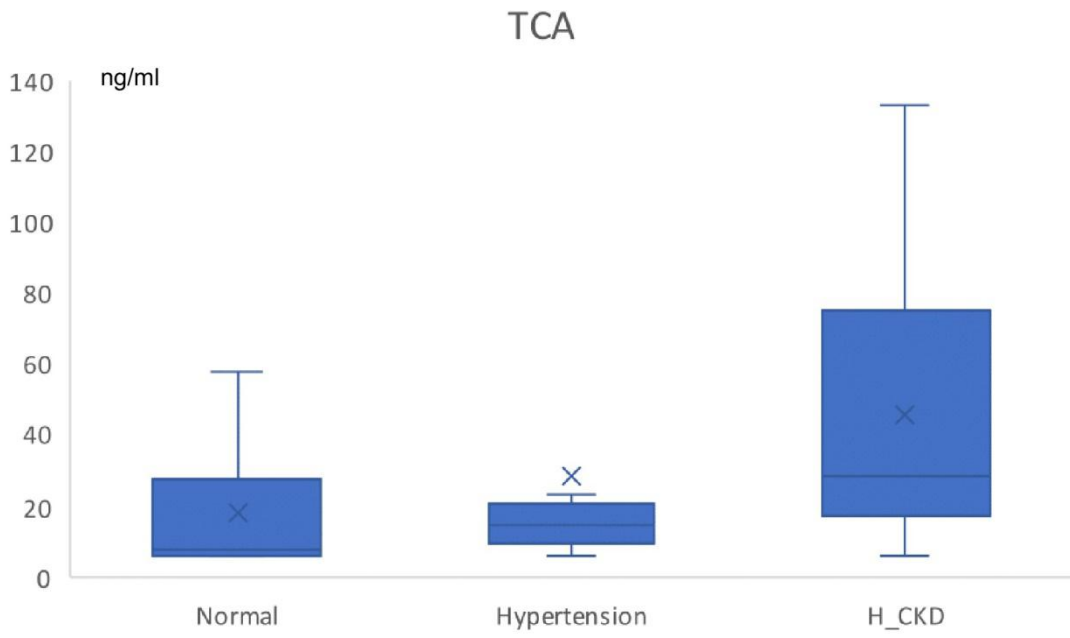


Figure 3c



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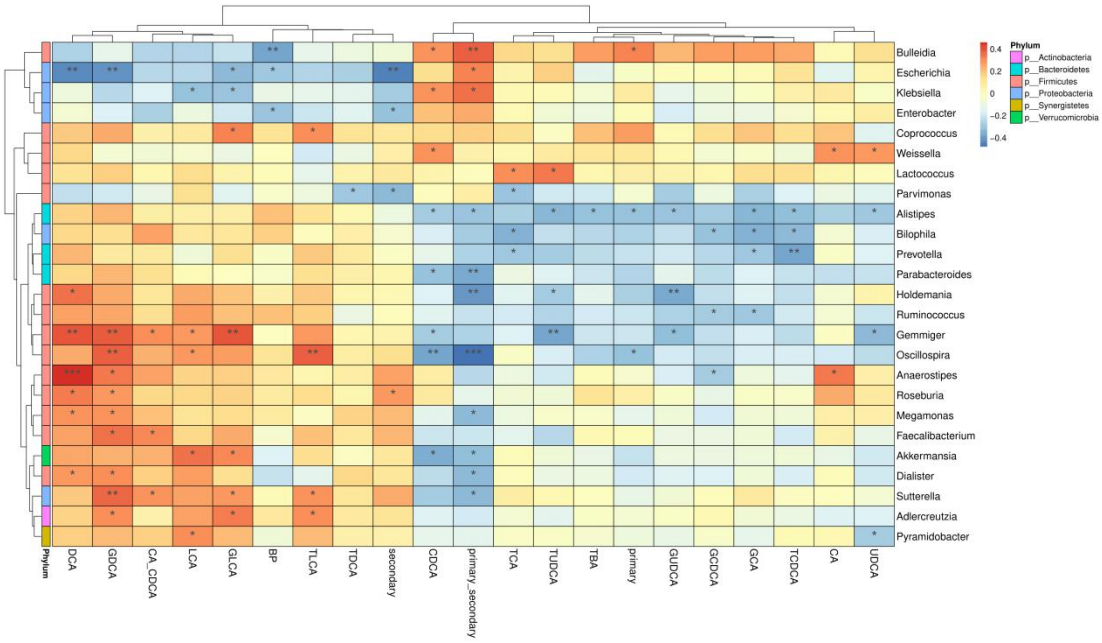

Figure 3: The Relationship between Gut Microbiota and Bile Acid Metabolism in H-CKD and Hypertension Groups.

(a) Different contents of bile acid metabolism among the Normal, H-CKD and Hypertension groups. (b) The concentration of CDCA is significantly different among groups by hypertension and the normal group was observed. (d) The heatmap shows that partial Spearman correlation coefficients between genera and bile acid metabolism. * ：0.01<p≤0.05，**：0.001<p≤0.01，***：p≤0.001.

- **Figure 4**

Figure 4a

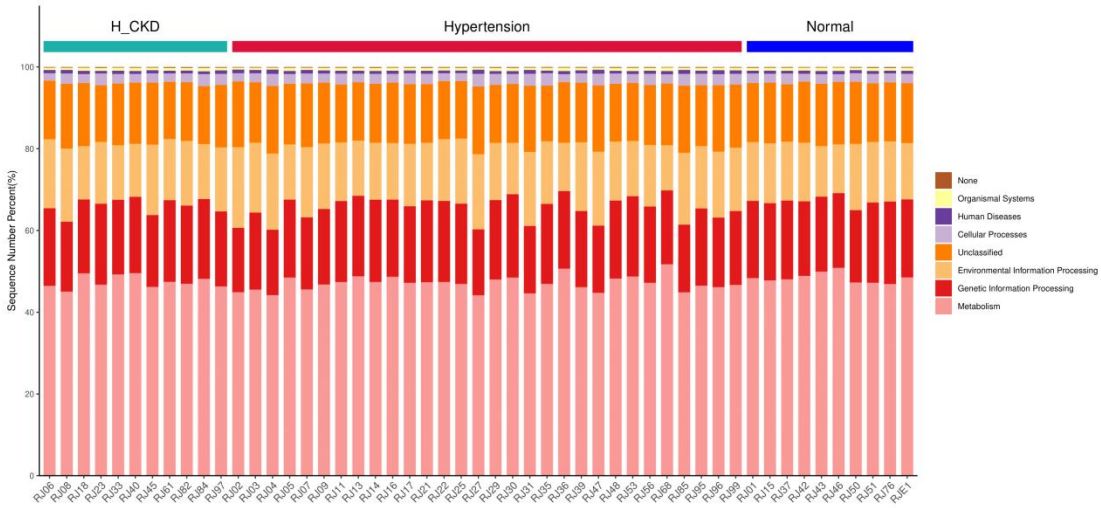

Figure 4b

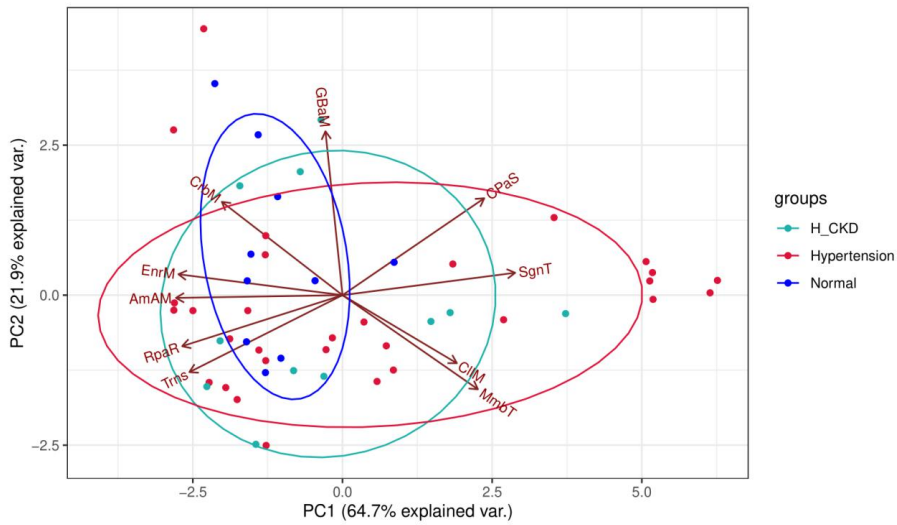

Figure 4: Predicted Metagenomes Functional Analysis

(a) Different relative abundance predicted function of gut bacteria in all individuals. (b) PC1 represents the first principal component, and the percentage represents the contribution of

the first principal component to the sample difference; PC2 represents the second principal component, and the percentage represents the contribution of the second principal component to the sample difference. The direction and length of the arrow represent the degree of dominance of the prediction function in the sample/group in that direction, the longer the length, the stronger the dominance.

- **Table 1. Characteristics information of Experimental Groups**

Characteristics	Normal n=10	HBP n=30	H-CKD n=11	P Normal vs HBP	P HBP vs H-CKD
Demographics					
Age,years median (Q1-Q3)	45 (42.0-51.5)	52.0 (40.0-65.3)	55.0(43.5-64.5)	0.109	0.116
Gender,female,n(%)	5(50.0%)	14(46.7%)	5（45.5%）	0.067	0.062
BMI,Kg/m^2, median,(Q1-Q3)	22.3(21.0-24.3)	24.1(22.9-25.8)	23.1(22.4-24.8)	0.055	0.054
Behavior					
Smoke,yes,n(%)	1(10.0%)	12(40.0%)	5(45.6%)	0.05	0.055
Alcohol,yes,n(%)	2(20.0%)	10(33.3%)	4(36.4%)	0.074	0.084
Hypertension					
History,yes,n(%)	3(33.3%)	23(76.7%)	8(63.9%)	0.001	0.001
Hgrade-1,n(%)	--	4(13.3%)	1(9.0%)	--	--
Hgrade-2,n(%)	--	6(20.0%)	6(54.5%)	--	--
Hgrade-3,n(%)	--	6(20.0%)	4(39.1%)	--	--
Drug,no,n(%)	--	9（30.0%）	6（54.5%）	--	0.032
Time, n(%)					
0-5years	--	22(73.3%)	7(63.6%)	--	0.010
5-10years	--	6(20.0%)	3(27.2%)	--	--
>10years	--	2(6.7%)	1(9.1%)	--	--

BMI: Body Mass Index; History: Family history of early-onset cardiovascular and cerebrovascular disease ; Hgrade：Hypertensive grade; Drug：Five categories of commonly used antihypertensive drugs; Time: Time of hypertension or H-CKD.

- **Table2. Abbreviation and Full Terms of Bile Acids**

Abbreviation Terms	Full Terms
CA	Cholic acid
DCA	Deoxycholic acid
CDCA	Chenodeoxycholic acid
LCA	Lithocholic acid
UDCA	Ursodeoxycholic acid
GCA	Glycocholic acid
GLCA	Glycolithocholic acid
GDCA	Glycodeoxycholic acid
GCDCA	Glycochenodeoxycholic acid
GUDCA	Glycoursodeoxycholic acid
TCA	Taurocholic acid
TLCA	Taurolithocholic acid
TDCA	Taurodeoxycholic acid,
TCDCA	Taurochenodeoxycholic acid
TUDCA	Tauroursodeoxycholic acid

Table3. Abbreviation and Full Terms of Metabolic Functions

Abbreviation Terms	Full Terms
CPaS	Cellular Processes and Signaling
Trns	Translation
EnrM	Energy Metabolism
RpaR	Replication and Repair
AmAM	Amino Acid Metabolism
CrbM	Carbohydrate Metabolism
MmpT	Membrane Transport
SgnT	Signal Transduction
MmbT	Membrane Transport
ClIM	Cell Motility
GBaM	Glycan Biosynthesis and Metabolism
EnrM:	Energy Metabolism

Figures

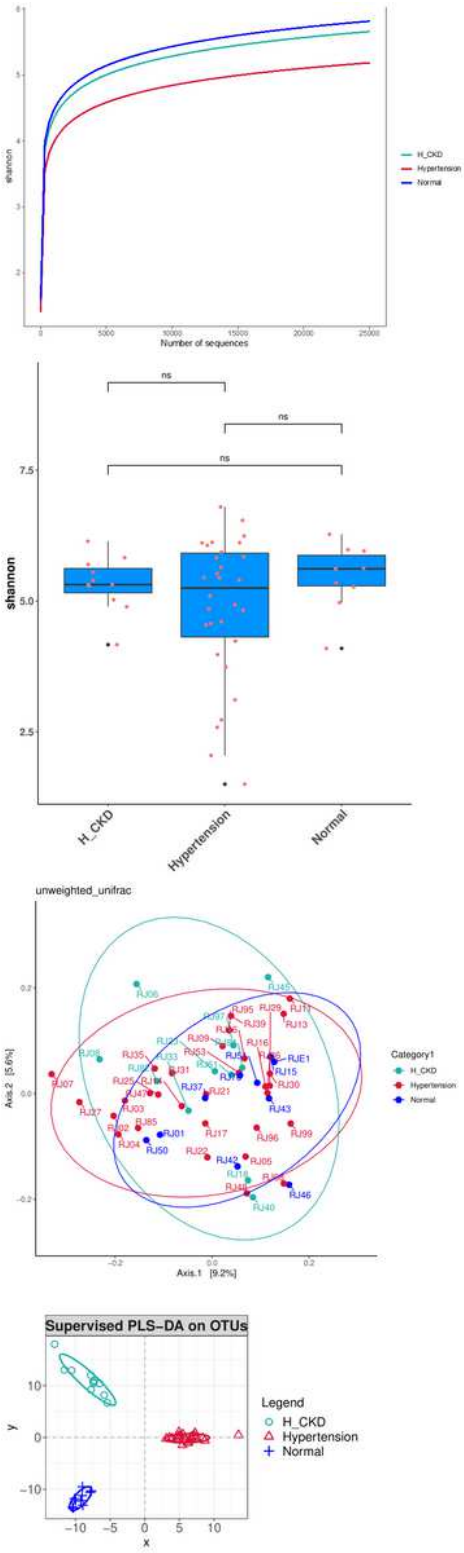

Figure 1

Comparisons of Alpha-diversity and Beta-diversity among the Groups of H-CKD, Hypertension and Normal (a) Rarefaction curves for the mean of Shannon index in the three groups. The curve in each group is near smooth when the sequencing data is abundant enough with few new OTUs undetected; (b) Comparison

of diversity in the three groups accessed by Shannon index. Compared with the control group tested by Kruskal-Wallis, the α diversity of H-CKD group and hypertensive groups did not significantly decrease at the genus level; (c) Based on the unweighted UniFrac matrix, PCoA showed that the overall fecal microbiota composition of the three groups was similar; (d) PL (b) S-DA shows the significant distinction among the three groups. OTU, operational taxonomic units; PCoA, principal coordinate analysis; PLS-DA, Partial Least Squares Discrimination Analysis.

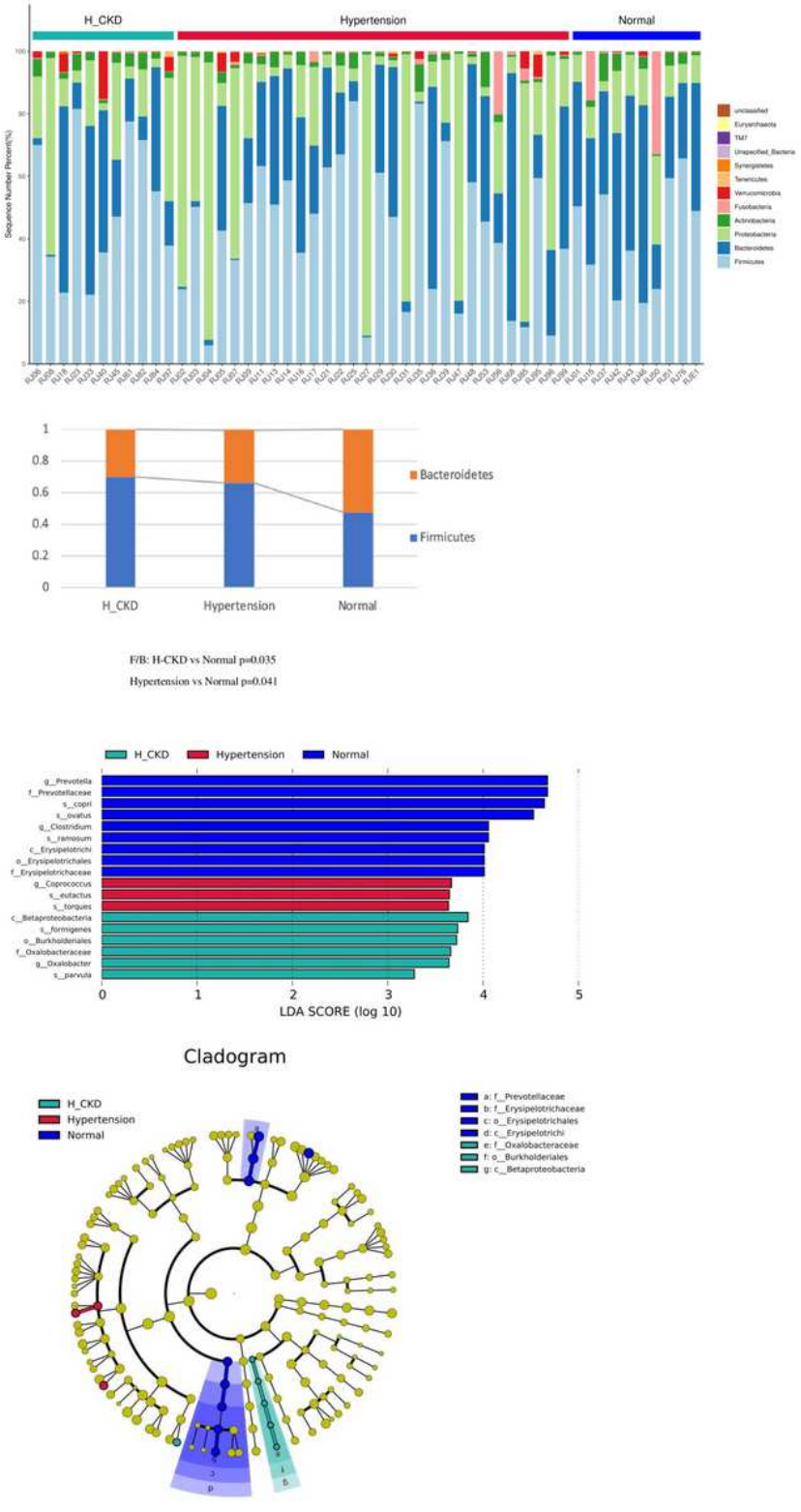

Figure 2

Variations of Fecal Microbiota in H-CKD, Hypertension and Normal Groups (a) Relative proportions of bacterial phyla in H-CKD (n=11), hypertension (n=30) and normal (n=10); (b) Rate of relative proportions of Firmicutes and Bacteroidetes (F/B). F/B in both HCKD and hypertensive groups is significantly lower than it in healthy controls; (c)(d) Linear discriminant analysis (LDA) effect size analysis revealed that the relative abundance of 19 bacteria were significantly different among H-CKD, hypertension and normal groups.

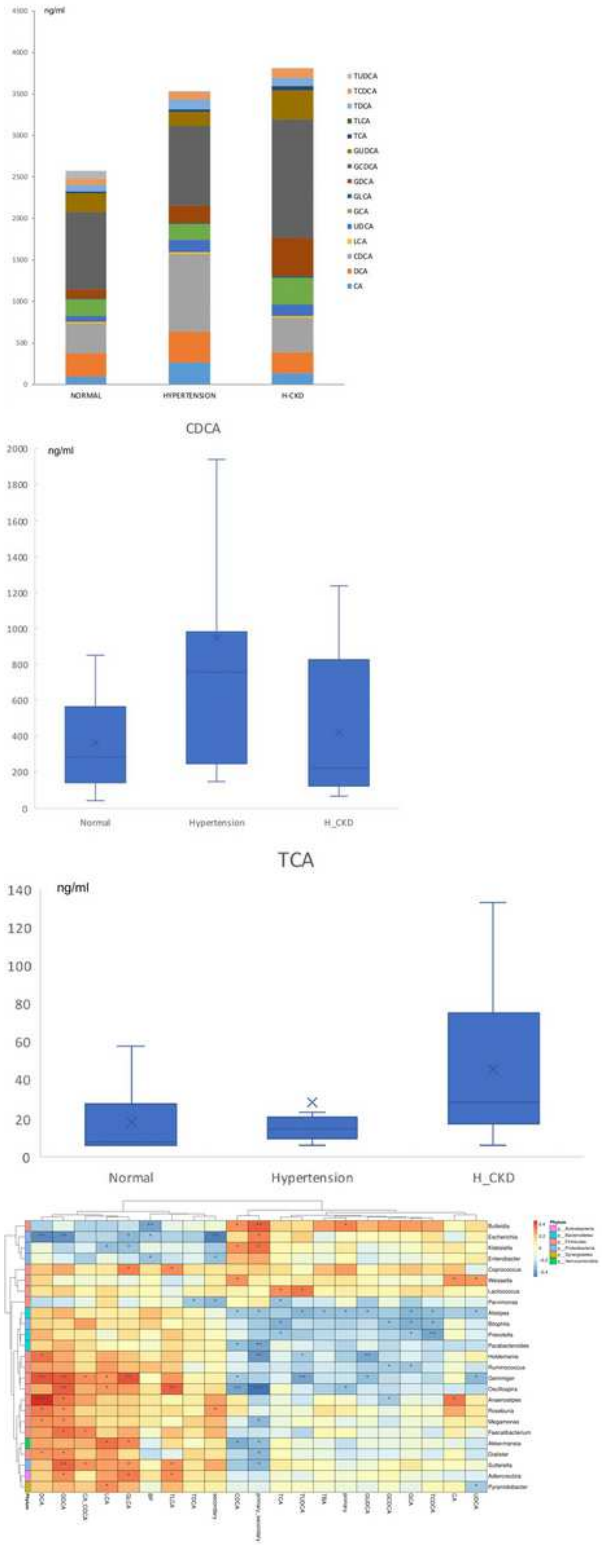

Figure 3

The Relationship between Gut Microbiota and Bile Acid Metabolism in H-CKD and Hypertension Groups. (a) Different contents of bile acid metabolism among the Normal, H-CKD and Hypertension groups. (b) The concentration of CDCA is significantly different among groups by hypertension and the normal group was observed. (d) The heatmap shows that partial Spearman correlation coefficients between genera and bile acid metabolism. * 0.01<p≤0.05 ** 0.001<p≤0.01 *** p≤0.001.

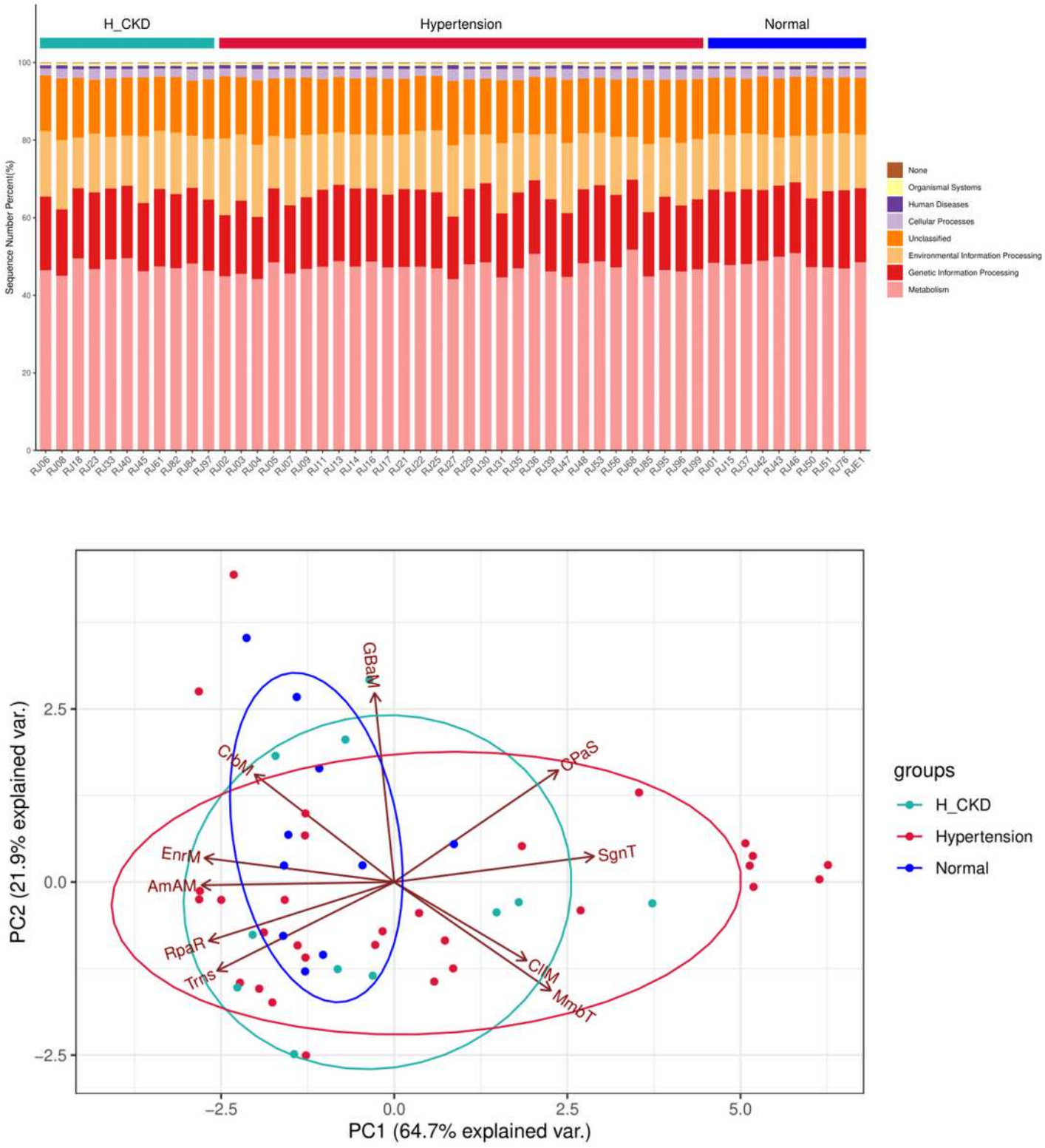

Figure 4

Predicted Metagenomes Functional Analysis (a) Different relative abundance predicted function of gut bacteria in all individuals. (b) PC1 represents the first principal component, and the percentage represents the contribution of the first principal component to the sample difference; PC2 represents the second principal component, and the percentage represents the contribution of the second principal component to the sample difference. The direction and length of the arrow represent the degree of dominance of the prediction function in the sample/group in that direction, the longer the length, the stronger the dominance.