

Relationship between Gut Microbiota and Phosphorus Metabolism in Hemodialysis Patients: A Preliminary Exploration

Yuan-Yi Miao¹, Cong-Min Xu², Min Xia¹, Huai-Qiu Zhu², Yu-Qing Chen¹

¹Renal Division, Department of Medicine, Peking University First Hospital, Institute of Nephrology, Peking University, Key Laboratory of Renal Disease, Ministry of Health of China, Key Laboratory of Chronic Kidney Disease Prevention and Treatment, Ministry of Education, Beijing 100034, China

²Department of Biomedical Engineering, State Key Laboratory for Turbulence and Complex Systems, College of Engineering, and Centre for Quantitative Biology, Peking University, Beijing 100871, China

Yuan-Yi Miao and Cong-Min Xu contribute to the paper equally.

Abstract

Background: Hyperphosphatemia is a risk factor associated with mortality in patients on maintenance hemodialysis. Gut absorption of phosphate is the major source. Recent studies indicated that the intestinal flora of uremic patients changed a lot compared with the healthy population, and phosphorus is an essential element of bacterial survival and reproduction. The purpose of this study was to explore the role of intestinal microbiota in phosphorus metabolism.

Methods: A prospective self-control study was performed from October 2015 to January 2016. Microbial DNA was isolated from the stools of 20 healthy controls and 21 maintenance hemodialysis patients. Fourteen out of the 21 patients were treated with lanthanum carbonate for 12 weeks. Thus, stools were also collected before and after the treatment. The bacterial composition was analyzed based on 16S ribosomal RNA pyrosequencing. Bioinformatics tools, including sequence alignment, abundance profiling, and taxonomic diversity, were used in microbiome data analyses. Correlations between genera and the serum phosphorus were detected with Pearson's correlation. For visualization of the internal interactions and further measurement of the microbial community, SparCC was used to calculate the Spearman correlation coefficient with the corresponding *P* value between each two genera.

Results: Thirteen genera closely correlated with serum phosphorus and the correlation coefficient was above 0.4 ($P < 0.05$). We also found that 58 bacterial operational taxonomic units (OTUs) were significantly different and more decreased OTUs were identified and seven genera ($P < 0.05$) were obviously reduced after using the phosphate binder. Meanwhile, the microbial richness and diversity presented downward trend in hemodialysis patients compared with healthy controls and more downward trend after phosphorus reduction. The co-occurrence network of genera revealed that the network complexity of hemodialysis patients was significantly higher than that of controls, whereas treatment with lanthanum carbonate reduced the network complexity.

Conclusions: Gut flora related to phosphorus metabolism in hemodialysis patients, and improving intestinal microbiota may regulate the absorption of phosphate in the intestine. The use of phosphate binder lanthanum carbonate leads to a tendency of decreasing microbial diversity and lower network complexity.

Key words: Gut Microbiota; Hemodialysis Patients; Lanthanum Carbonate; Phosphorus

INTRODUCTION

Hyperphosphatemia is a common comorbidity in patients on maintenance dialysis. Higher level of plasma phosphorus associated with increased vascular calcification, mineral bone disease cardiovascular events, and all-cause mortality.^[1,2] Food is the major source of phosphorus,^[3] and phosphate is the major form absorbed in small intestine. Absorption

Address for correspondence: Dr. Yu-Qing Chen,
Renal Division, Department of Medicine, Peking University First Hospital,
No. 8, Xishiku Street, Beijing 100034, China
E-Mail: cyq@bjmu.edu.cn

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of phosphate depends on passive diffusion through the epithelial junction and active transport via luminal epithelial sodium phosphate cotransporter (NaPi-IIb).^[4,5]

Previous report suggested significant differences in the abundance of 190 bacterial operational taxonomic units (OTUs) between end-stage renal disease (ESRD) and control groups.^[6] Phosphorus is an important element in the survival of microflora. A recent study on pigs indicated that phosphorus might influence the composition and activity of intestinal microflora.^[7] The excessive phosphate can be stored as polyphosphates in some types of bacterial cells and used as energy or phosphorus source for metabolism.^[8] Thus, it is interesting to know whether gut microbiota participates in phosphorus metabolism in patients with ESRD.

The phosphate-binding agent can bind with phosphate, lower the burden of phosphate in the intestinal lumen, finally reduce phosphate absorption. On the other hand, these medicines may influence the distribution of microbiota in the gut, due to complicated interactions between phosphorus and microbiota. Thus, we took advantage of the cohort who use phosphate-binding agent, to test and analyze stool microbiota of the patients, to investigate the relationship between gut bacteria and phosphate level.

METHODS

Ethical approval

The study was approved by the Ethics Committee of Peking University First Hospital and all individuals signed informed consent.

Study cohort and patient characteristics

From October, 2015 to January, 2016, 21 patients (including 12 men and 9 women, mean age 53.0 ± 9.0 years) with ESRD on maintenance hemodialysis for at least 3 months were recruited from the Hemodialysis Center at Peking University First Hospital. The 21 patients were in a cohort study observing the effect of lanthanum carbonate on lowering serum phosphate level. During the study, seven patients withdrew from the using of lanthanum carbonate because of gastrointestinal side effect. Fourteen patients were treated with the phosphate binder for 12 weeks. Their serum phosphorus levels were over 1.78 mmol/L before taking lanthanum carbonate. The patients have been on regular dialysis three times a week, 4 h each time and single pool urea clearance index (spKt/V) ≥ 1.2 on regular monitor. All clinical data were collected by standard procedures. Patients with digestive tract diseases, cancer, heart failure, and allergic to lanthanum carbonate were excluded. Moreover, none of them had received lanthanum carbonate within the last 3 months before enrollment. We also got stool samples from volunteers ($n = 20$) as controls. Mean age was 31.7 ± 9.1 years, including 10 males and 10 females. All these persons are staffs of the unit and had accepted the annual routine medical examination within one year. They had negative urinalysis and normal renal function.

Feces sample collection and DNA extraction

Feces samples were collected from 20 controls, 21 dialysis patients before using lanthanum carbonate, and 14 dialysis patients provided second feces samples after 12 weeks of lanthanum carbonate treatment. The seven patients withdrawn from the drug use did not provide the second feces samples. DNA from the fresh stool samples was extract with QIAamp DNA stool kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. DNA concentration was estimated by absorbance at 260 nm, and the purity of DNA was detected by the A260/A280 ratio. The extracted DNA was used for polymerase chain reaction (PCR) amplification and 454 pyrosequencing.

Polymerase chain reaction amplification

DNA from the stool sample was amplified using primers for the V1-V3 regions of the 16S rRNA gene. The PCR procedure was set by the following cycle conditions: 94°C for 5 min, then 15 cycles at 94°C for 45 s, 55°C for 45 s, 72°C for 45 s followed by a final step of 72°C for 10 min, then stored at 4°C. After the PCR was completed, the products were purified using the QIAquick PCR purification kit (Qiagen Valencia, CA, USA).

Sequencing result estimation

The 454-pyrosequencing data were analyzed in a well-designed pipeline. The sequencing reads were filtered using the Ribosomal Database Project (RDP). OTUs were clustered at 97% sequence identity with CD-HIT,^[9] resulting in 44,613 OTUs. After constructing a sample-OTU count matrix, Shannon index was calculated to estimate the species diversity.

Shannon-Weiner curve of different sample groups with increasing number of sequences illustrates the species diversity at different sequencing depth. A smooth curve indicates a good sequencing depth to illustrate the species composition of all the sample groups. In addition, OTUs with statistically significantly different abundance between HC and IBS-D were picked to form a phylogenetic tree by unweighted pair-group method with arithmetic means, and the tree was drawn by iTOL.^[10,11]

Taxonomic composition analysis

We further give taxonomic assignment to every sequence by searching RDP resources with BLAST software,^[12,13] where the e -value threshold was set 10^{-5} . The counts of sequences at different phylogenetic levels (i.e. genus, family, order, class, and phylum) were then generated into a matrix to characterize the microbial composition of each sample. In addition, the Euclidean distance was calculated and mapped into a heat map to further reveal the different abundance distribution of different sample groups.

Statistical analysis

Correlations between genera and the serum phosphorus were detected with Pearson's correlation. The correlation efficient (R) with $P < 0.05$ was acceptable; otherwise, R was manually set to be zero. Wilcoxon rank sum test was

used to compare the abundance distribution of different taxonomic compositions. *P* values were adjusted with false discovery rate, and *Q* < 0.05 were statistically significant. For visualization of the internal interactions and further measurement of the microbial community, SparCC^[14] was used to calculate the Spearman correlation coefficient with the corresponding *P* value between each two genera. The co-occurrence network was then visualized by Circos^[15] with the nodes denoting the genera, and connections representing the existence of correlation meeting a given criteria.

RESULTS

General data

Clinical, biochemical traits were compared before and after the use of lanthanum carbonate in patients on maintenance hemodialysis. The serum phosphorus decreased after using lanthanum carbonate for 12 weeks (2.56 ± 0.45 vs. 1.86 ± 0.36, *P* = 0.002). There was no difference in other biochemical traits, such as serum calcium, potassium, sodium, white blood cell, albumin, blood sugar, triglyceride, creatinine, and urea nitrogen [Table 1].

To characterize the bacterial richness, Shannon-Wiener curve was made by random samples to estimate the total gene numbers that could be identified from these samples. The curve in each group was near saturation, suggesting the sequencing data was enough to reflect the microbial information in the samples [Supplementary Figure 1].

Abundance profiling

In this study, there were significant variations in the composition of gut microbiota before and after using lanthanum carbonate. At the phylum level, more than 70.00% microbiota was Firmicutes in both groups, but an obvious reduction, from 80.59% to 74.89%, was identified in the patients after using lanthanum carbonate. *Bacteroides* and *Proteobacteria* were also decreased after treatment (9.90–8.38% and 2.57–1.01%). At the same time, *Actinobacteria* increased after the use of lanthanum carbonate from 1.09% to 4.66% as well

as other bacteria (5.84–11.06%). No obvious difference was identified between control and patients before using lanthanum carbonate [Figure 1].

At the OTU level, 58 OTUs were different before and after the use of phosphate binder. More decreased OTUs were identified after using the phosphate binder [Figure 2].

At the genus level, seven genera were obviously reduced (*P* < 0.05), including *Centipeda*, *Chryseobacterium*, *Gemella*, unclassified *Rhodocyclaceae*, *Pelomonas*, *Curvibacter*, and *Parvimonas* [Figure 3].

Phosphorus-associated genera in gut microbiome

To further examine the relationship between phosphorus and gut microbiome, correlation analysis showed that 13 genera were closely correlated with serum phosphorus and the correlation coefficient was above 0.4 (*P* < 0.05). Among them, 11 genera were positively related to serum phosphorus and two genera were negatively related to serum phosphorus [Table 2].

Taxonomic diversity

Shannon index based on the genera profile was calculated to estimate diversity. The microbial diversity showed decreasing trends in hemodialysis patients compared with healthy controls and declined further after the phosphate binder therapy [Figure 4].

Co-occurrence network of genera

We constructed the co-occurrence networks of genera in the three different sample groups. Only the strong connection between different genera with the absolute value of the Spearman correlation coefficient larger than 0.6 and the corresponding *P* < 0.05 was considered valuable and visualized on [Figures 5 and Figure 6]. Compared to the healthy controls, the co-occurrence network of microbial genera in hemodialysis patients gut trend to be more complex [Figure 6]. Similarly, after 12 weeks of treatment with lanthanum carbonate, the co-occurrence network of microbial genera in patients was simplified when compared with that without treatment [Figure 5].

Table 1: General data before and after using lanthanum carbonate in the cohort of hemodialysis patients

Parameters	Before (n = 14)	After (n = 14)	Z	P
Ca (mmol/L)	2.19 ± 0.14	2.22 ± 0.13	-1.320	0.187
P (mmol/L)	2.56 ± 0.45	1.86 ± 0.36	-3.045	0.002
K (mmol/L)	5.16 ± 0.53	5.26 ± 0.64	-0.408	0.683
Na (mmol/L)	136.49 ± 2.74	137.01 ± 3.24	-0.722	0.470
WBC (× 10 ⁹ /L)	6.23 ± 2.13	6.28 ± 1.88	-0.220	0.826
ALB (g/L)	42.17 ± 3.50	41.37 ± 2.01	-0.754	0.451
GLU (mmol/L)	6.93 ± 3.37	6.81 ± 2.32	-0.345	0.730
TG (mmol/L)	1.33 ± 0.48	1.42 ± 0.48	-0.280	0.779
SF (ng/ml)	434.82 ± 188.18	424.78 ± 255.97	-0.408	0.683
Scr (μmol/L)	996.41 ± 203.58	962.19 ± 190.78	-0.973	0.331
BUN (mmol/L)	26.33 ± 4.22	24.44 ± 3.66	-1.099	0.272

Values were presented as mean ± SD. Ca: Calcium; P: Phosphorus; K: Potassium; Na: Sodium; WBC: White blood cell; ALB: Albumin; GLU: Glucose; TG: Triglyceride; SF: Serum ferritin; Scr: Serum creatinine; BUN: Blood urea nitrogen; SD: Standard deviation.

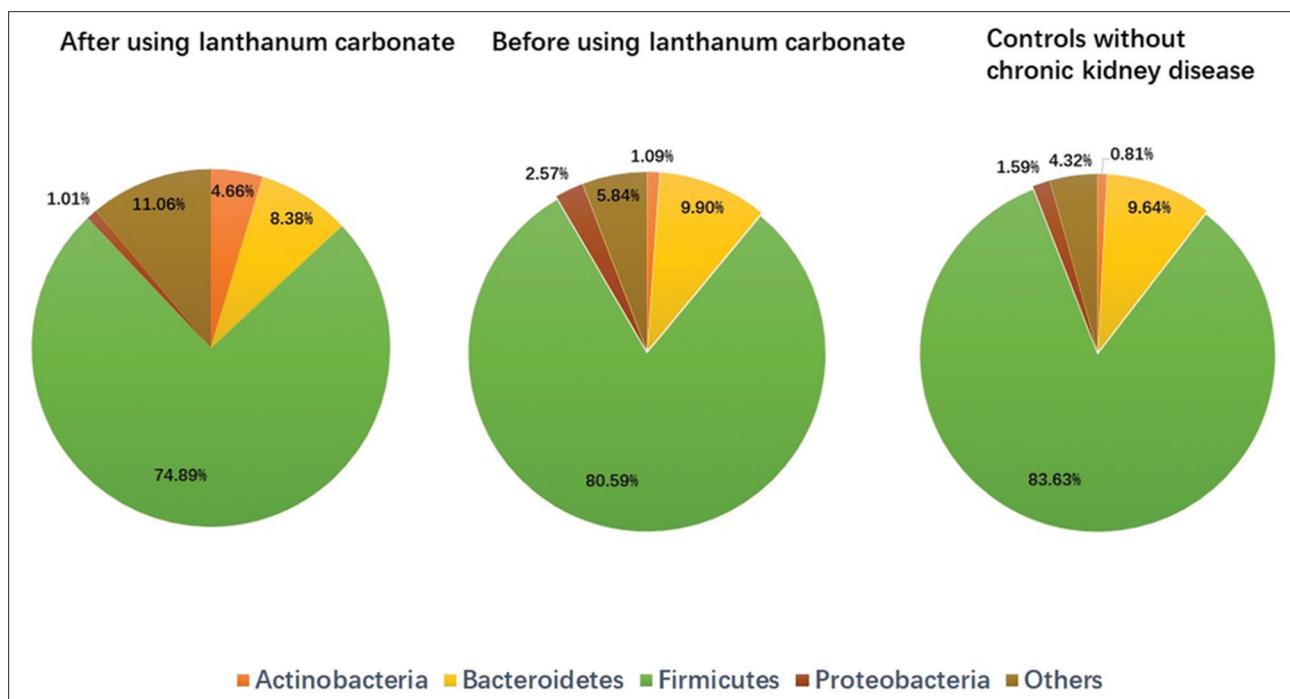


Figure 1: Pie chart based on pyrosequencing analysis showing the percentage of phyla before ($n = 14$) and after ($n = 14$) using lanthanum carbonate and normal controls ($n = 20$) without chronic kidney disease.

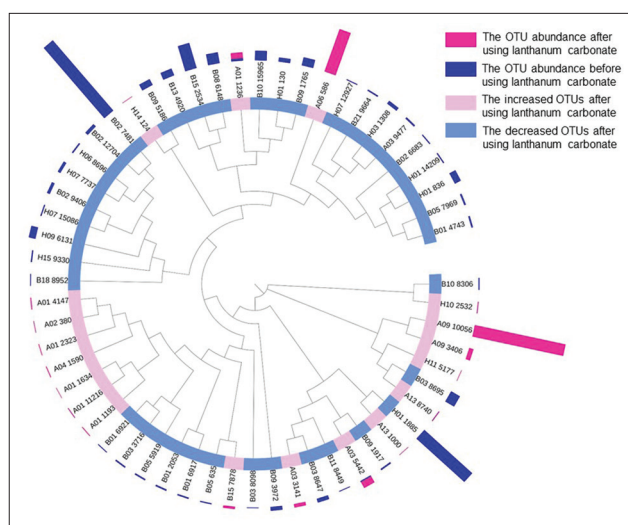


Figure 2: Phylogenetic tree based on 16S rRNA gene sequences of bacterial taxa showing significantly different relative abundances before and after using lanthanum carbonate. Branches of the tree are color coded, blue and red bars display the OTU abundance. Light red and pale blue indicate increased and decreased OTUs, respectively, after phosphorus reduction. There were 58 OTUs that were significantly different. OTU: Operational taxonomic units.

DISCUSSION

Phosphorus is an essential element for life and phosphates (containing the phosphate ion, PO_4^{3-}) are components of DNA, RNA, ATP, and the phospholipids.^[16] In certain types of bacteria, phosphates can be stored as polyphosphates in cells and can be used as energy or phosphorus source to meet metabolic demand.^[17,18] The storage process is commonly described as enhanced biological phosphorus

removal (EBPR). EBPR was a well-established process and has been used widely to remove phosphorus from wastewater.^[19] Several studies suggested that changes in dietary phosphorus could influence intestinal microbial composition and activity in pigs and chickens.^[7,20,21] On the other side, bacteria who stores phosphate can decrease the phosphate ion burden of the gut epithelium. However, none of the above studies explored whether there was association between gut microbiota and phosphorus level in population.

Phosphate-binding agents can change the intestine phosphate burden by forming phosphate complex. The formation of phosphate complex decreased the phosphate ion level and changes the intestinal microenvironment. Because of the decreased phosphate, the bacteria living on phosphorus may decrease and trigger chain reactions and finally change the community of gut microbiota. Thus, it is possible to find bacteria flora associated with phosphorus metabolism with the model of using phosphate binder. In this study, we took advantage of the model of “phosphate-binder therapy” to perform a longitudinal study, to observe the change of microflora. Elements influencing bacteria flora, such as diet, other drugs and antibiotics, were controlled in the 14 patients before and after the use of phosphate binder.

In this study, we identified seven reduced genera after the use of phosphate binder for 12 weeks, compared with the same cohort of patients before the use of the drug. The result suggested that these genera may have a higher demand for phosphorus, and thus may store more phosphate in the intestinal lumen. We did correlation analysis between genera and the serum phosphorus level, and found 11 genera, positively related to serum phosphorus,

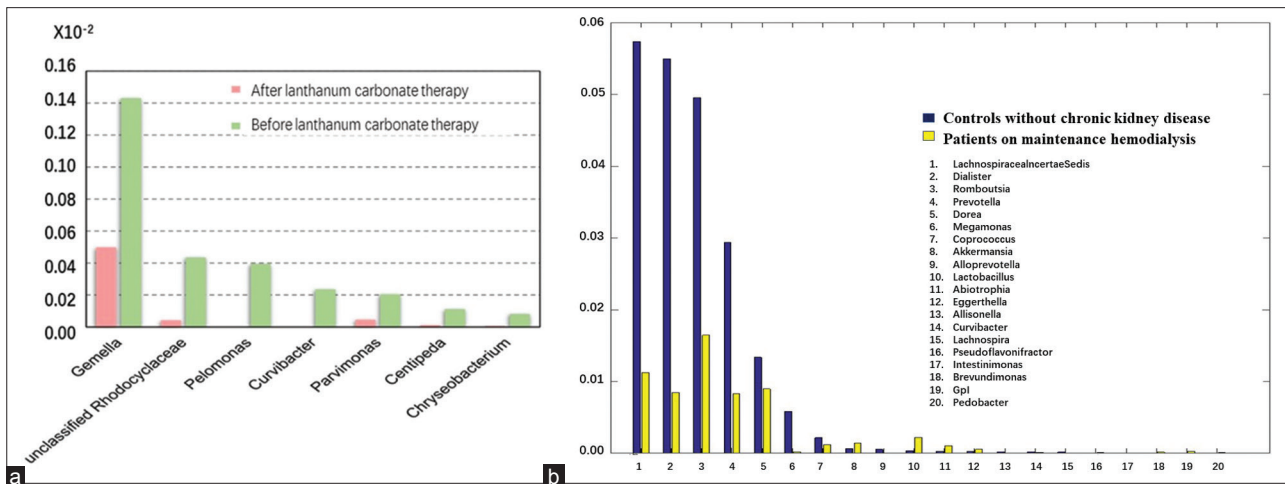


Figure 3: Bar plots showing the relative abundances of bacterial groups at the genus level. (a) Genus levels in patients before and after using lanthanum carbonate. Pink bar and green bar represent after and before using lanthanum carbonate, respectively. (b) Genus levels in controls and patients on maintenance hemodialysis. Blue and yellow bar represent controls and patients on hemodialysis.

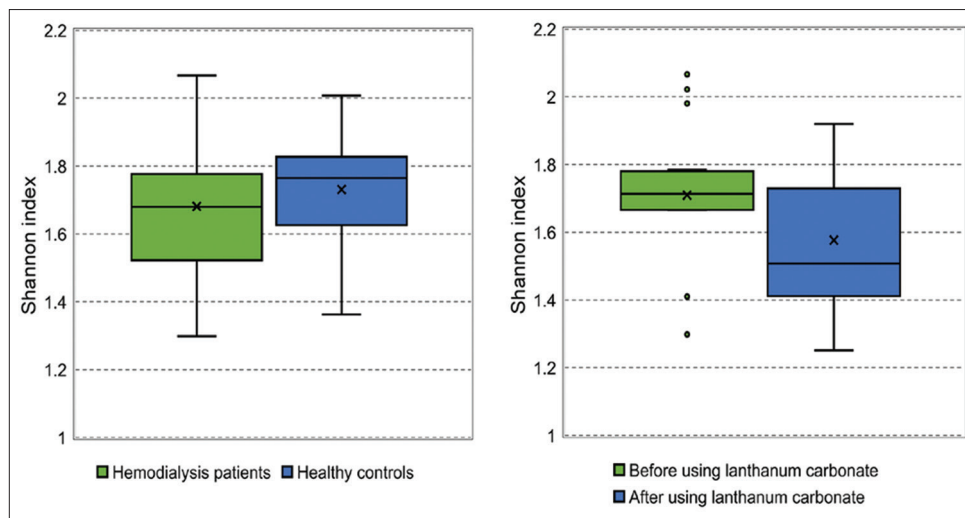


Figure 4: Comparison of the microbial gene diversity (as accessed by Shannon index) based on the genus abundance in the groups. The gene diversity has downward trend after using lanthanum carbonate ($n = 14$ in each group), $P = 0.131$; the gene diversity has downward trend in hemodialysis patients ($n = 21$) compared with healthy controls ($n = 20$), $P = 0.429$.

Table 2: Genus closely correlated with phosphorus in the cohort of hemodialysis patients ($n = 21$)

Genus	R (correlation coefficient)	P
Anaerovibrio	0.458	0.021
Bergeriella	0.458	0.021
Clostridium XIX	0.432	0.031
Georgenia	0.462	0.020
Hydrogenophilus	0.458	0.021
Leptotrichia	0.458	0.021
Murdochiella	0.458	0.021
Proteiniborus	-0.404	0.045
Robinsoniella	-0.427	0.033
Selenomonas	0.509	0.009
Streptococcus	0.458	0.021
Unclassified_Bifidobacteriaceae	0.462	0.020
Unclassified_Moraxellaceae	0.506	0.010

indicating that survival of the 11 genera was closely related to phosphorus level in intestinal microenvironment. Among the 11 genera, we identified one genera, *Clostridium* XIX. Similarly, Metzler-Zebeli *et al.* found increased numbers of *Clostridium* cluster XIVa in the distal ileum of pigs fed with high-phosphate diet.^[22,23] *Clostridium* XIX and *Clostridium* XIVa belong to the same genus, suggested that *Clostridium* may correlate with phosphate concentration in the intestine. We found two genera negatively related to serum phosphorus also. Previous studies showed that the intestinal microbiota was a complex ecological network closely related with the microbiology of the host and its homeostasis.^[24,25] The microbes interacted with each other competed for some growth factors and led to increase of some microbes and decrease of others.

With the two main analysis approaches, we found some possible intestinal bacteria associated with serum phosphate

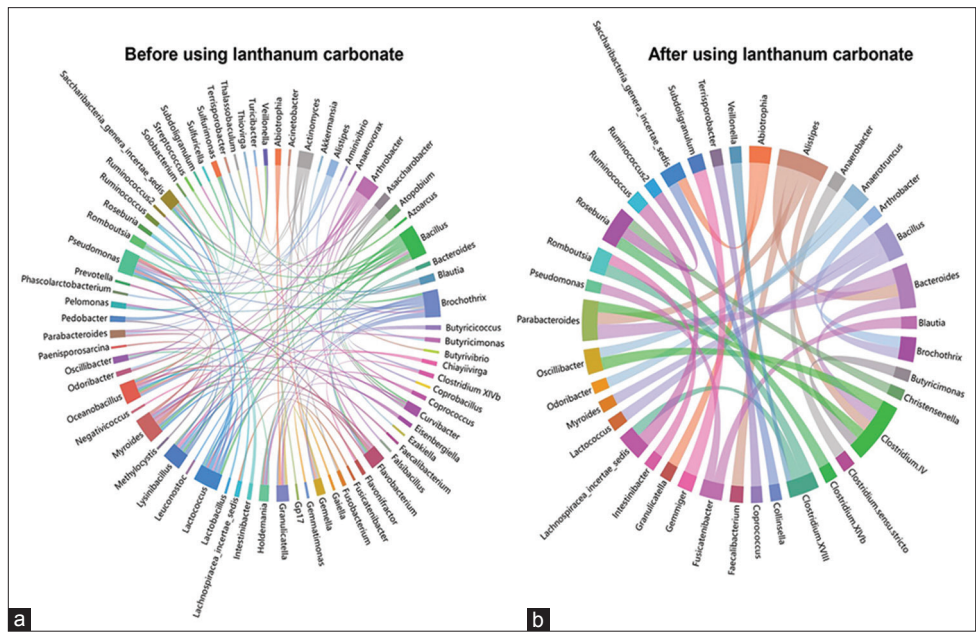


Figure 5: Co-occurrence network of genera in patients before ($n = 14$; a) and after lanthanum carbonate ($n = 14$; b) treatment. The size of circular arc represents the activity of the node and the relative width of the connection ribbon indicates the connection strength between the pair of nodes.

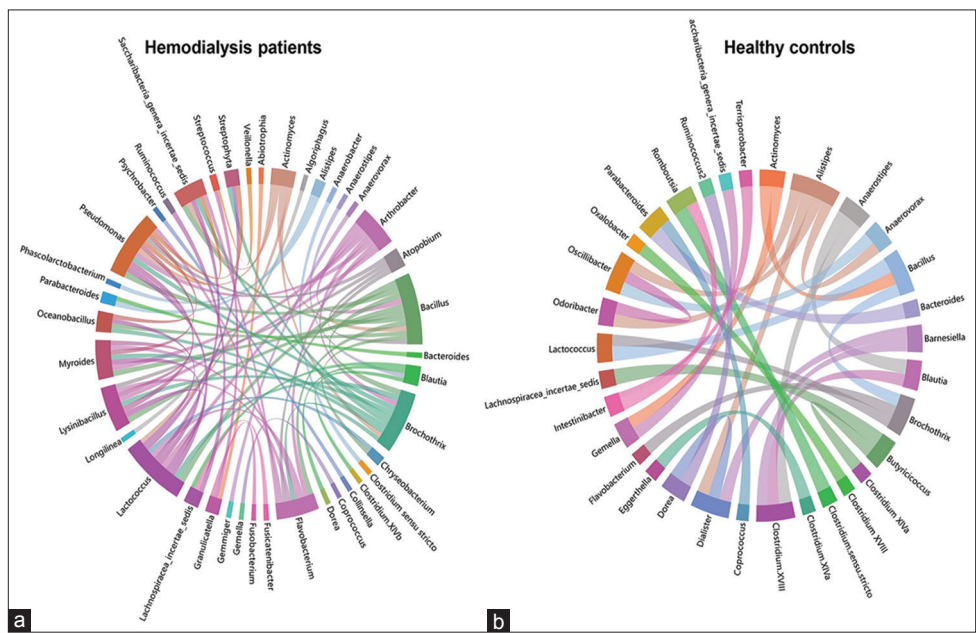


Figure 6: Co-occurrence network of genera in hemodialysis patients ($n = 21$; a) and healthy controls ($n = 20$; b). The genus nodes are sorted in alphabetical order. The size of circular arc represents the activity of the node and the relative width of the connection ribbon indicates the connection strength between the pair of nodes.

level in population. Meta-genomic sequencing is needed to confirm bacterial at the species level and genes participating in phosphorus metabolism.

Other factors, such as phosphorus transformation, absorption, and utilization contribute to the metabolism of phosphorus. Some microbial flora associated with inflammation and epithelial dysfunction, lead to higher permeability of intestinal epithelial cells and destroyed cell junction so that phosphates were easily to get into the blood.^[26,27] *Parvimonas* among the genera identified in this study has been reported

causes of inflammatory diseases, including spinal infections, septic arthritis and periapical periodontitis.^[28-30]

We also found that the co-occurrence network of genera was more complex in patients with ESRD before use of phosphorus-binder compared with normal controls, but the complexity declined after phosphorus binder therapy. Gut microbiota plays a vital role in health and disease,^[31] and bacterial network complexity was reported to be associated with various diseases.^[32,33] The gut microenvironment changed a lot due to high uremic toxins in ESRD patients,

and the network of bacteria also changed to adapt to the environment. The use of phosphorus binder accompanied by an obviously decreased complexity of microbiota. The clinical value of this change needs to be further investigated.

Advanced renal failure can alter the microbial flora composition due to selection pressures from uremic status. The previous study found marked differences in the abundance of 190 bacterial OTUs between the ESRD and control groups.^[6] In our study, we also identified significant differences in the abundance of OTUs. However, in addition to uremia, differences in the underlying diseases therapy and dietary intervention could modify the gut microbiome in the ESRD patients. It is less meaningful to compare the difference between ESRD and normal controls in the gut microbiome.

In summary, our study found that gut flora was related to phosphorus metabolism in hemodialysis patients. The use of phosphate binder lanthanum carbonate leads to decreased microbial diversity and lower network complexity. Our findings indicated a new clue to the therapy of hyperphosphatemia. Since our study was a pilot study with a small sample size. More studies with more samples and different phosphorus binders are needed.

Supplementary information is linked to the online version of the paper on the Chinese Medical Journal website.

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Conflicts of interest

There are no conflicts of interest.

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血液透析患者肠道菌群和磷代谢相关性的初步探讨

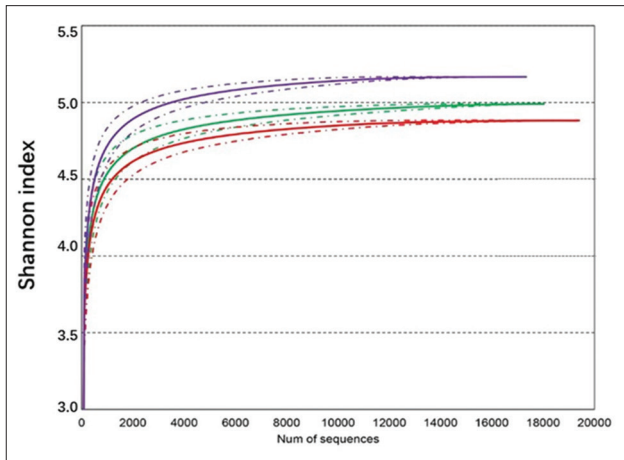
摘要

背景：高磷血症是维持性血液透析患者死亡相关的高危因素。肠道对磷的吸收是主要的来源。近期的研究显示尿毒症患者的肠道菌群与健康人群相比发生了较大改变。研究显示磷元素是细菌生存和繁殖的基本元素。该研究的主要目的是探讨血液透析患者肠道菌群在磷代谢中的作用。

方法：该研究为前瞻性自身对照研究，于2015年10月到2016年1月进行。维持性血液透析患者21人参加了碳酸镧降低磷负荷的治疗研究，其中14例透析患者完成了12周的治疗，7例因胃肠道反应和低钙血症中途退出。收集患者降磷前的粪便样本和14例完成治疗的患者治疗后的粪便标本，同时收集了20例健康志愿者的粪便标本。提取样本中的DNA，进行16S rRNA基因测序，对肠道菌群的分布情况和血磷之间的关系进行分析。分析过程包括数据拆分、长度过滤等数据预处理，以及OTU聚类、 α 多样性分析、Shannon指数计算、群落结构分析、菌门和菌属丰度分析。用Pearson相关性分析菌属与血清磷的相关性，P值小于0.05被认为是有意義的。为了进一步分析菌群内部之间的相互作用，SparCC用于计算菌属之间Spearman相关系数及对应的P值

结果：13种肠道菌和血磷显著相关，相关系数在0.4以上（ $P < 0.05$ ）。降磷治疗后，58个操作分类单元（OTU）有明显差异，减少的OTU比增加的OTU多；并且有7种菌属显著减少。健康人的菌群多样性有高于血透患者的趋势，同一批患者进行降磷后菌群多样性有进一步下降的趋势。血透患者菌属之间的网络复杂度高于健康人，患者降磷后菌属之间的网络复杂度降低。

结论：肠道菌群与血透患者磷代谢有相关性，改善肠道菌群有可能调节磷的肠道吸收。肠道磷结合剂的使用，使肠道菌群的多样性和网络复杂度下降



Supplementary Figure 1: Shannon curves for gene number. The curve in each group is near smooth when the sequencing data are good enough. Green line: Before lanthanum carbonate therapy; Red line: After lanthanum carbonate therapy; Purple line: Healthy controls.