The gut microbiome affects the responses of heart valve replacement patients to the anticoagulant warfarin

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Abstract

Backgrounds: Numerous algorithms based on patient genetic variants have been established with the aim of reducing the risk of GI bleeding and thromboembolism during warfarin administration. However, approximately 35% of individual warfarin sensitivity remains unexplained. Gut microbiota composition related to the vitamin K generation should be taken into account.

Methods : Faecal samples were collected from 200 inpatients undergoing heart valve replacement (HVR). Ultimately, faecal samples from 80 inpatients (27 low responders (LRs), 27 high responders (HRs) and 26 normal responders (NRs) were analyzed for microbiota composition through 16S rDNA sequencing. Fifteen samples (5 LRs, 5 HRs and 5 NRs) were also analyzed through metagenomic whole-genome shotgun (WGS) sequencing. We validated the results from 10 LRs, 10 HRs and 10 NRs.

Results: Significant differences were observed in the diversity and composition of the gut microbiome among the three groups. The genera Bacteroides, Escherichia-Shigella and Klebsiella were enriched in the LRs, while the genus Enterococcus was enriched in the HRs. WGS sequencing indicated that the abundance of enzymes, modules and KO associated with bacterial vitamin K biosynthesis was significantly higher in LRs than in HRs or NRs. The 12 optimal microbial markers were identified through tenfold cross-validation with a random forest model.

Conclusions: This study characterized gut microbiome in the different response to warfarin of HVR patients and suggested that gut microbiome might play an important role in the clinical warfarin anticoagulation therapy.

1. Introduction

Warfarin, a vitamin K antagonist,[1] is prescribed to nearly one million patients with prosthetic heart valves worldwide.[2] Although warfarin is highly effective in reducing thromboembolism, its use is limited by its narrow therapeutic index and the required frequent monitoring of the international normalized ratio (INR).[3] An INR of 1.5 to 2.5 is recommended in East Asian patients with heart valve replacement (HVR), consistent with the guidelines announced by the American College of Chest Physicians in 2008.[4] Overall, patients taking warfarin have significantly more bleeding events than patients taking other anticoagulants (P<0.001).[5-7]

Numerous algorithms that are based on patient factors and genetic variants have been established by multivariate linear regression with the aim of reducing the risk of bleeding during the initial days of warfarin administration.[8]

In 2007, we[9,10] created an algorithm for daily warfarin dose requirements so that appropriate warfarin maintenance doses could be calculated easily.[11] This tool has verifiably improved the decision-making process for warfarin dosing in HVR patients at our hospital, but approximately 35% of individual variability remains unexplained[10].

All types of vitamin K(VK) used by the human body are obtained from food or are synthesized by bacteria.[12] It has been stated that up to 50% of the human requirement for vitamin K is fulfilled by the intestinal production.[13,14] However, vitamin K concentrations in the human gut appear highly variable and are associated with gut microbiota composition.[15] To date, there has been no systematic study on the effect of bacterial VK on warfarin anticoagulation in HVR patients with a low dietary intake of vitamin K.

The aim was to establish possible differences in gut microbiota composition and functionality among warfarin low responder (LR), high responder (HR) and normal responder (NR) using next-generation metagenomics sequencing techniques. In the discovery phase, we characterized the gut microbiomes of different groups, analyzed the content of vitamin K in the stool with LC-MS/MS, and studied the mechanism through which the gut microbiome might influence the effects of anticoagulation therapy. Furthermore, a validation cohort was used to evaluate the potential of the gut microbiome as a noninvasive biomarker, which showed the importance of gut microbiome in the clinical warfarin anticoagulation therapy (Figure 1).

2. Material And Methods

2.1Ethics and patients

Ethical permission for this study was obtained from the Health Authority Ethics Committee of the First Affiliated Hospital of Soochow University (Suzhou, Jiangsu, China). All patients provided written informed consent in accordance with the Declaration of Helsinki. Stable inpatients who had undergone HVR surgery and taken warfarin sodium tablets (Shanghai Xinyi Pharmaceutical Co., Ltd., Shanghai, China) during hospitalization from July 2017 to December 2018 were recruited. All patients were given written consent prior to their participation in the study.

The inpatients recruited for this study (Supplementary Table S1) were those who had first undergone heart valve replacement and had used second generation cephalosporin antibiotics during the operation to prevent infection（Supplementary Methods 1.1）.

2.2Fecal sample collection and stool moisture measurement

Each inpatient was provided with fresh stool specimen collection systems during hospitalization and asked to collect all stools produced after cardiac valve surgery (Supplementary Methods 1.2). Five aliquots of 5 g were obtained from the homogenate and immediately stored at -80°C.

Stool moisture content was determined in duplicate from frozen homogenized faecal material (−80°C) as the percentage of stool mass loss after lyophilization.

2.3DNA extraction, PCR amplification and MiSeq sequencing

Bacterial DNA was extracted from stool samples, and the V3-V4 partial 16S rDNA gene sequences were amplified (Supplementary Methods 1.3). The raw reads were deposited into the NCBI Sequence Reads Archive (SRA) database (PRJNA518710). Raw fastq files were demultiplexed and quality filtered using the QIIME package version 1.17 (Quantitative Insights Into Microbial Ecology, Flagstaff, AZ, USA).

2.416S rDNA microbial profiling analysis

The QIIME software suite (http://qiime.org/scripts/assign_taxonomy.html) and the related 16S database SILVA, V.128 (http://www.arb-silva.de) were used for taxonomic classification of Operational Taxonomic Units (OTUs). All OTUs for all samples in the discovery sets were collected (Supplementary Table S3; Supplementary Methods 1.4).

A rarefaction curve was developed using the R language and was constructed using the number of sequences extracted and the diversity index of the corresponding OTUs. Based on the microbial profiles, we calculated the alpha diversity in the discovery phase to estimate the richness using the Chao index, and the diversity was calculated using the Shannon index and Simpson index. A large value of the Chao and Shannon indexes indicated a higher degree of diversity in the sample. These results were used to analyse effects of different phenotype factors.

A Venn diagram can be used to represent the number of common and unique species (such as OTUs) among the LR, HR and NR groups. We created a Venn diagram using an R language tool.

A beta diversity analysis of interindividual variability was performed using the principal coordinate analysis (PCoA) method based on weighted UniFrac and Bray-Curtis dissimilarity at the genus level.

2.5Shotgun metagenomics analysis of fecal samples

We used microPITA (microbiomes: Picking Interesting Taxonomic Abundance)[16] to select representative samples (n=15; 5 LR samples, 5 HR samples, and 5 NR samples) with specific characteristics or biological criteria for follow-up shotgun metagenomics analysis based on the 16S rDNA microbial profiles. MicroPITA open-source software is available for download and for online use through Galaxy[17] at https://www.i-sanger.com; the program provides the benefits of a full population survey in the first stage and guaranteed targeting of samples in the second stage.

Bacterial DNA was extracted from stool samples as detailed in the Supplementary Methods 1.5. All predicted genes with 95% sequence identity (90% coverage) were clustered using CD-HIT[18] (http://www.bioinformatics.org/cd-hit/). The longest sequences from each cluster were selected as representative sequences to construct a non-redundant gene catalogue. After quality control, reads were mapped to the representative sequences with 95% identity using SOAPaligner[19] (http://soap.genomics.org.cn/), and the gene abundance in each sample was evaluated.

Representative sequences of the non-redundant gene catalogue were aligned to the NCBI NR database with e-value cutoff of $1e^{-5}$ using BLASTP (version 2.2.28+, http://blast.ncbi.nlm.nih.gov/Blast.cgi) for

taxonomic annotations. Cluster of Orthologous Groups (COG) of proteins annotation for the representative sequences was performed using BLASTP against the eggNOG database[20,21] (version 4.5) with an e-value cutoff of $1e^{-5}$. KEGG annotation was conducted using BLASTP (version 2.2.28+) against the Kyoto Encyclopedia of Genes and Genomes database[22] (http://www.genome.jp/keeg/) with an e-value cutoff of $1e^{-5}$. The metagenomics raw reads were deposited into the NCBI SRA database (PRJNA520777 and PRJNA520743.).

Organism-specific gene hits were annotated with the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology (KO) database. Comparisons of pathway enrichment across the LR, HR and NR groups showed differences in metabolic functions, especially menaquinone biosynthesis.

2.6UPLC-APCI-MS analysis for vitamin K 2 in fecal samples

Aliquots of plasma and fecal samples were freeze-dried to a constant weight and homogenized by using a mortar and pestle. 500 μL plasma sample was analyzed for vitamin K1 concentration, and approximately 40 mg dried fecal sample were analyzed for vitamin K2 content. Samples were measured using published methods with minor modifications [23,24]. Briefly, we changed the amount of protein precipitation and extraction agent as well as the proportion of mobile phase. 600 μL acetonitrile was added to the mixture of 200 μL fecal sample suspension and 5 μL internal standard (1.6 μg/mL MK4-d7) to precipitate the protein, and the mixture was extracted with 900 μL cyclohexane. The cyclohexane phase was separated and evaporated to dryness. The residue was reconstituted with 100 μL mobile phase, and then 10 μL was injected into a LC-30 UPLC (Shimadzu Corporation)–AB SCIEX Triple Quad 5500 mass spectrometry system for identification and quantification of vitamin K2. A series of Vitamin K2 were separated before mass spectrometry by UPLC by using a reverse-phase C18 analytic column (Acquity uplcrbeh: 1.7μm, 50 mm×2.1 mm; Waters).MK5–MK13 were detected at the following mass-to-charge ratios: m/z 450.6 (VK1), m/z 444.5 (MK4), m/z 513.4 (MK5), m/z 581.4 (MK6), m/z 649.5 (MK7), m/z 717.6 (MK8), m/z 785.2(MK9), m/z 853.8 (MK10), m/z 922.4 (MK11), m/z 990.6 (MK12),and m/z 1058.7 (MK13). The mobile phase was methanol-water with gradient conditions. No significant interferences caused by endogenous compounds were observed. Validation of analytical method for VK2 showed that the chosen method was precise and accurate with a linear response range of 0.5–50 ng/mL. Some fecal samples, whose concentrations were beyond the upper limit of the standard curve, were diluted with blank samples to the range of 0.03–30 μg/mL and prepared.

Because we only obtained calibration standards for MK5, MK6, MK7 and MK9 at the time of analysis, and MK8, MK10-MK13 were not available, we could not confidently quantify precise fecal MK8, MK10–MK13 concentrations. We calculated the VK1, MK4, MK5, MK6, MK7 and MK9 amounts, while fecal MK8, MK10–MK13 amounts were statistically analyzed by using the AUC derived from the UPLC–mass spectrometry chromatogram.

2.7OTU biomarker identification

We developed a random forest-based classification approach to perform faecal source identification[25] using microbial community data from the discovery cohort and validation cohort (n=90; 30 LRs, 30 HRs and 30 NRs). Tenfold cross-validation was performed on a random forest model (R 3.4.1, random Forest 4.6-12 package) using the OTU abundance profile of the training cohort, including 27 LRs, 27 HRs and 26 NRs, as previously described. Using five trials of the tenfold cross-validation, we obtained the cross-validation error curve (Supplementary figure S3A). The point with the minimum cross-validation error was viewed as the cut-off point. We listed all sets (≤50) of genus markers with an error less than the cut-off value (Supplemental table S14) and chose the set with the smallest number of OTUs as the optimal set(Supplementary Methods 1.6).

2.8Statistical analysis

Continuous variables are expressed as the mean±SD. Discrete variables are expressed as percentages. The average relative abundances of specific taxa and KEGG functions are also expressed as percentages. The Kruskal-Wallis H test of variance was used to evaluate differences among the three groups. Continuous variables were compared between two groups using the Wilcoxon rank sum test. Statistical analyses were performed using SPSS V20.0 (IBM),considering P values<0.05 as statistically significant.

3. Results

3.1Participant characteristics

The 200 enrolled HVR patients' demographics, indications for anticoagulation therapy, and genotypes are shown in Supplementary Table S1. According to the inpatients' warfarin doses and INRs, we found that 20% of the patients were oversensitive to warfarin and were prone to excessive anticoagulation, while 15% of the patients exhibited low sensitivity to warfarin. These groups of patients (20% and 15%) were defined as HR group and LR group, respectively. The others (65%) were defined as NR group. The warfarin sensitivity index (WSI)[26] was significantly different among the LR, HR and NR groups (P=0.0004), while characteristics including age, height, weight, body mass index (BMI), body surface area (BSA), hospitalization time and vitamin K intake were not significantly different among the three groups(P>0.05). There was no statistically significant difference in the distribution of CYP2C9 and VKORC1 gene types among the three groups (P>0.05). The daily dietary intake of vitamin K (P=0.637, range 41.8-96.8 μg/day) was stable over the observation period(Table 1).

This finding indicated that at the beginning of anticoagulation therapy, three groups' clinical characteristics were all equivalent, but the difference of WSI among the three groups was still very significant (P<0.001).

3.2Stool moisture and form scale

Stool moisture and form are strongly associated with gut microbiota richness and composition.[27] In terms of stool moisture, there were no significant differences among LRs, HRs and NRs in the discovery

phase (*P*=0.213), as shown in Table 1 and Supplementary Table S2. Moreover, all stool samples had a soft consistency. With regard to stool color, most of the stool samples were yellow, and there were no significant color differences among the stools LRs, HRs and NRs.

Biodiversity of fecal microbiota

To profile the differences in the structure of the gut microbiota among HVR patients, we performed Illumina MiSeq sequencing of the V3-V4 region of the bacterial 16S rDNA gene for 80 fecal samples collected from the three groups (27 LRs, 27 HRs, and 26 NRs). In total, 2 557 695 high-quality 16S rDNA sequences were generated. After subsampling to identify the lowest number of reads observed in a single sample (28 003 reads) and clustering, 660 OTUs with 97% identity were obtained, with the number of OTUs ranging from 34 to 286 per sample.

Rarefaction analysis showed that the estimated OTU richness approached saturation in each group (Figure 2A). The estimated sample coverage (Good's coverage) was approximately 0.999, and the correlation between duplicate samples was more than 99.5%, which indicated that the accuracy and reproducibility of the sequences were good (Figure 2B). Compared with the NRs (n=26) and LRs (n=27), the HRs showed markedly reduced fecal microbial diversity as estimated by the Shannon index (*P*=0.0088 and 0.0060, respectively), while there was no significant difference between the LR group and the NR group in this respect (*P*=0.875) (Figure 2C).

Moreover, a Venn diagram displaying the overlap between groups showed that 378 of the overall 660 OTUs were shared among the three groups. (Figure 2D, Supplementary Table S4). To measure the microbiome differences between samples, we calculated beta diversity using Bray-Curtis dissimilarity, and principal coordinate analysis (PCA) indicated a symmetrical distribution of the fecal microbial community among all samples (Figure 2E).

Based on these data, we examined the diversity of the gut microbiomes in HVR inpatients and found that the alpha diversity of the gut microbiome was significantly higher in LRs (n=27) than in HRs (n=27) according to sobs, shannon, simposon, ace, chao indices (*P*=0.006, Supplementary Table S5).

3.3Microbiota composition

The bacterial genera *Enterococcus*, *Bacteroides*, *Escherichia-Shigella, Klebsiella, Streptococcus, Lactobacillus, Prevotella_9, Bifidobacterium*, *Veillonella* and *Parabacteroides* were the top 10 dominant populations in the three groups (Figure 3A; Supplementary Table S6). Two genera (Figure 3B), namely, *Enterococcus* (*P*=1.249e^{-11}) and *Escherichia-Shigella* (*P*=3.189e^{-11}), exhibited significantly different abundance among the three groups. The proportion of *Enterococcus* sequences was significantly higher in the HR group (50.15%±14.26%, Figure 3C) but lower in the LR group (3.99%±1.23%) than in the NR group(15.44%±5.55%). The genera *Escherichia-Shigella* was markedly more abundant in the LR group(27.50%±4.57%) than in the HR group (0.51%±0.11%) (Figure 3D).

To further explore these findings, we performed high-dimensional class comparisons through LEfSe (Supplementary Figure S1A, 1B). A representative cladogram of fecal microbiota structures and predominant bacteria showed the great differences in taxa among the LR, HR and NR groups (Supplementary Figure S1C). These data again demonstrated that there were differences in the abundance of bacteria in the fecal microbiome of the three groups with different responses to anticoagulation therapy; *Escherichia-Shigella* was enriched in the LR group, and *Enterococcus* was enriched in the HR group.

3.4Functional analysis of fecal microbiota through shotgun metagenomics

Next, we sought to gain further insight into the mechanism through which the gut microbiome may influence the response to anticoagulation therapy. We first conducted functional genomic profiling of gut microbiome samples via metagenomic WGS sequencing (n=15) of LR (n=5), HR (n=5) and NR (n=5) samples.

We performed unbiased WGS sequencing of total DNA extracted from 15 fecal samples, obtaining an average of 95 540 367 raw reads per sample (range: 87 579 090-107 493 398). The WGS reads were filtered multiple times to remove host components before sequence assembly and gene prediction (Supplementary Table S7, S8); then, the resulting sequences were aligned to datasets containing 3 167 461 bacterial genes.

We annotated the genes using the KEGG functional database (V.59) to investigate whether certain microbial functions were associated with menaquinone biosynthesis. Pathway id:ko00360, phenylalanine metabolism (Supplementary Figure S2) suggesting that bacterial metabolic pathways related to vitamin K2 present in the KEGG database were involved in responses to warfarin anticoagulation therapy. We found the highest abundance in the LRs among the three groups, and the lowest abundance in the HRs, especially for enzymes 5.4.4.2, 4.2.99.20, 4.2.1.113, 4.1.3.36, 3.1.2.28 and 2.1.1.163. The greater the enrichment of those enzymes, the more bacterial vitamin K is produced (Figure 4 A,Supplementary Table S9).[28,29]

The modules M00022, M00023, M00024, M00025, M00095, M00096, M00116 and M00117 are associated with menaquinone biosynthesis. The abundance of those modules is positively correlated with the quantity of bacterial vitamin K.[30] In Figure 4B and Supplementary Table S10, the LR group contained higher level modules than the other two groups, and the levels of M00023, M00095, M00096 and M00116 were significantly increased.

The 16 KO functions from the available KEGG functions are equal to the enzymes listed above. The LR group showed a higher abundance of these K0s than the other two groups (Figure 4C, Supplementary Table S11).

Overall, the LR group had low selectivity for the anticoagulant warfarin, maybe because these patients are enriched with those enzymes, modules and KOs, which are all positive factors related to bacterial vitamin

K biosynthesis; the reverse was found for the HR group.

3.5Vitamin K content and correlation analysis

It exists naturally as two distinct molecular species, vitamin K1 and vitamin K2[30]. The later is also named as menaquinone (MK). Depending on the number of isoprene units, a series of VK2 are denoted as MK-n[31].

Both VK1 and MK4 concentrations in plasma were derived from dietary VK intake [28]. There was a large difference between VK1 and MK4 concentrations in human plasma, but there was no significant difference in the average distribution of VK1 and MK4 concentrations in LR group, HR group and control group (P>0.05, Table 2).

MK5-MK13 are synthesized in bacteria and are closely associated with the inner cytoplasmic membrane and function under reduced (anaerobic) conditions as redox compounds in bacterial respiration[32]. The amount of MK5, MK6, MK7 and MK9 in stool is different (Table 3). Although the MK7 and the amount of MK9 no obvious difference in the three groups of patients, but the amount of four VK2 is a very significant difference (P< 0.01), The amount of VK2 synthesized by intestinal flora in LR group, NR group and HR group was (8.76±5.55) μg, (4.44±3.89) μg and (3.04±2.14) μg, respectively, showing a trend of LR group >NR group >HR group.

Bivariate Correlation analysis provided by SPSS was used to explore relations between gut microbiota composition and stool vitamin K content. The results showed that the correlation coefficient between *Enterococcus* abundance and the total amount of MK in feces was -0.397 (P=0.037), indicating a significant negative Correlation. The correlation coefficient between the abundance of *Escherichia-shigella* and the total amount of MK in feces was 0.353 (P=0.012), indicating a significant positive correlation.

3.6Identification of microbial OTU-based markers of HVR inpatient responses to anticoagulation therapy

To illustrate the diagnostic value of the fecal microbiome in determining responses to warfarin anticoagulation, we constructed a random forest classifier model that could specifically identify LRs, HR and NRs. Based on the 80 HVR patients in the discovery phase, bacterial genera corresponding to 50 OTU markers were selected as the optimal marker set (Supplementary Table S12). The cross-validation error curve (Supplementary Figure S3A) was calculated using the set of 50 identified optimal OTUs for both the discovery cohort and the validation cohort. The 12th solid point represents the point with the lowest error rate. Then, 12 genera were selected for the optimal marker set by random forest models.

In the discovery phase, the ROC curve between the LR and HR groups had an AUC value of 0.87 with a 95% confidence interval (CI) of 0.73 to 1 between LR and HR cohorts (Supplementary Figure S3B), that between the HR and NR had an AUC value of 0.78 with a 95% CI of 0.56 to 1 (Supplementary Figure S3C), and that between the LR and NR had an AUC value of 0.75 with a 95% CI of 0.56 to 0.91 (Supplementary

Figure S3D). These data suggested that the random forest module based on microbial OTU markers had high diagnostic value for the LR, HR and NR groups.

In the validation phase, 30 LRs, 30 HRs and 30 NRs were used to validate the diagnostic efficacy for the LR and HR groups. The ROC curve between the LR and HR groups had an AUC value of 0.81 (95% CI: 0.62 to 0.99) (Supplementary Figure S3E), the AUC value between the HR and NR was 0.72 (95% CI: 0.48 to 0.92) (Supplementary Figure S3F), and the AUC value between the LR and NR was 0.69 (95% CI: 0.44 to 0.93), which indicated significant diagnostic potential for both groups.

4. Discussion And Conclusions

It has previously been suggested that the potential role of microbiota in vitamin K synthesis should be considered in selecting warfarin regimens[33]. Given the samples were from the first replacement surgery hospitalized patients taking warfarin anticoagulant therapy, vitamin k intake is limited, the diet daily intake of vitamin k and plasma concentrations of VK1 and MK4 were all have no significant difference in the three groups of patients, so this study focused on the intestinal bacteria groups for the synthesis of VK2 pathways and gene abundance.

Recent studies[15,32] have reported that several forms of vitamin K are synthesized by *Bacteroides, Enterobacter, Veillonella,* and *Eubacterium lentum,* which are typical members of the intestinal microflora. If the number of vitamin K-synthesizing bacteria in the microbiota increases for any reason, vitamin K levels will be elevated[33]. This study found the abundance of *escherichia-shigella,* which was positively correlated with vitamin K production, were significantly different among three groups (P<0.001) and showed LR group >NR group > HR group.

This study is the first report to illustrate gut microbial characteristics in HVR patients at the beginning of warfarin anticoagulation therapy. Dawson *et al* reported that the biosynthesis of VK in gut microbiota was catalyzed and regulated by 16 enzymes encoded by the cluster of *Men* genes[24].We found the abundance of these enzymes positively correlated with VK biosynthesis was the highest in LR group, the lowest in HR group. The 16 KO abundance and 4 modules corresponding to the above enzyme also present the phenomenon of LRs>NRs>HRs in the three groups. These results indicated a significant global shift in gut microbiota from HRs to LRs; this altered microbial community might play an important role during the initiation of anticoagulation therapy. Thus, greater richness or diversity of vitamin K-synthesizing bacteria, *Escherichia-Shigella,* in the gut microbiota in our cohort was a sign of poorer warfarin response. In addition, our results suggest that HVR patients with overgrowth of *Enterococcus* might show high sensitivity to warfarin anticoagulation therapy.

Some limitations should be considered when interpreting our results. First, only the relative abundances of single taxa, not the absolute numbers, were considered for statistical analyses. The relative abundance approach may not reflect substantial inter-individual differences in fecal microbial loads, producing possible bias. Additionally, the sample size in the study is relatively small. we will also include other types of patients needing warfarin for anticoagulation therapy in subsequent research

Declarations

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The paired-end (PE) amplicon libraries were constructed and sequencing was performed (using the Illumina MiSeq platform) at Majorbio Bio-pharm Technology Co., Ltd., Shanghai, China.

Author's contribution

LY.M and ZY.S developed the concept for the study; L.W, and L.Z collected the samples. XX.L performed bioinformatics analyses. LS.L performed statistical analyses. L.W and LY.M wrote the manuscript with input from all authors. M.X and CRH provided intellectual contribution.

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Availability of data and materials

The raw Illumina read data for all samples were deposited in the National Center for Biotechnology Information (NCBI) database under accession numbers PRJNA518710, PRJNA520777 and PRJNA520743.

Ethics approval and consent to participate

Ethical permission for this study was obtained from the Health Authority Ethics Committee of the First Affiliated Hospital of Soochow University (Suzhou, Jiangsu, China). All patients provided written informed consent in accordance with the Declaration of Helsinki.

Consent for publication

Yes.

Competing interests

The authors declare no competing interests.

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References

1 Bell RG, Matschiner JT. Warfarin and the inhibition of vitamin k activity by an oxide metabolite. Nature 1972;237(5349): 32-33.

2 Martin MH, Meadows J, McElhinney DB, Goldstein BH, Bergersen L, Qureshi AM, Shahanavaz S, Aboulhosn J, Berman D, Peng L, Gillespie M, Armstrong A, Weng C, Minich LL, Gray RG. Safety and feasibility of melody transcatheter pulmonary valve replacement in the native right ventricular outflow tract: A multicenter pediatric heart network scholar study. JACC Cardiovascular interventions 2018;11(16): 1642-1650.

3 Wadelius M, Pirmohamed M. Pharmacogenetics of warfarin: Current status and future challenges. The pharmacogenomics journal 2007;7(2): 99-111.

4 Salem DN, O'Gara PT, Madias C, Pauker SG. Valvular and structural heart disease: American college of chest physicians evidence-based clinical practice guidelines (8th edition). Chest 2008;133(6 Suppl): 593S-629S.

5 Hussain S, Siddiqui AN, Baxi H, Habib A, Hussain MS, Najmi AK. Warfarin use increases bleeding risk in hemodialysis patients with atrial fibrillation: A meta-analysis of cohort studies. Journal of gastroenterology and hepatology 2019.

6 Feagins LA, Weideman RA. Gi bleeding risk of doacs versus warfarin: Is newer better? Digestive diseases and sciences 2018;63(7): 1675-1677.

7 Riley TR, Gauthier-Lewis ML, Sanchez CK, Riley TT. Evaluation of bleeding events requiring hospitalization in patients with atrial fibrillation receiving dabigatran, warfarin, or antiplatelet therapy. Journal of pharmacy practice 2017;30(2): 214-218.

8 Zhao L, Chen C, Li B, Dong L, Guo Y, Xiao X, Zhang E, Qin L. Verification of pharmacogenetics-based warfarin dosing algorithms in han-chinese patients undertaking mechanic heart valve replacement. PloS one 2014;9(4): e94573.

9 Miao L, Yang J, Huang C, Shen Z. Contribution of age, body weight, and cyp2c9 and vkorc1 genotype to the anticoagulant response to warfarin: Proposal for a new dosing regimen in chinese patients. European journal of clinical pharmacology 2007;63(12)**:** 1135-1141.

10 Xue L, Holford N, Ding XL, Shen ZY, Huang CR, Zhang H, Zhang JJ, Guo ZN, Xie C, Zhou L, Chen ZY, Liu LS, Miao LY. Theory-based pharmacokinetics and pharmacodynamics of s- and r-warfarin and effects on international normalized ratio: Influence of body size, composition and genotype in cardiac surgery patients. British journal of clinical pharmacology 2017;83(4)**:** 823-835.

11 Kim DJ, Kim HS, Oh M, Kim EY, Shin JG. Cost effectiveness of genotype-guided warfarin dosing in patients with mechanical heart valve replacement under the fee-for-service system. Applied health economics and health policy 2017;15(5)**:** 657-667.

12 Rost S, Fregin A, Ivaskevicius V, Conzelmann E, Hortnagel K, Pelz HJ, Lappegard K, Seifried E, Scharrer I, Tuddenham EG, Muller CR, Strom TM, Oldenburg J. Mutations in vkorc1 cause warfarin resistance and multiple coagulation factor deficiency type 2. Nature 2004;427(6974)**:** 537-541.

13 Beulens JW, Bots ML, Atsma F, Bartelink ML, Prokop M, Geleijnse JM, Witteman JC, Grobbee DE, van der Schouw YT. High dietary menaquinone intake is associated with reduced coronary calcification. Atherosclerosis 2009;203(2)**:** 489-493.

14 Gast GC, de Roos NM, Sluijs I, Bots ML, Beulens JW, Geleijnse JM, Witteman JC, Grobbee DE, Peeters PH, van der Schouw YT. A high menaquinone intake reduces the incidence of coronary heart disease. Nutrition, metabolism, and cardiovascular diseases : NMCD 2009;19(7)**:** 504-510.

15 Karl JP, Meydani M, Barnett JB, Vanegas SM, Barger K, Fu X, Goldin B, Kane A, Rasmussen H, Vangay P, Knights D, Jonnalagadda SS, Saltzman E, Roberts SB, Meydani SN, Booth SL. Fecal concentrations of bacterially derived vitamin k forms are associated with gut microbiota composition but not plasma or fecal cytokine concentrations in healthy adults. The American journal of clinical nutrition 2017;106(4)**:** 1052-1061.

16 Tickle TL, Segata N, Waldron L, Weingart U, Huttenhower C. Two-stage microbial community experimental design. The ISME journal 2013;7(12)**:** 2330-2339.

17 Blankenberg D, Von Kuster G, Coraor N, Ananda G, Lazarus R, Mangan M, Nekrutenko A, Taylor J. Galaxy: A web-based genome analysis tool for experimentalists. Current protocols in molecular biology 2010;Chapter 19**:** Unit 19 10 11-21.

18 Fu L, Niu B, Zhu Z, Wu S, Li W. Cd-hit: Accelerated for clustering the next-generation sequencing data. Bioinformatics 2012;28(23)**:** 3150-3152.

19 Li R, Li Y, Kristiansen K, Wang J. Soap: Short oligonucleotide alignment program. Bioinformatics 2008;24(5)**:** 713-714.

20 Jensen LJ, Julien P, Kuhn M, von Mering C, Muller J, Doerks T, Bork P. Eggnog: Automated construction and annotation of orthologous groups of genes. Nucleic acids research 2008;36(Database issue): D250-254.

21 Tatusov RL, Fedorova ND, Jackson JD, Jacobs AR, Kiryutin B, Koonin EV, Krylov DM, Mazumder R, Mekhedov SL, Nikolskaya AN, Rao BS, Smirnov S, Sverdlov AV, Vasudevan S, Wolf YI, Yin JJ, Natale DA. The cog database: An updated version includes eukaryotes. BMC bioinformatics 2003;4: 41.

22 Xie C, Mao X, Huang J, Ding Y, Wu J, Dong S, Kong L, Gao G, Li CY, Wei L. Kobas 2.0: A web server for annotation and identification of enriched pathways and diseases. Nucleic acids research 2011;39(Web Server issue): W316-322.

23 Karl JP, Fu X, Wang X, Zhao Y, Shen J, Zhang C, Wolfe BE, Saltzman E, Zhao L, Booth SL. Fecal menaquinone profiles of overweight adults are associated with gut microbiota composition during a gut microbiota-targeted dietary intervention. The American journal of clinical nutrition 2015;102(1): 84-93.

24 Ma L, Cao Y, Chen L, Xiao Q, Yang J, Yang J. Pharmacokinetics and pharmacodynamics of two vitamin k1 formulations in rats. Drug research 2015;65(3): 125-132.

25 Roguet A, Eren AM, Newton RJ, McLellan SL. Fecal source identification using random forest. Microbiome 2018;6(1): 185.

26 Lubetsky A, Dekel-Stern E, Chetrit A, Lubin F, Halkin H. Vitamin k intake and sensitivity to warfarin in patients consuming regular diets. Thrombosis and haemostasis 1999;81(3): 396-399.

27 Vandeputte D, Falony G, Vieira-Silva S, Tito RY, Joossens M, Raes J. Stool consistency is strongly associated with gut microbiota richness and composition, enterotypes and bacterial growth rates. Gut 2016;65(1): 57-62.

28 Conly JM, Stein K, Worobetz L, Rutledge-Harding S. The contribution of vitamin k2 (menaquinones) produced by the intestinal microflora to human nutritional requirements for vitamin k. The American journal of gastroenterology 1994;89(6): 915-923.

29 Hill MJ. Intestinal flora and endogenous vitamin synthesis. European journal of cancer prevention : the official journal of the European Cancer Prevention Organisation 1997;6 Suppl 1: S43-45.

30 Shearer MJ, Newman P. Metabolism and cell biology of vitamin k. Thrombosis and haemostasis 2008;100(4): 530-547.

31 Aydin S. Can vitamin k synthesis atered by dysbiosis of microbiota be blamed in the etiopathogenesis of venous thrombosis? Bioscience of Microbiota,Food and Health 2017;36(3): 2.

32 Card DJ, Gorska R, Cutler J, Harrington DJ. Vitamin k metabolism: Current knowledge and future research. Molecular nutrition & food research 2014;58(8): 1590-1600.

33 Woods K, Douketis JD, Kathirgamanathan K, Yi Q, Crowther MA. Low-dose oral vitamin k to normalize the international normalized ratio prior to surgery in patients who require temporary interruption of warfarin. Journal of thrombosis and thrombolysis 2007;24(2): 93-97.

Tables

Table 1 Clinical characteristics of HVR inpatients in three groups (n=80)

Clinical characteristics	LR group (n=27)	HR group (n=27)	NR group (n=26)	*P* value
Gender(Male/Female)	14/13	15/12	13/13	
Age(year)[±SD]	58.1±9.2	62.1±10.5	58.9±11.1	0.611
range	41～76	37～76	42～75	
Height(cm) [±SD]	161.5±8.5	162.8±5.9	159.3±7.3	0.377
range	145～177	153～172	148～170	
Weight(kg) [±SD]	60.0±17.4	57.9±9.2	58.3±9.3	0.874
range	42.5～115	41～75	47～76	
BMI(kg/m^2) [±SD]	22.7±4.6	21.7±2.7	22.9±2.8	0.573
range	17.5～36.7	16.6～28.6	18.3～28.3	
BSA(m^2) [±SD]	1.6±0.3	1.6±0.2	1.6±0.2	0.885
range	1.3～2.4	1.3～1.9	1.4～1.9	
WSI($mg/day/m^2$) [±SD]	0.44±0.17	1.55±0.78	0.87±0.66	0.000***
range	0.20～0.80	0.40～2.90	0.20～2.20	
VK intake(ug/d)[±SD]	61.7±10.2	59.8±9.5	56.8±6.6	0.637
range	41.8～96.8	45.6～76.3	47.1～75.2	
Hospitalization(d)[±SD]	61.7±10.2	59.8±9.5	59.6±10.4	0.798
range	51～96	50～82	50～90	
Stool moisture(%)[±SD]	73.0±5.5	75.1±3.8	75.4±2.8	0.213
range	58.9～80.1	68.6～80.3	68.4～79.2	
Type of valve MVR/AVR/MVR+AVR/TVR/MVR+TVR/MVR+AVR+TVR	18/5/4/0/0	16/4/5/1/1/0	14/6/3/2/0/1	
CYP2C9 genotype(n) *1*1/*1*3	26/1	26/1	24/2	
VKORC1 genotype(n) AA/GA/GG	22/5/0	22/4/1	20/4/2	

One-way analysis of variance was used to evaluate the difference among the three groups.
HVR, heart valvular replacement; LR, low warfarin responders; HR, high warfarin responders; NR,normal warfarin responders; BMI,body mass index; BSA, body surface area; WSI, Warfarin sensitivity index; VK,vitamin K; MVR, mitral valve replacement; AVR, aortic valve replacement; TVR, tricuspid valve replacement.
*0.01≤p<0.05；**0.005≤p<0.01；***0.001≤p<0.005

Table 2 The distribution of vitamin K in plasma in HVR patients（*n*=80）

Vitamin K	LR group (*n*=27)	HR group (*n*=27)	NR group (*n*=26)	*P* value
VK1(ng·mL^{-1})[±SD]	14.7±4.2	16.1±9.5	15.9±4.1	0.213
range	0～19.8	3.7～26.6	4.2～22.2	
MK4 (ng·mL^{-1})[±SD]	16.5±3.5	16.8±5.9	15.9±7.3	0.377
range	4.5～21.7	5.3～27.2	4.8～31.0	

Table 3 The distribution of vitamin K in stool in HVR patients (*n*=80)

Vitamin K	LR group (n=27)	HR group (n=27)	NR group (n=26)	*P* value
MK5 (μg)[±SD]	1.60±1.27	0.33±0.44	0.59±0.59	0.000***
MK6 (μg)[±SD]	1.31±1.51	0.23±0.29	0.46±0.29	0.003***
MK7 (μg)[±SD]	3.74±5.54	1.27±0.56	2.02±1.79	0.107
MK9 (μg)[±SD]	2.11±1.03	1.21±1.61	1.37±2.07	0.236
MK5-MK9 (μg)[±SD]	8.76±5.55	3.04±2.14	4.44±3.89	0.005**

*0.01≤p<0.05，**0.005≤p<0.01，***0.001≤p<0.005

Figures

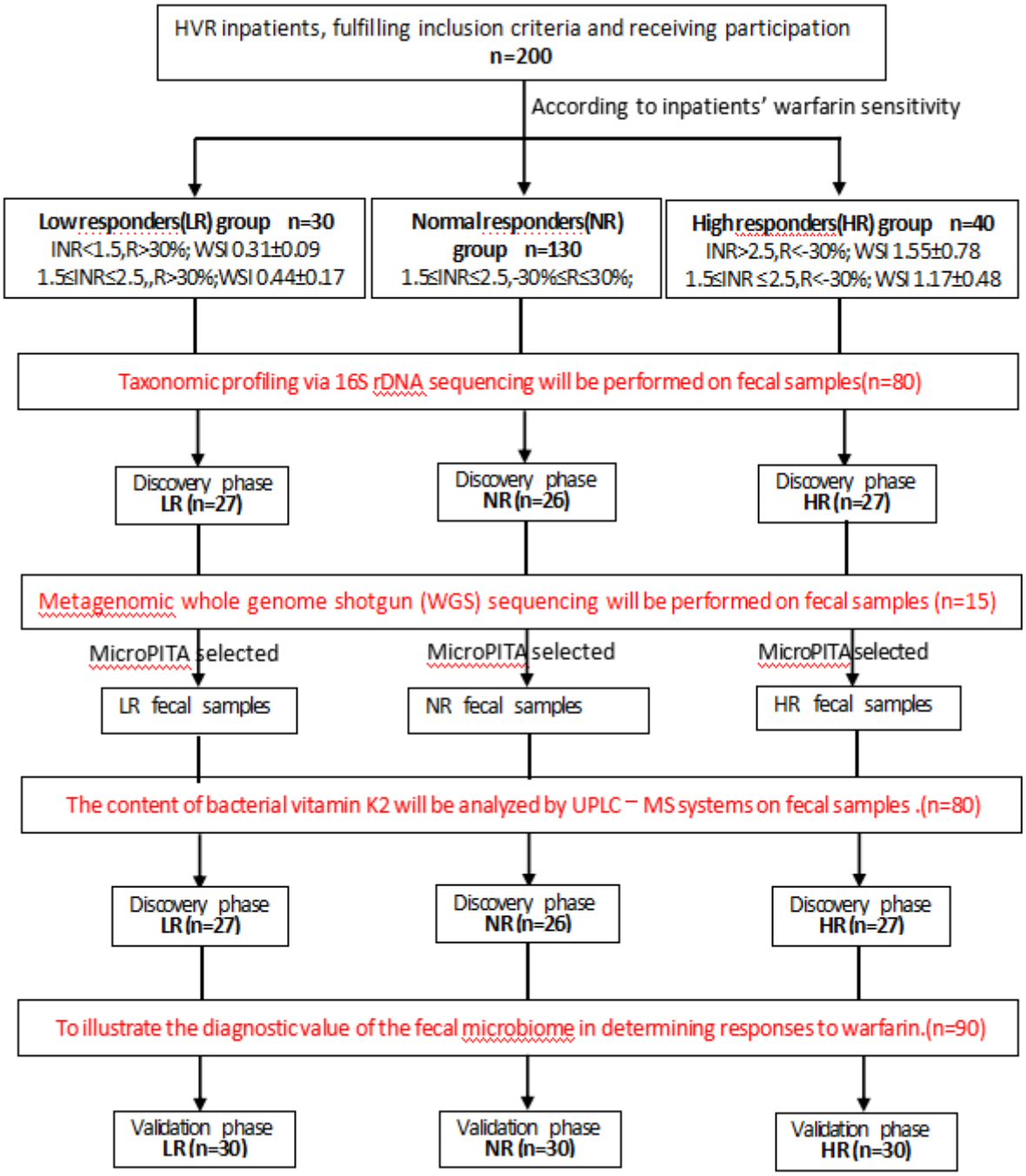

Figure 1

Study design and flow diagram. A total of 200 HVR inpatients fulfilled our inclusion criteria and participated in the study. According to their warfarin sensitivity, patients were classified as low responders (LR, n=30), high responders (HR, n=40) and normal responders (NR n=130). In the discovery phase, we characterized the gut microbiome of 27 LRs, 26 NRs, and 27 HRs, analyzed the bacterial vitamin K2, identified microbial markers and constructed a warfarin response classifier using a random forest model for HVR inpatients. We sought to gain insight into the mechanism by which the gut microbiome may influence responses to warfarin anticoagulation therapy. Therefore, we conducted functional genomic profiling of the gut microbiome via metagenomic whole genome shotgun (WGS) sequencing of 15 fecal

samples (5 LRs, 5 NRs, 5 HRs) selected by MicroPITA analysis. In the validation phase, 30 LRs, 30 NRs and 30 HRs were used to validate the diagnostic efficacy of the warfarin response classifier. HRV, heart valve replacement; INR, international normalized ratio; R, the difference rate between a patient's actual warfarin dose and the theoretical warfarin dose calculated by the formula generated in this study; WSI, warfarin sensitivity index; MicroPITA, microbiomes: Picking Interesting Taxonomic Abundance.

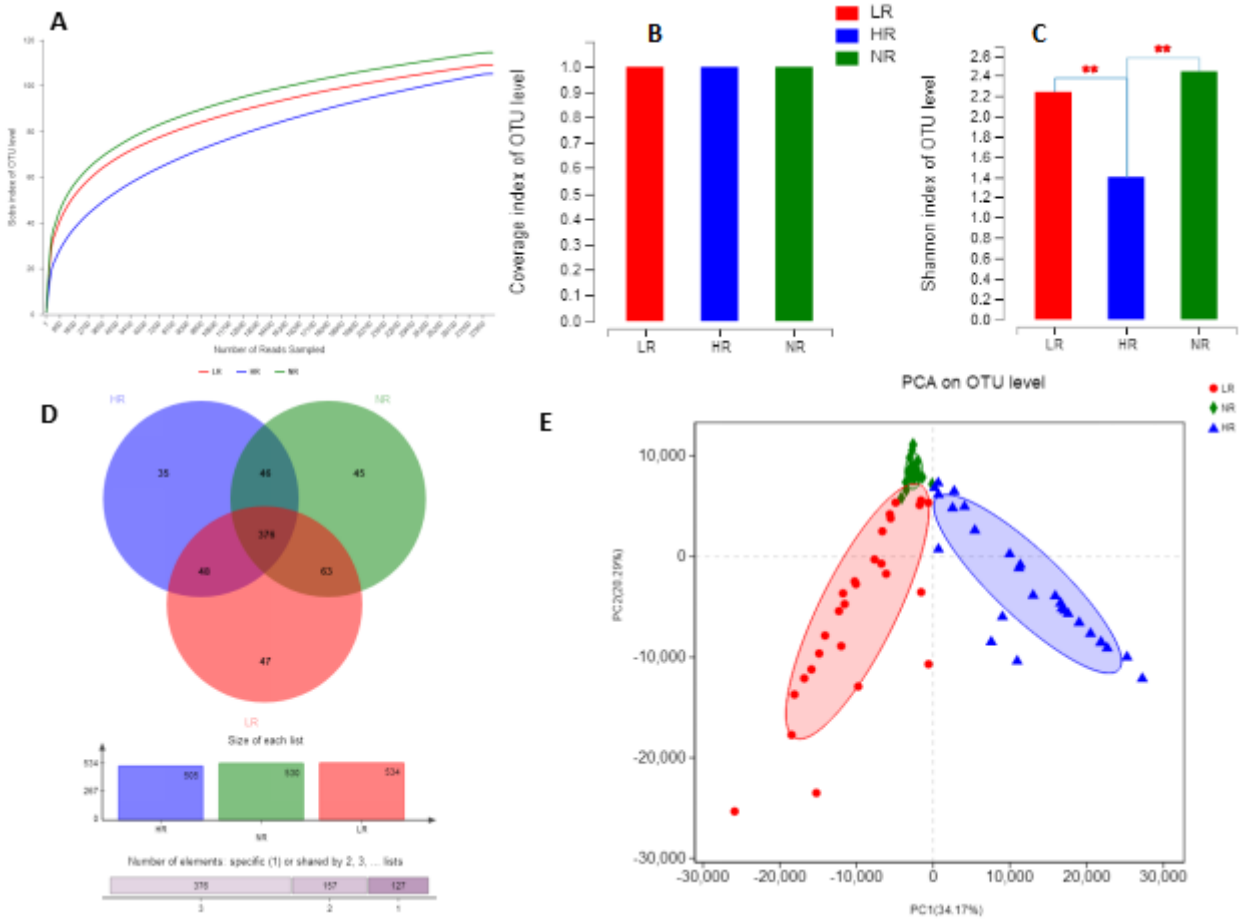

Figure 2

Biodiversity of fecal microbiota from 80 HVR patients in the discovery phase. (A) Rarefaction analysis showed that the estimated OTU richness approached saturation in each group. (B) The estimated sample coverage (Good's coverage) was approximately 0.999. (C) Fecal microbial diversity, as estimated by the Shannon index, was markedly decreased in HRs (depicted in blue) (P=0.0088 and P=0.0060, respectively), while no significant difference was found between the LRs and NRs (P=0.875). (D) A Venn diagram displaying the overlap. (E) Beta diversity indicated a symmetrical distribution of the fecal microbial community among all the samples. * 0.01<P≤0.05, ** 0.001< P≤ 0.01, *** P≤0.001

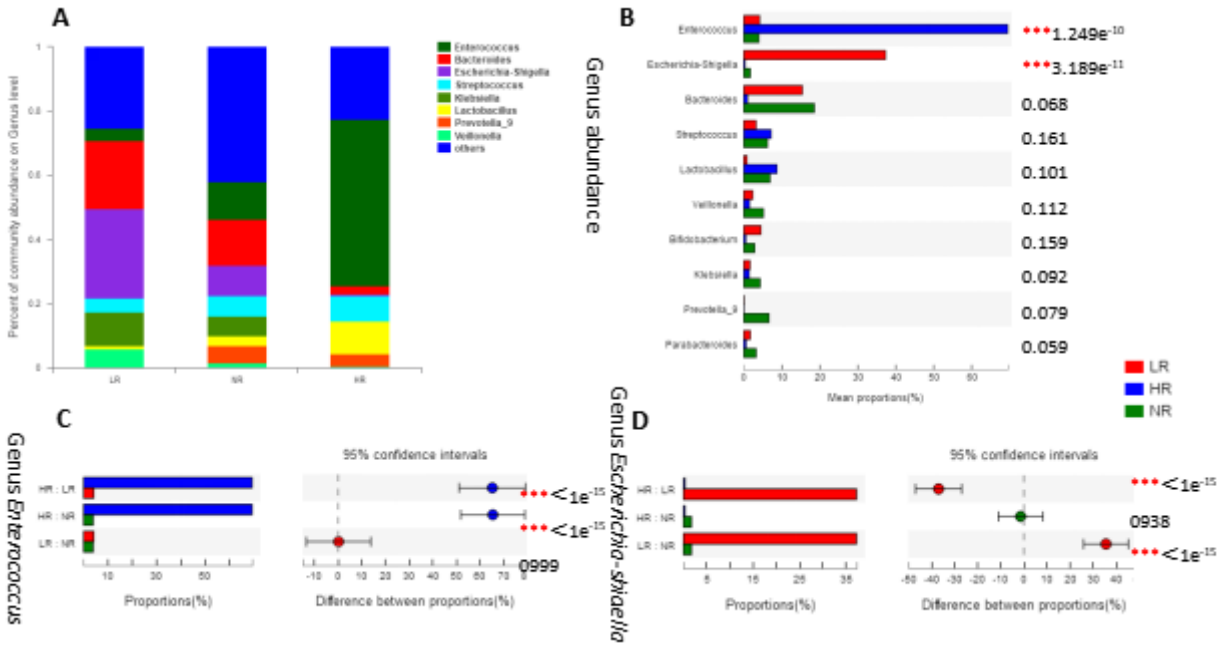

Figure 3

Compositional differences in the gut microbiome from 80 HVR patients in the discovery phase. The composition of fecal microbiota at the genus level (A) among LR patients (n=27, depicted in red), HR patients (n=27, depicted in blue) and NRs (n=26, depicted in green) is shown. (B) The compositions of Enterococcus and Escherichia-Shigella were all significantly different among the three groups (P=1.249e-11, and P=3.189e-11, respectively). Compared to NRs, species of the Enterococcus genus were significantly increased in the HR group (C), but decreased in the LR group. The markedly increased microbial organisms at the genus Escherichia-Shigella in the LR group, while such microbe was significantly decreased in the HR group (D). * 0.01<P≤0.05, ** 0.001< P≤0.01, *** P≤0.001

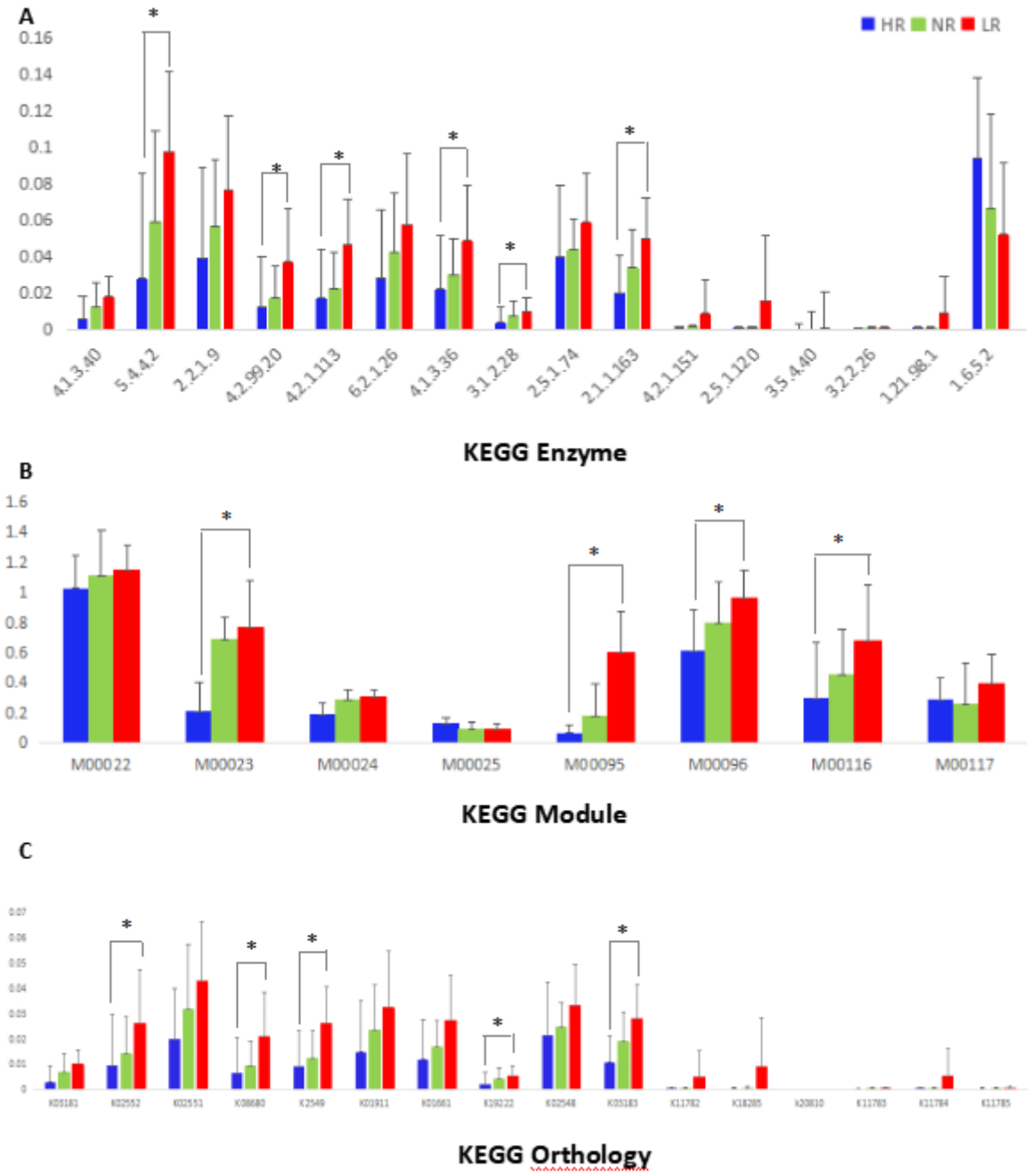

Figure 4

Abundance of differences in KEGG functions within the gut microbiome among the LR (red), HR (blue) and NR (green) groups via metagenomic WGS sequencing. (A) Comparison of the abundances of 18 types of KEGG enzymes among the three groups by the Kruskal-Wallis H test. (B) Comparison of the abundances of 12 types of KEGG modules among the three groups by the Kruskal-Wallis H test. (C) Comparison of the abundances of 8 types of KEGG Orthology (KO) groups among the three groups by the Kruskal-Wallis H test. * $0.01<P\leq0.05$, ** $0.001<P\leq 0.01$, *** $P\leq0.001$

Supplementary Files

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