Restoration of the gut microbiota is associated with a decreased risk of hepatic encephalopathy after TIPS

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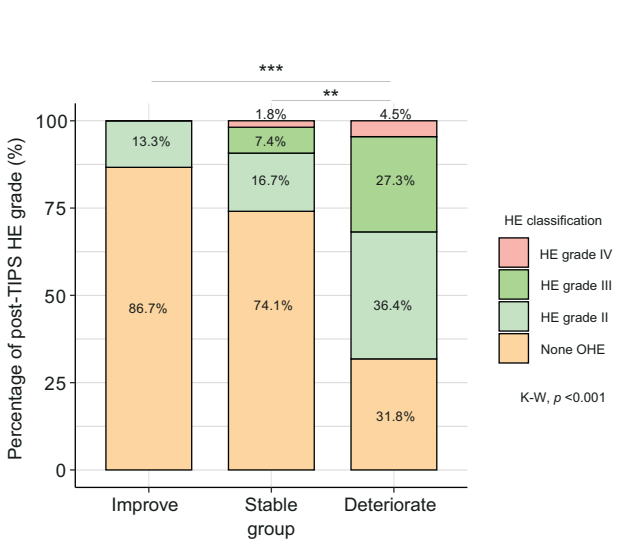
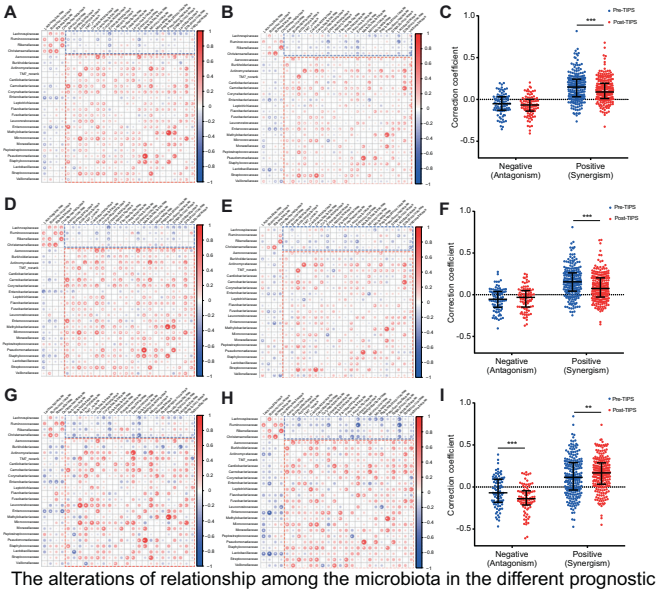
Graphical abstract



The incidence of post-TIPS HE in patients with improved microbiota was about one-fifth of that in patients with deteriorated microbiota.



The alterations of relationship among the microbiota in the different prognostic groups were opposite.

Highlights

- Gut dysbiosis of the patients without HE showed significant improvement after TIPS.
- Expansion of autochthonous taxa after TIPS was negatively correlated with the occurrence and severity of HE.
- The changes of relationship among the microbiota in different prognostic groups were opposite.
- Pre-TIPS gut microbiota and certain clinical indices were associated with survival.

Lay summary

Alterations in the gut microbiota after transjugular intrahepatic portosystemic shunt (TIPS) and the relationship between such alterations and post-TIPS hepatic encephalopathy (HE) remain unclear. We therefore performed this study and found that after TIPS, restoration of the gut microbiota, mainly characterised by expansion of autochthonous taxa, depletion of harmful taxa, and weakening of synergism among harmful bacteria, was inversely related to the occurrence and severity of post-TIPS HE.

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Research article

JHEP|Reports

Restoration of the gut microbiota is associated with a decreased risk of hepatic encephalopathy after TIPS☆

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Background & Aims: Hepatic encephalopathy (HE) is a major complication after transjugular intrahepatic portosystemic shunt (TIPS) and is primarily influenced by the gut microbiota. We aimed to evaluate alterations in the microbiota after TIPS and the association between such alterations and HE.
Methods: We conducted a prospective longitudinal study of 106 patients with cirrhosis receiving TIPS. Faecal samples were collected before and after TIPS, and the gut microbiota was analysed by 16S ribosomal RNA sequencing.
Results: Among all patients, 33 developed HE (HE+ group) within 6 months after TIPS and 73 did not (HE- group), and 18 died during follow-up. After TIPS, the autochthonous taxa increased, whereas the potential pathogenic taxa decreased in the HE- group, and the autochthonous taxon Lachnospiraceae decreased in the HE+ group. Furthermore, synergism among harmful bacteria was observed in all patients, which was weakened in the HE- group (p <0.001) but enhanced in the HE+ group (p <0.01) after TIPS. Variations of 5 autochthonous taxa, namely, *Coprococcus, Ruminococcus, Blautia, Ruminococcaceae_uncultured*, and *Roseburia*, were negatively correlated with the severity of HE. Notably, increased abundances of *Coprococcus* and *Ruminococcus* were protective factors against HE, and the incidences of HE in patients with improved, stable, and deteriorated microbiota after TIPS were 13.3, 25.9, and 68.2%, respectively. Higher total bilirubin level, Child–Pugh score, model for end-stage liver disease score, *Granulicatella*, and *Alistipes* and lower *Subdoligranulum* before TIPS were the independent risk factors for death.
Conclusions: Alterations in gut dysbiosis were negatively related to the occurrence and severity of post-TIPS HE, and the pre-TIPS microbiota were associated with death, suggesting the gut microbiota could be a promising potential biological target for screening suitable patients receiving TIPS and prevention and treatment of post-TIPS HE.
Lay summary: Alterations in the gut microbiota after transjugular intrahepatic portosystemic shunt (TIPS) and the relationship between such alterations and post-TIPS hepatic encephalopathy (HE) remain unclear. We therefore performed this study and found that after TIPS, restoration of the gut microbiota, mainly characterised by expansion of autochthonous taxa, depletion of harmful taxa, and weakening of synergism among harmful bacteria, was inversely related to the occurrence and severity of post-TIPS HE.
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Introduction

Transjugular intrahepatic portosystemic shunt (TIPS) is recommended by guidelines for complications of cirrhosis and portal hypertension.[1–3] TIPS may aggravate or induce hepatic encephalopathy (HE),[1,4–6] characterised by a series of neuropsychiatric abnormalities ranging from subclinical changes to coma and affecting cognitive performance, consciousness, and motor function.[4,7,8] HE is closely associated with gut microbiome dysbiosis in cirrhosis, specifically depletion of autochthonous taxa and expansion of potential pathogenic taxa.[9–17] The so-called gut–liver–brain axis[16,18] has been modulated in recent years using either engineering of the microbiota[19] or faecal microbiota transplantation (FMT) from a healthy donor, which thereby could treat or prevent HE.[20,21] In addition, FMT was able to prevent complications of liver cirrhosis,[20,21] as it is known that intestinal microbiome dysbiosis is aggravated with the progression of cirrhosis and the development of adverse events.[11,20,22] The profiles of the gut microbiota and some specific taxa (*e.g.*

Keywords: Cirrhosis; Portal hypertension; Gut microbiota; Hepatic encephalopathy; Transjugular intrahepatic portosystemic shunt.
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Pasteurellaceae) seem to be associated with mortality in patients with decompensated cirrhosis,[12,15] especially in patients with acute-on-chronic liver failure (ACLF).[23] However, the profiles may vary between studies, and additional longitudinal studies are needed.[24] Indeed, in patients with decompensated cirrhosis, portal hypertension and systemic inflammation are the relevant pathomechanisms.[25–27] TIPS is the most effective measure to treat portal hypertension in cirrhosis,[28,29] but the relationship of microbiota profiles and changes in portal pressure is not yet clear. The aims of this prospective study are to assess alterations in the microbiota addressing their relationship (i) with HE after TIPS and (ii) with overall outcome measures.

Materials and methods

Study design and sample collection

This prospective observational study was conducted at Xijing Hospital, a Chinese tertiary university-affiliated hospital. Patients were included if they met the following criteria: (1) confirmed liver cirrhosis (detected by clinical signs, laboratory tests, imaging features, or liver biopsies), (2) planned to receive TIPS for prophylaxis for portal hypertensive variceal rebleeding, and (3) aged 18–75 years. Exclusion was based on the following criteria: (1) active bleeding, (2) previous history of HE, (3) ineligible or unavailable stool samples, (4) concomitant hepatocellular carcinoma or other malignancies, (5) gastrointestinal surgery within 3 months before enrolment, (6) presence on the waiting list for liver transplantation, (7) uncontrolled infection or sepsis, (8) previous TIPS or surgical shunting, (9) stent dysfunction during postoperative review, (10) pregnancy or lactation, or (11) refusal. After admission, recent exposure to antibiotics and proton pump inhibitor (PPI) was recorded. In addition, antibiotics were used to prevent infection 24 h before and after surgery. The faecal samples were collected 1–3 days before and 1 month (range 31–40 days) after the operation and were preserved at -80°C within 3 h after sampling. Statistics and clinical variables were collected at the sampling time.

TIPS procedure, follow-up, and endpoints

The TIPS procedure was performed as described in a previous study, and an 8-mm stent was applied in all patients.[30] Scheduled follow-ups were performed at 1, 3, and 6 months and then every 6 months thereafter or whenever the patient had a serious adverse event requiring hospital admission, including for clinical and laboratory information collection, Doppler ultrasound, and CT evaluations. Patients were followed up until death or when the last enrolled patient had been followed up for 1 year. The primary endpoint was the occurrence of HE within 6 months after TIPS, defined according to the West Haven criteria.[7,8] Patients were grouped into the HE+ group and HE- group according to whether HE occurred. The secondary endpoints were orthotopic liver transplantation (OLT)-free survival and 1-year mortality.

DNA extraction and 16S rRNA gene sequencing

All samples were subjected to the same procedures for DNA extraction and PCR amplification by the same laboratory staff. The samples were suspended in 790 μl of sterile lysis buffer (4 M guanidine thiocyanate; 10% N-lauroyl sarcosine; 5% N-lauroyl sarcosine–0.1 M phosphate buffer [pH 8.0]) in a 2 ml screw-cap tube containing 1 g glass beads (0.1 mm BioSpec Products, Inc., Bartlesville, OK, USA). This mixture was vortexed vigorously and then incubated at 70°C for 1 h. After incubation by bead beating for 10 min at maximum speed, DNA was extracted with an E.Z.N.A.® Stool DNA Kit (Omega Biotek, Inc., Norcross, GA, USA) and stored at -20°C for further analysis. Then the extracted DNA was used as the template to amplify the V3∼V4 region of the 16S ribosomal RNA (rRNA) genes.

The primers F1 and R2 (5′-CCTACGGGNGGCWGCAG-3′ and 5′-GACTACHVGGGTATCTAATCC-3′) corresponding to positions 341–805 in the *Escherichia coli* 16S rRNA gene were used to amplify the V3∼V4 region of each sample by PCR, which was run in an EasyCycler 96 PCR system (Analytik Jena Corp., AG, Jena, Gemany) using the following programme: 3 min of denaturation at 95°C followed by 21 cycles of 0.5 min at 94°C (denaturation), 0.5 min for annealing at 58°C, and 0.5 min at 72°C (elongation), with a final extension at 72°C for 5 min. The products from different samples were indexed and mixed at equal ratios for sequencing by Shanghai Mobio Biomedical Technology Co., Ltd. (Shanghai, China), using the Miseq platform (Illumina Inc., San Diego, CA, USA) according to the manufacturer's instructions.

Bioinformatic analysis

The 16S rRNA sequencing data were processed by the Quantitative Insights Into Microbial Ecology platform (http://qiime.org/scripts/assign_taxonomy.html). Sequencing reads were demultiplexed and filtered. Operational taxonomic units (OTUs) were classified based on 97% similarity after chimeric sequences were removed using UPARSE (version 7.1; http://drive5.com/uparse/). The phylogenetic affiliation of each 16S rRNA gene sequence was analysed by the Ribosomal Database Project (RDP) Classifier (http://rdp.cme.msu.edu/) against the Silva (SSU123) 16S rRNA database using confidence threshold of 70%. Rarefaction was performed on the OTU table to minimise differences in sequencing depth among samples. Alpha diversity was estimated using the Chao1 and Shannon diversity indices. Beta diversity was measured using the Bray–Curtis distance matrix and was visualised with principal coordinate analysis (PCoA). The taxa with median relative abundance >0.01% in either group were included in the comparison analysis.

Based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) functional pathways, the metagenomes of the gut microbiome were inferred from the 16S rRNA sequences using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) with an estimated accuracy of 0.8. Only the pathways with median relative abundance >0.1% in either group were included in the comparison analysis.

Statistical analysis

All statistical analyses and graph preparation were performed using the SPSS V.19.0 (SPSS, Inc. Chicago, IL, USA) and R V.3.6.0 (R Foundation for Statistical Computing, Vienna, Austria; http://www.R-project.org) software packages. Quantitative variables are presented as the median (IQR) and were compared using the Wilcoxon rank sum test, Wilcoxon matched-pair rank test, or Kruskal–Wallis test as appropriate. Qualitative variables were expressed as numbers and were compared using the X^2 test. Linear discriminant analysis effect size (LEfSe) was used to identify differentially abundant taxa or pathways. Redundancy analysis (RDA) was used to evaluate the influence of confounding factors on the composition of the microbiome, and a Mantel test was performed to determine the significance of this influence. Statistical significance of sample grouping for beta diversity was performed using the analysis of similarities (ANOSIM) method (999 permutations). Spearman's rank test was performed for

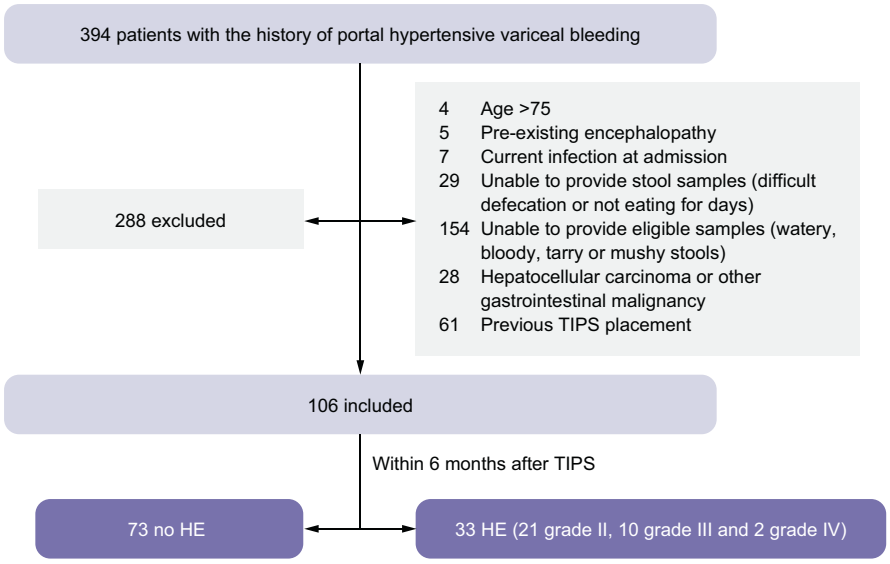

Fig. 1. Participant flow in the study. HE, hepatic encephalopathy; TIPS, transjugular intrahepatic portosystemic shunt.

correlation analysis. Propensity score matching (PSM) was performed with a caliper of 0.1 to match patients in the HE+ group and HE- group. Logistic regression tests and multiple linear regression analysis were used to adjust for potential confounding factors such as age, sex, ascites, BMI, aetiology, or antibiotic use. Multivariable stepwise logistic regression analysis was used to identify independent key factors for postoperative HE. OLT-free survival was evaluated with Kaplan–Meier curves and log-rank tests. A Cox regression model was used to identify independent predictors, and death during follow-up was encoded as a time-dependent variable when included in the analyses. Variables with p <0.10 in univariate analyses were used for the subsequent multivariate analysis. The p value was adjusted using the Benjamini and Hochberg false discovery rate (FDR), and differences with p <0.05 were considered statistically significant.

Ethics

The study protocol was approved by the ethics committee of the hospital, under approval number KY20172061-1, and all patients signed informed consent documentation. The study was registered in the Chinese Clinical Trial Registry (ChiCTR, www.chictr.org.cn), under registration number ChiCTR2100053664, and the data were deposited in the China National Microbiology Data Center (NMDC, nmdc.cn), under accession number NMDC10017852.

Results

Characteristics of the study population

From May 2017 to July 2019, 394 consecutive patients treated with TIPS were initially considered for the study, among whom 288 patients were excluded (Fig. 1), and 106 patients were ultimately eligible (212 samples). The final follow-up was completed in July 2020. Among these patients, 33 patients developed HE (66 samples, HE+ group) within 6 months after TIPS, whereas the remaining 73 did not (146 samples, HE- group). All HE patients had been successfully treated by lactulose, rifaximin, or ammonia-lowering therapy. Their demographic and clinical characteristics are shown in Table 1. Seventy-five patients had cirrhosis caused by HBV or HCV infection (50 in the HE- group and 25 in the HE+ group), and 31 as a result of other causes, such as alcohol use, autoimmunity, or biliary cirrhosis (23 in the HE- group and 8 in the HE+ group). The numbers of patients in Child–Pugh A, B, and C liver function were 27, 39, and 7 in the HE- group and 14, 16, and 3 in the HE+ group, respectively. In addition, all patients had a history of PPI use, and 56 had exposure to antibiotics within 1 month before pre-TIPS sampling (39 in the HE- group and 17 in the HE+ group). The preoperative data were comparable between the HE- and HE+ groups (all p >0.05, Wilcoxon rank sum test). Portal hypertension was resolved after TIPS in all patients, whereas liver function deteriorated with worsening function tests except for gamma-glutamyl transpeptidase and albumin levels (all p <0.05, Wilcoxon matched-pair rank test). In comparison with those in the HE- group, the albumin level was significantly lower and the total bilirubin level was higher in the HE+ group, resulting in a higher model for end-stage liver disease (MELD) score.

Diversity of the gut microbiota after TIPS placement

After TIPS, the alpha diversity of the microbiota in the entire cohort was not significantly changed (Fig. 2A and B). However, when the cohort was stratified into 2 groups according to the occurrence of HE after TIPS, in the HE+ group, the Chao1 and Shannon indices showed a decreasing tendency (p = 0.09 and 0.08, respectively, Wilcoxon matched-pair rank test). In contrast, the Chao1 index in the HE- group showed an increasing trend (p = 0.06), whereas the Shannon index was significantly elevated (p = 0.006). Additionally, the beta diversity for patients in the entire cohort changed significantly (ANOSIM, p = 0.008, Fig. 2C). In subgroup analyses, beta diversity was also obviously changed in the HE- group (ANOSIM, p = 0.001, Fig. 2D) but not in the HE+ group (ANOSIM, p = 0.64, Fig. 2E).

Specific alterations of the gut microbiota after TIPS

The microbiota of all patients was dominantly composed of 5 phyla: Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Verrucomicrobia (Fig. S1). There was no significant change at the phylum level in all patients or in either subgroup after surgery, whereas 9 differential taxa at the family level and 18 at the genus

Table 1. Study population's characteristics.

Variables	All patients (n = 106)		HE- group (n = 73)		HE+ group (n = 33)	
	Pre-TIPS	Post-TIPS	Pre-TIPS	Post-TIPS	Pre-TIPS	Post-TIPS
Age (years), median (IQR)	51 (45–58)		52 (45–58)		51 (44–58)	
Sex, male, n (%)	65 (61.3)		46 (63.0)		19 (57.6)	
BMI (kg/m^2), median (IQR)	20.8 (19.05–22.83)		20.5 (18.8–22.7)		21.4 (20.2–23.1)	
Antibiotic use, n (%)	56 (52.8)		39 (53.4)		17 (51.5)	
Aetiology, (HBV + HCV), n (%)	75 (70.8)		50 (68.5)		25 (75.8)	
PPG (mmHg), median (IQR)	23 (20–26.33)	9 (6–10.48)*	23 (20–26.35)	8.7 (5.9–10)†	23 (20–26.5)	9.2 (6.15–11.2)‡
MELD score, median (IQR)	11.59 (9.34–14.08)	14.7 (12.49–17.72)*	10.9 (8.81–13.78)	14.34 (12.15–16.47)†	12.33 (9.83–14.78)	16.84 (13.65–19.27)‡,§
Child–Pugh score, median (IQR)	7 (6–8)	7 (6–8)	7 (6–8)	7 (6–8)	7 (6–8)	8 (7–11)‡,§
Child–Pugh class‡,§, n (%)						
A (5–6)	41 (38.7)	37 (34.9)	27 (37.0)	34 (46.6)	14 (42.4)	3 (9.1)
B (7–9)	55 (51.8)	56 (52.8)	39 (53.4)	35 (47.9)	16 (48.5)	21 (63.6)
C (10–13)	10 (9.5)	13 (12.3)	7 (9.6)	4 (5.5)	3 (9.1)	9 (27.3)
Ascites¶, n (%)						
Mild	27 (25.4)	11 (10.3)	16 (21.9)	7 (9.6)	11 (33.3)	4 (12.1)
Moderate	48 (45.3)	4 (3.8)	35 (47.9)	3 (4.1)	13 (39.4)	1 (3.0)
Massive	3 (2.8)	1 (0.9)	2 (2.7)	1 (1.4)	1 (3.0)	0 (0)
HGB (g/dl), median (IQR)	85 (75–99.25)	97 (89.75–110)*	86 (75.5–97)	97 (90–109.5)†	84 (71.5–106)	98 (83–112)‡
Albumin (g/dl), median (IQR)	35.6 (32.3–38.7)	35.45 (32.88–38)	35.6 (32.15–38.7)	35.9 (33.1–38.15)	35.4 (32.4–39.75)	33.3 (29.85–36.95)‡,§
INR, median (IQR)	1.33 (1.18–1.5)	1.52 (1.32–1.72)*	1.3 (1.18–1.5)	1.48 (0.94–2.63)†	1.41 (1.2–1.53)	1.64 (1.4–1.86)‡,§
TB (μmol/L), median (IQR)	24.1 (15.25–32.8)	44.75 (28.4–68.53)	19.6 (14.7–31.7)	39.5 (26.3–63.4) †	26.3 (18.75–33.6)	45.8 (35.85–95.55)‡,§
ALT (U/L), median (IQR)	23.5 (16–33.25)	32.5 (26–46.25)*	24 (17.5–34)	33 (11–237)†	21 (15–31)	30 (27–40.5)‡
AST (U/L), median (IQR)	29 (23–41.25)	47.5 (36–57.25)*	30 (22.5–41)	46 (20–387)†	27 (23–45.5)	49 (38.5–60)‡
ALP (U/L), median (IQR)	82.5 (67–115.5)	109 (84–142.25)*	83 (67–116)	111 (84.5–142.5)†	82 (70–112)	95 (80–141.5)‡
GGT (U/L), median (IQR)	29.5 (19.75–56.25)	26 (16.75–39.5)*	32 (19–56.5)	25 (17–40)†	27 (20.5–58.5)	26 (15–39.5)‡
BUN (mg/dl), median (IQR)	4.74 (3.8–6.07)	4.01 (3.1–4.82)*	4.64 (3.77–5.83)	4.01 (3.09–4.74)†	5 (4.09–6.18)	4.01 (3.23–5.22)
Cr (mg/dl), median (IQR)	75.5 (64.75–96.25)	69 (55–80.25)*	76 (64–98.5)	69 (55–82)†	74 (65.5–96.5)	67 (55–78.5)‡
Venous ammonia** (μg/dl), median (IQR)	45 (31–57)	60 (40.5–88.25)*	48 (27.75–57.75)	60 (40.25–82.75)	36 (31.5–53)	56.5 (41–100.25)‡

Wilcoxon matched-pair rank test for longitudinal comparison and Wilcoxon rank sum test for cross-sectional comparison. ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; Cr, creatinine; GGT, gamma-glutamyl transpeptidase; HE, hepatic encephalopathy; HGB, haemoglobin; INR, international normalised ratio; MELD, model for end-stage liver disease; PPG, portosystemic pressure gradient; TB, total bilirubin; TIPS, transjugular intrahepatic portosystemic shunt.

* For comparison between pre-TIPS and post-TIPS in all patients (statistical difference between groups, p <0.05).
† For comparison between pre-TIPS and post-TIPS in the HE- group (statistical difference between groups, p <0.05).
‡ For comparison between pre-TIPS and post-TIPS in the HE+ group (statistical difference between groups, p <0.05).
§ For comparison between the HE- and HE+ groups in post TIPS (statistical difference between groups, p <0.05).
¶ Concurrent ascites resolved in most patients after TIPS (all groups, p <0.05).
** The data about venous ammonia were missing for some patients. The number of patients with data had 26 pairs (40 before TIPS and 47 after TIPS).

level in the HE- group were observed (linear discriminant analysis [LDA] score >2.0, p <0.05, Fig. 3). The relative abundances of the indigenous taxon Ruminococcaceae and its subordinate genera *Flavonifractor*, *Ruminococcus*, *Ruminococcaceae_uncultured*, and *Faecalibacterium* were significantly increased. Although another indigenous taxon, Lachnospiraceae, had no obvious change, *Anaerostipes, Blautia, Coprococcus, Roseburia*, and *Pseudobutyrivibrio*, which belong to Lachnospiraceae, increased significantly. Additionally, the potential pathogenic bacteria Carnobacteriaceae, Actinomycetaceae, and Streptococcaceae and their subordinates were significantly depleted. Unexpectedly, the HE-over-represented bacteria Veillonellaceae and the subordinates *Dialister*, *Megasphaera*, and *Veillonella* increased. The alterations in the gut microflora in all patients were similar to those in the HE- group (Fig. S2A).

However, in the HE+ group, 6 bacteria at the family level and 5 at the genus level were altered (Fig. S2B). The potential pathogenic bacteria Actinomycetaceae, Peptostreptococcaceae, *Gemella*, and Streptococcus decreased, whereas Carnobacteriaceae and *Granulicatella* increased. The beneficial taxon Lachnospiraceae and its subordinates, *Lachnospiraceae_incertae_sedis and Coprococcus*, also decreased.

The association between the gut microbiota and post-TIPS HE

Comparison of the microbiota between the HE- and HE+ groups revealed that pre-TIPS alpha diversity was comparable between the 2 subgroups, whereas post-TIPS Shannon diversity in the HE- group was higher than that in the HE+ group (p = 0.006, Wilcoxon rank sum test, Fig. 2B). The overall microbial composition did not differ between the 2 groups regardless of TIPS (Fig. S3A and B). After adjustment for the covariates, including age, sex, BMI, ascites, aetiology, and antibiotic use, there were no distinguishing taxa between the 2 groups before TIPS, and only Lachnospiraceae (LDA = 4.29, p = 0.028) and *Pseudobutyrivibrio* (LDA = 3.65, p = 0.049, belonging to Lachnospiraceae) were enriched in the HE- group after surgery.

The intestinal microbiota in the HE- group but not in the HE+ group was significantly changed after TIPS. We compared the fold change of taxa post-TIPS (relative abundance post-TIPS/pre-TIPS) between the 2 groups with correction for confounding factors to explore the difference of bacterial alterations. At the family level, alterations in the abundances of Lachnospiraceae and Ruminococcaceae in the HE- group were significantly higher (Fig. S4A). There were 8 bacteria at the genus level, and 7 of them remained significant after multiple testing correction (Fig. S4C). Four of

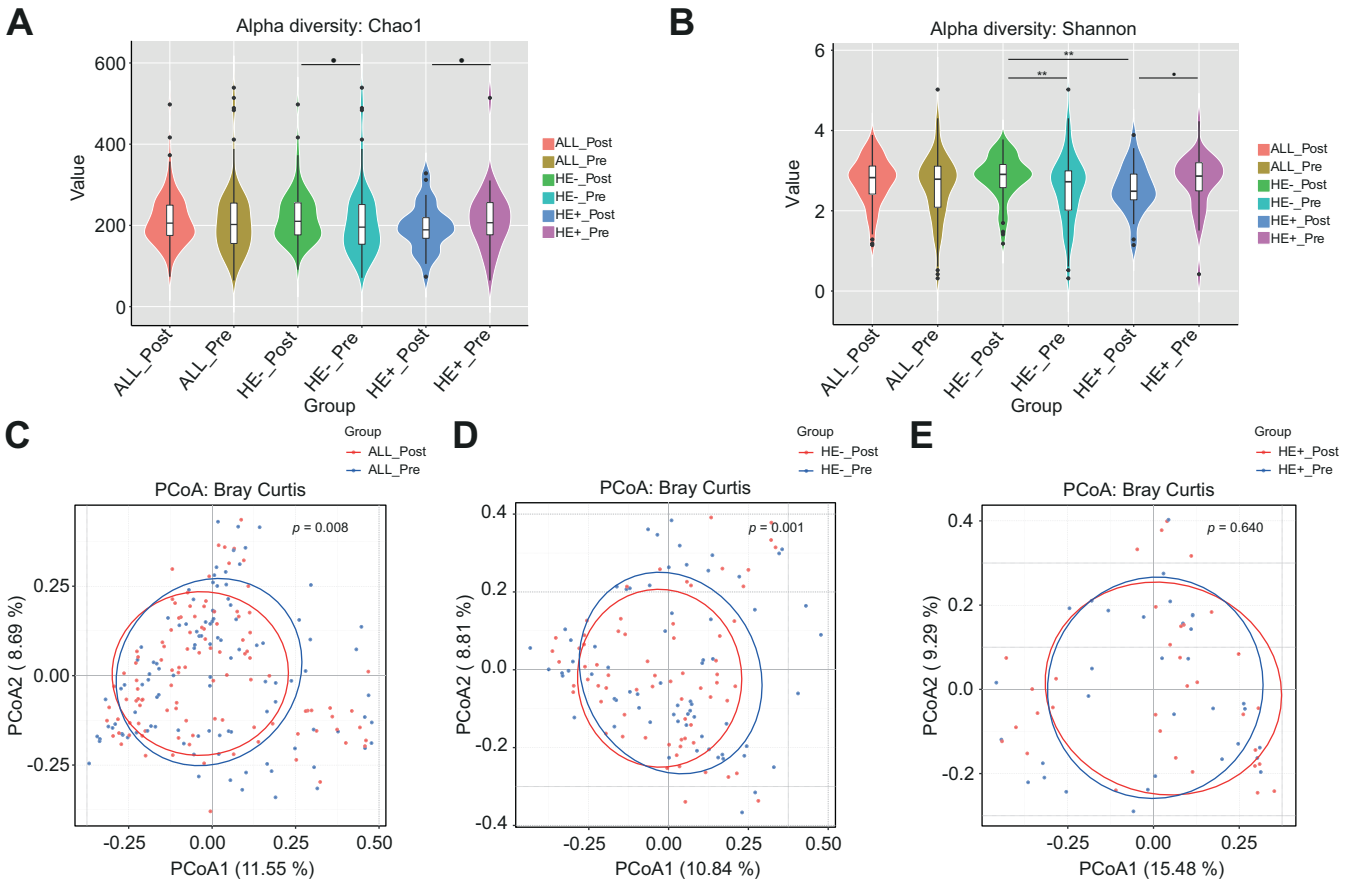

Fig. 2. Comparisons of alpha and beta diversity between pre- and post-TIPS in the overall patients and in the HE- and HE+ groups. Alpha diversity was illustrated by the (A) Chao1 and (B) Shannon indices (Wilcoxon matched-pair rank test for longitudinal comparison and Wilcoxon rank sum test for cross-sectional comparison). Beta diversity was assessed by PCoA of Bray–Curtis distance. Each sample was coloured according to the study group; the ANOSIM method was used for comparisons between pre-TIPS and post-TIPS in (C) the overall patients and the (D) HE- and (E) HE+ groups. $^{\cdot}p$ <0.10; $^{*}p$ <0.05; $^{**}p$ <0.01. ALL_Pre group, pre-TIPS samples in the overall patients; ALL_Post group, post-TIPS samples in the overall patients; HE-_Pre group, pre-TIPS samples in the HE- group; HE-_Post group, post-TIPS samples in the HE- group; HE+_Pre group, pre-TIPS samples in the HE+ group; HE+_Post group, pre-TIPS samples in the HE+ group. ANOSIM, analysis of similarity; HE, hepatic encephalopathy; PCoA, principal coordinate analysis; TIPS, transjugular intrahepatic portosystemic shunt.

them belonged to Lachnospiraceae (*Coprococcus*, *Blautia, Anaerostipes*, and *Roseburia*), 2 belonged to Ruminococcaceae (*Ruminococcaceae_uncultured* and *Ruminococcus*), and 1 belonged to Veillonellaceae (*Dialister*). After FDR correction and adjusting for confounding variables, variations in *Coprococcus, Blautia, Ruminococcus, Ruminococcaceae_uncultured*, and *Roseburia* abundances were obviously different for different degrees of post-TIPS HE (Fig. S5). Notably, multiple stepwise logistic regression analysis revealed that the increased abundance of *Coprococcus* (odds ratio [OR] 0.702, 95% CI 0.589–0.838, $p = 0.045$) and *Ruminococcus* (OR 0.730, 95% CI 0.635–0.839, $p = 0.024$) after TIPS were protective factors against post-TIPS HE (Table S1).

According to changes in *Coprococcus* and *Ruminococcus* abundances, the entire cohort was divided into 3 groups, Improve group (patients with both taxa increased), Deteriorate group (both taxa decreased), and Stable group (remaining patients). The incidences of post-TIPS HE in these groups were 13.3% (4/30), 68.2% (15/22), and 25.9% (14/54), respectively (Fig. 4). Strikingly, the incidence in the Improve group was only one-fifth of that in the Deteriorate group.

Because the clinical data in the HE+ and HE- groups were comparable before TIPS but showed significant differences after TIPS, PSM was performed at a ratio of 1:1 to match patients in the 2 groups according to age, sex, BMI, aetiology, postoperative ascites severity, and other differential indices including international normalised ratio (INR), albumin level, total bilirubin level, and MELD score. Twenty-seven pairs of patients were identified (Table S2). We found alterations in the gut microbiota in the 2 matched-groups after TIPS (Fig. S2C and D), and the differences in such alterations between them were consistent with those in the HE- and HE+ groups (Fig. S4A–D).

Alterations in the relationship among the HE-associated microbiota constituents after TIPS

We performed correlation test on a total of 26 taxa that were reported to be associated with HE in previous research (Table S3), including autochthonous bacteria, healthy-enriched bacteria (Lachnospiraceae, Ruminococcaceae, Rikenellaceae, and Christensenellaceae, beneficial taxa),[11,18,20,22,31] HE-enriched bacteria, and potential pathogenic bacteria (the remaining bacteria, harmful taxa).[9–17] Before TIPS, we detected significant positive correlations, suggesting a synergistic relationship among harmful bacteria in all patients or in either subgroup (red frame in Fig. 5). Notably, in all patients and in the HE- group, the

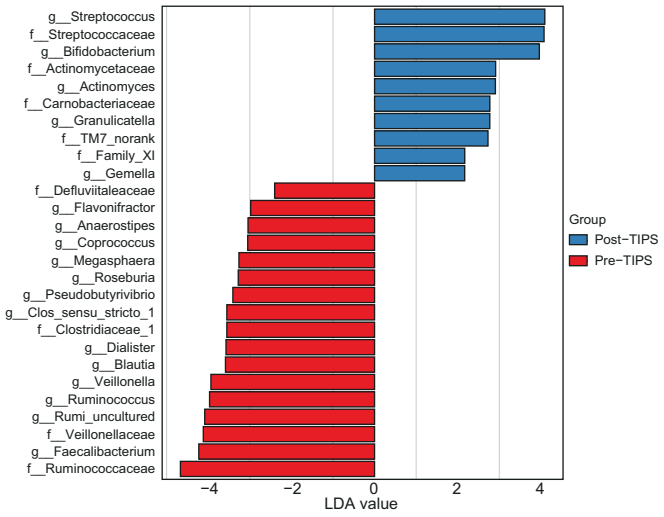

Fig. 3. Alterations in the gut microbiota in the HE- group after TIPS. LDA effect size revealed that the relative abundance of 9 taxa at the family level and 18 taxa at the genus level were altered significantly. Rumi_uncultured, Ruminococcaceae_uncultured; Clos_sensu_stricto_1, Clostridium_sensu_stricto_1. HE, hepatic encephalopathy; LDA, linear discriminant analysis; TIPS, transjugular intrahepatic portosystemic shunt.

synergism was markedly weakened after TIPS (both p <0.001, Wilcoxon matched-pair rank test) but was enhanced significantly in the HE+ group (p <0.01, Fig. 5A–F). Furthermore, a post-TIPS negative correlation showing antagonism between the beneficial and harmful bacteria was obvious in the HE+ group (blue frame in Fig. 5) but was not apparent in the HE- group, overall patients, or HE+ group pre-TIPS (Fig. 5G–I).

Microbial function alteration after TIPS

PICRUSt was used to predict the functional composition from the sequencing data, revealing that 3 pathways (Fig. S6), namely, Pantothenate and CoA biosynthesis (LDA = 2.03, p = 0.049); Arginine and proline metabolism (LDA = 2.25, p = 0.035); and Replication, recombination, and repair proteins (LDA = 2.31, p = 0.029), which have been previously reported as healthy-enriched pathways,[15,32,33] were enriched after TIPS.

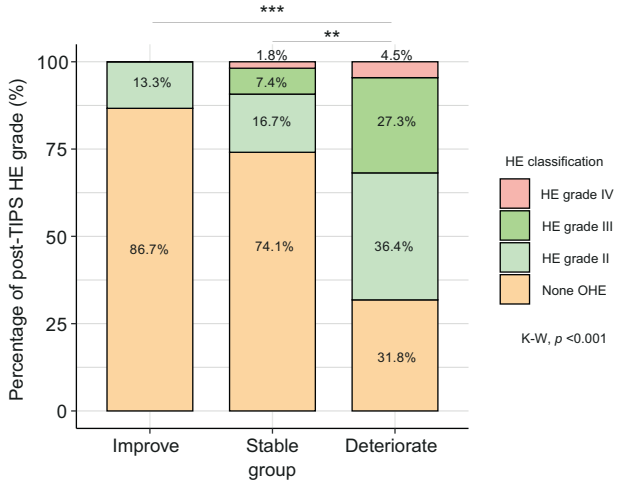

Fig. 4. Percentage bar chart showing post-TIPS HE grade. The post-TIPS HE grades were compared with the K-W test and were significantly different among the 3 groups, and the Wilcoxon rank sum test was used for comparisons between any 2 of the 3 groups. **p <0.01; ***p<0.001; K-W, Kruskal–Wallis test. HE, hepatic encephalopathy; TIPS, transjugular intrahepatic portosystemic shunt.

Gut microbiota constituents associated with mortality

During follow-up, none of the patients received orthotopic liver transplantation and 18 patients (17.0%) died, and the reasons are listed in Table 2. There were 9 deaths each in the HE- (9/73, 12.3%) and HE+ (9/33, 27.3%) groups. OLT-free survival in the HE- group was higher than that in the HE+ group (log-rank p = 0.046, Fig. 6). The total 1-year mortality was 10.4% (11/106). Higher total bilirubin level, Child–Pugh score, MELD score, *Granulicatella,* and *Alistipes* and lower *Subdoligranulum* before TIPS were identified as the independent risk factors for death using the Cox logistic regression (Table S4A–C).

Effects of antibiotics on the gut microbiota

RDA was used to evaluate the influence of confounding factors on the composition of microbiota, and we found that only antibiotic use had a weak influence on the composition of microbiota (Mantel test, p = 0.044, Fig. S7). Patients who had received antibiotics (n = 56, ANTI+ group) within 1 month before sampling and those who had not (n = 50, ANTI- group) were grouped. The alpha diversity was comparable between the 2 groups (Table S5). Despite a significant difference in pre-TIPS beta diversity (ANOSIM, p = 0.007, Fig. S3C), the significance did not remain consistent after TIPS (ANOSIM, p = 0.471, Fig. S3D). After adjustment for age, sex, BMI, ascites, and aetiology, Erysipelotrichaceae, Clostridiaceae_1, *Clostridium_sensu_stricto_1*, and *Klebsiella* were different between the 2 groups before TIPS, whereas only Ruminococcaceae was differential after TIPS. Moreover, we compared the alteration in the abundances of taxa (relative abundance post-TIPS/pre-TIPS) between the 2 groups with correction for confounding factors and found no difference. Remarkably, the altered taxa after TIPS in the patients not receiving antibiotics and the overall patients were found to be almost identical (Table S6).

Discussion

We conducted a prospective longitudinal study with a large sample size using 16S rRNA sequencing and found that (i) gut dysbiosis improved significantly in the HE- group after TIPS but remained poor in the HE+ group, (ii) the increased abundance of autochthonous taxa were negatively correlated with the occurrence and severity of post-TIPS HE, (iii) synergism existed among harmful bacteria that was weakened after TIPS in the HE- group but was enhanced in the HE+ group, and (iv) pre-TIPS microbiota information had a close association with death.

The strengths and novelties of the current study lie in the following: (i) conducting a real-world study based on a large sample size and delineating the alteration in the microbiota after TIPS and its relationship with post-TIPS HE and death for the first time; (ii) investigating the correlation among microbiota constituents and changes in the correlation after TIPS, including the beneficial and harmful bacteria related to HE reported in previous research rather than the differentially microbiota constituents found in our study to ensure objectiveness and accuracy of the conclusion; (iii) evaluating the effect of antibiotics on post-TIPS alterations in the gut microbiota; and (iv) identifying the

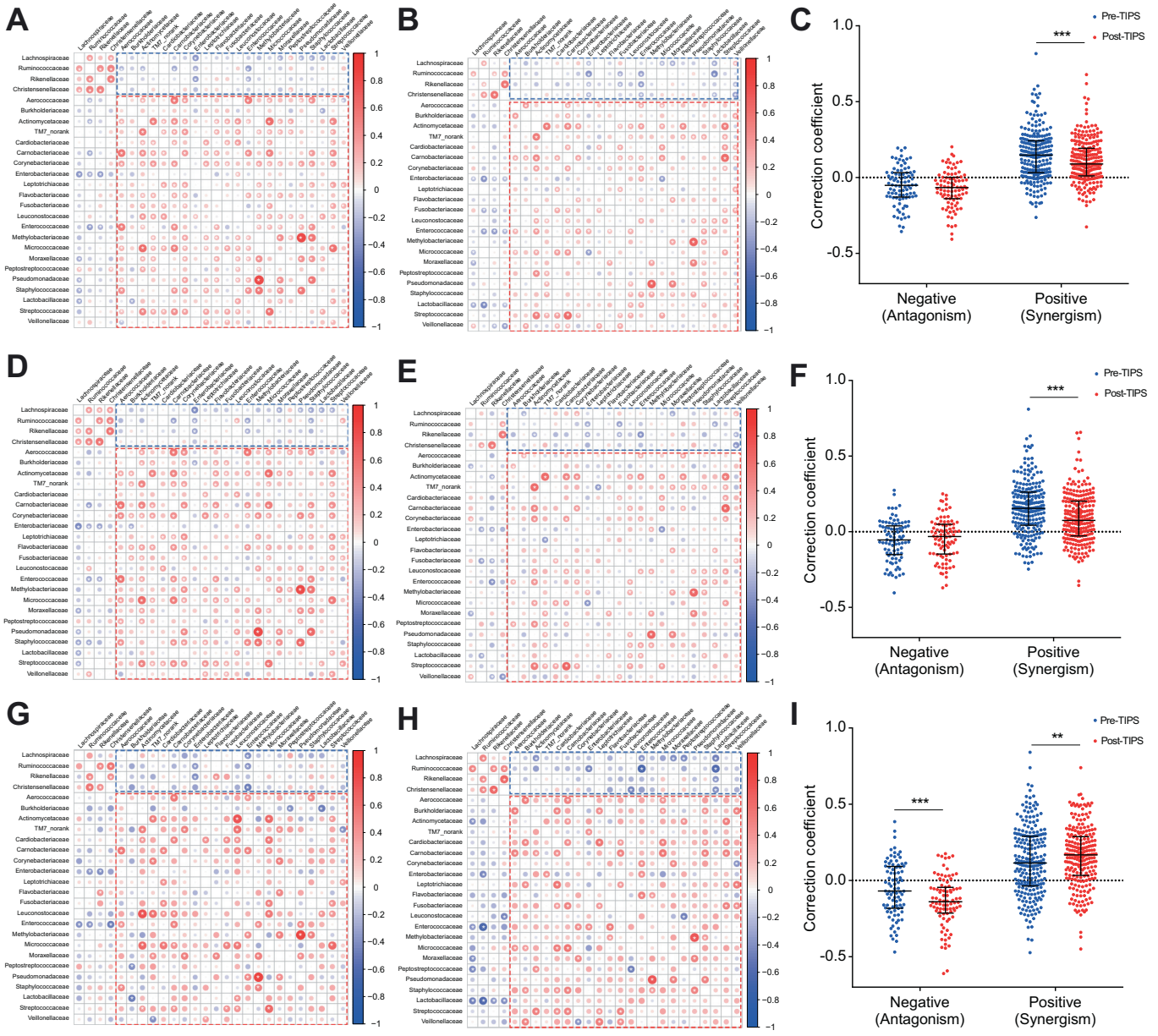

Fig. 5. Correlations among taxa in the entire cohort and the HE- and HE+ groups. Correlation analysis: overall patients, (A) pre-TIPS and (B) post-TIPS; HE- group, (D) pre-TIPS and (E) post-TIPS; and HE+ group, (G) pre-TIPS and (H) post-TIPS. The Spearman correlation test was used, and the results were visualised in the form of a correlation matrix. In the correlation matrix, the first 4 taxa were autochthonous, and the others were harmful taxa; '*' with white colour in the dot's centre indicates that the correlation was statistically significant; red frame: the synergism among the harmful bacteria; blue frame: the antagonism or no relationship between the autochthonous and harmful taxa. The comparisons between pre-TIPS and post-TIPS correlation coefficients were performed using the Wilcoxon matched-pair rank test in (C) the overall patients and the (F) HE- and (I) HE+ groups. $^{**}p$ <0.01; $^{***}p$ <0.001. HE, hepatic encephalopathy; TIPS, transjugular intrahepatic portosystemic shunt.

microbiota constituents possibly associated with post-TIPS HE and death.

Our study revealed that post TIPS, the overall bacterial composition in the HE- group was markedly altered, and the indigenous taxa Ruminococcaceae, *Ruminococcaceae_uncultured*, *Faecalibacterium*, *Flavonifractor*, and *Ruminococcus* and the subordinate bacteria of Lachnospiraceae, *Anaerostipes, Blautia, Coprococcus, Roseburia*, and *Pseudobutyrivibrio*, were significantly enriched. Studies had confirmed that indigenous bacteria participated in the production of short-chain fatty acids (SCFAs), which regulated the colonic pH and reduced colonic inflammation.[34–36] In contrast, the autochthonous flora competed with pathogenic bacteria and produced antimicrobial peptides, potentially strengthening the integrity of the gut barrier,[11,37,38] which had been shown in studies on Crohn disease and cirrhosis.[39,40] In addition, the potential pathogenic bacteria Carnobacteriaceae, Actinomycetaceae, and Streptococcaceae, whose enrichment was associated with higher blood ammonia levels, inflammation, increased MELD scores, and poorer cognitive performance, were significantly reduced.[11,17,18] Unexpectedly, the

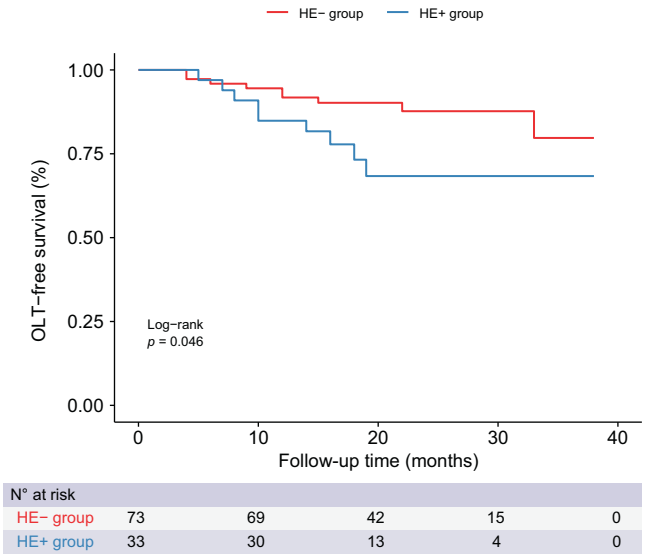

Fig. 6. Kaplan–Meier curves of post-TIPS survival between the HE- and HE+ groups. Log-rank test was employed. HE, hepatic encephalopathy; OLT, orthotopic liver transplantation; TIPS, transjugular intrahepatic portosystemic shunt.

HE-enriched flora constituent Veillonellaceae that was reported to be positively correlated with inflammation was found expanded.[14] In acute HE, *Veillonella* was over-represented,[15] and *Veillonella* spp. were implicated in several severe inflammatory diseases, such as autoimmune hepatitis, recurrent Crohn's disease, and endocarditis.[32,41,42] We speculated that TIPS intervention, differing from some antibiotics that could specifically select bacteria, was more likely to change the intestinal microenvironment by decompression of portal hypertension, consequently affecting the gut microbiota. Some studies also reported the taxa could be anti-inflammatory; for example, coculture of *Streptococcus* and *Veillonella* could result in a reduction in inflammatory cytokine production.[43,44]

Although there was also a decrease in the abundance of harmful bacteria in the HE+ group, the depletion of Lachnospiraceae, *Coprococcus*, and *Lachnospiraceae_incertae_sedis* was more noticeable. Strikingly, we compared the differences in microbial alterations between the HE- and HE+ groups and found that the beneficial taxa, *Blautia*, *Roseburia*, *Coprococcus*, *Anaerostipes*, *Ruminococcus*, and *Ruminococcaceae_uncultured*, had a greater expansion in the HE- group. Variations in some of them were negatively correlated with the severity of postoperative HE. One study on the intestinal mucosal microbiota in patients with HE demonstrated that *Blautia*, *Roseburia*, *Fecalibacterium*, and *Dorea* were associated with better cognition and lower levels of inflammatory markers.[13] Multiple stepwise logistic regression analysis revealed that the increased abundance of *Coprococcus* (pertaining to Lachnospiraceae) and *Ruminococcus* (pertaining to Ruminococcaceae) were protective factors against postoperative HE. Patients with both taxa increased had a lower incidence of post-TIPS HE than other patients. In fact, faecal transplantation using material from a healthy donor enriched in Lachnospiraceae and Ruminococcaceae could effectively reduce the recurrence of HE and the occurrence of severe adverse events,[20,21] underscoring that the alteration of autochthonous taxa had a strong impact on post-TIPS HE. In the HE+ group, the microbiota showed no significant change after TIPS, suggesting that the lack of improvement of microbiota signature after TIPS was associated with HE, whereas improvement of gut microbiota after TIPS lowered the risk of HE.

Table 2. Causes of death.

Cause	HE- group	HE+ group	Total
Rebleeding	5 (27.7%)	1 (5.6%)	6 (33.3%)
Ascites	0 (0%)	1 (5.6%)	1 (5.6%)
Liver failure	2 (11.1%)	2 (11.1%)	4 (22.2%)
Renal failure	1 (5.6%)	2 (11.1%)	3 (16.7%)
Brain failure	1 (5.6%)	1 (5.6%)	2 (11.1%)
Unknown	0 (0%)	2 (11.1%)	2 (11.1%)
Total	9 (50%)	9 (50%)	18 (100%)

HE, hepatic encephalopathy.

In addition, differences in liver function after TIPS might have a potential influence on the occurrence of HE, so PSM was performed, and we found that alterations in the gut microbiota in matched 2 groups and the differences in such alterations between them were consistent with those in the original groups; thus, such a possible influence was excluded.

While investigating the correlation among microbiota constituents and the alteration of that correlation after TIPS, we detected a synergic effect among the harmful bacteria, which was similar to the findings of a study about primary biliary cholangitis.[33] Notably, the synergism was attenuated significantly in the HE- group but strengthened in the HE+ group after surgery. Postoperative antagonism between beneficial and harmful bacteria was observed in the HE+ group, which was not apparent in these patients before TIPS and in other patients, indicating that the bacterial status of the HE+ group remained poor, whereas that of the HE- group improved significantly.

The altered microbial function pathways, namely, Pantothenate and CoA biosynthesis; Arginine and proline metabolism; and Replication, recombination, and repair proteins, were elevated in the HE- group, and they had been reported to be enriched in healthy controls in recent studies about different liver diseases,[15,32,33] suggesting that alterations of bacterial functions were consistent under different background liver diseases. We did not perform this analysis in the entire cohort or in the HE+ group, because some of these patients had received anti-HE treatment such as rifaximin, lactulose, or ammonia-lowering treatment before the second sampling, which might have minimal impact on the microbial composition but could significantly alter microbial function.[14,45–47]

Microbiota dysbiosis was linked with death, but different studies had found diverse microbiota constituents that might be associated with different disease states in enrolled patients. Our study revealed that pre-TIPS microbiota information regarding *Granulicatella, Alistipes*, and *Subdoligranulum* and clinical indicators total bilirubin level, Child–Pugh score, and MELD score were the key factors for postoperative death. *Alistipes* was more abundant in patients with cirrhosis than in healthy people and was related to HE.[13] *Granulicatella* was a normal part of the oral flora but an opportunistic pathogen in the intestinal flora and was involved in many invasive infections in humans. *Subdoligranulum* was a subordinate genus of the autochthonous taxon Ruminococcaceae, and its relative abundance showed a progressive decrease in healthy people and patients without and with HE.[11,13]

Because many patients in this study were exposed to antibiotics before pre-TIPS sampling, which was the condition within

the real-world setting, we analysed the effect of antibiotics on our conclusion rather than excluding these patients. The results showed that antibiotic use had an influence on the composition of the microbiota, but the influence was weak. Actually, we found only 3 differentially abundant taxa between patients with and without exposure to antibiotics, and antibiotic use had no influence on the development of post-TIPS HE. This was presumably because patients with decompensated cirrhosis possessed a very poor microbial status that might mask the effect of antibiotics. Moreover, we found that the alteration of taxa had no significant difference between the 2 groups and that the altered microbiota caused by TIPS in the ideal patients and the real-world patients kept generally consistent, indicating that compared with that of TIPS intervention, the influence of antibiotics on the microbiota was negligible.

There were several other limitations. First, the use of 16S rRNA sequencing limited further analysis of microbial composition and function; thus, metagenomics sequencing was warranted in future studies. Second, we collected stool rather than mucosal samples, but the faecal microbiota could not fully represent mucosal microbiota[13]; however, the collection of faecal samples was more pragmatic for clinical application. Third, the exclusion criteria, for example, ineligible samples, might be a potential screening bias for enrolment confounding the results. Nevertheless, it was unavoidable in the real-world setting. Moreover, it was noteworthy that this study established association rather than causation. Finally, as a single-centre study, our study needed to be validated by multicentre researches with larger sample sizes.

We conclude that restoration of the gut microbiota after TIPS, reflected in expansion of autochthonous taxa, depletion of harmful taxa, and weakened synergism among harmful bacteria, is inversely correlated with the occurrence and severity of post-TIPS HE and that the increase of autochthonous microbiota constituents may have a pivotal effect. Moreover, the pre-TIPS microbiota and clinical information have a close association with death. This study reveals the gut microbiota as a potential source of biomarkers for the prevention and treatment of post-TIPS adverse events.

Abbreviations

ACLF, acute-on-chronic liver failure; ALP, alkaline phosphatase; ALT, alanine aminotransferase; ANOSIM, analysis of similarities; AST, aspartate aminotransferase; BUN, blood urea nitrogen; ChiCTR, Chinese Clinical Trial Registry; Cr, creatinine; FDR, false discovery rate; FMT, faecal microbiota transplantation; GGT, gamma-glutamyl transpeptidase; HE, hepatic encephalopathy; HGB, haemoglobin; KEGG, Kyoto Encyclopedia of Genes and Genomes; LDA, linear discriminant analysis; LEfSe, linear discriminant analysis effect size; MELD, model for end-stage liver disease; NMDC, China National Microbiology Data Center; OLT, orthotopic liver transplantation; OR, odds ratio; OTU, operational taxonomic unit; PCoA, principal coordinate analysis; PICRUSt, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; PPG, portosystemic pressure gradient; PPI, proton pump inhibitor; PSM, propensity score matching; RDA, redundancy analysis; RDP, Ribosomal Database Project; rRNA, ribosomal RNA; SCFA, short-chain fatty acid; TB, total bilirubin; TIPS, transjugular intrahepatic portosystemic shunt.

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Conflicts of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Study concept and design: ML, KL, ST, YL, QW, JT, GH. Acquisition of data: ML, ST, KL, YL, QW, ZW, YZ, WG, JN, WB, EW, DX, ZW, BL, XL, JY. TIPS surgery: WG, WB, ZY, GH. Funds collection: JT, GH. Statistical analysis: ML. Analysis and interpretation of data: ML, KL, ST, YL, QW, GH. Drafting of the manuscript: ML. Critical revision of the manuscript for important intellectual content: KL, JT, GH.

Data availability statement

Data supporting the findings of this study are available from the corresponding author upon reasonable request.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhepr.2022.100448.

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Author names in bold designate shared co-first authorship.

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