Journal for ImmunoTherapy of Cancer

Characterizations of multi-kingdom gut microbiota in immune checkpoint inhibitor-treated hepatocellular carcinoma

Chengpei Zhu,[1,2] Chenchen Zhang,[3] Shanshan Wang,[1] Ziyu Xun,[1] Dongya Zhang,[3] Zhou Lan,[3] Longhao Zhang,[1] Jiashuo Chao,[1] Yajun Liang,[3] Zilun Pu,[3] Cong Ning,[1] Xinting Sang,[1] Xiaobo Yang,[1] Hanping Wang,[1,4] Xianzhi Jiang,[3] Haitao Zhao [1]

To cite: Zhu C, Zhang C, Wang S, *et al*. Characterizations of multi-kingdom gut microbiota in immune checkpoint inhibitor-treated hepatocellular carcinoma. *Journal for ImmunoTherapy of Cancer* 2024;**12**:e008686. doi:10.1136/jitc-2023-008686

► Additional supplemental material is published online only. To view, please visit the journal online (https://doi.org/10.1136/jitc-2023-008686).

CZhu, CZha, SW and ZX contributed equally.

CZhu, CZha, SW and ZX are joint first authors.

Accepted 17 May 2024

Check for updates

© Author(s) (or their employer(s)) 2024. Re-use permitted under CC BY-NC. No commercial re-use. See rights and permissions. Published by BMJ.

For numbered affiliations see end of article.

Correspondence to
Dr Haitao Zhao;
zhaoht@pumch.cn

Dr Xianzhi Jiang;
jxz@moonbio.com

Dr Hanping Wang;
Wanghp@pumch.cn

Dr Xiaobo Yang;
yangxiaobo67@pumch.cn

ABSTRACT

Background The association between gut bacteria and the response to immune checkpoint inhibitors (ICI) in hepatocellular carcinoma (HCC) has been studied; however, multi-kingdom gut microbiome alterations and interactions in ICI-treated HCC cohorts are not fully understood.

Methods From November 2018 to April 2022, patients receiving ICI treatment for advanced HCC were prospectively enrolled. Herein, we investigated the multi-kingdom microbiota characterization of the gut microbiome, mycobiome, and metabolome using metagenomic, ITS2, and metabolomic data sets of 80 patients with ICI-treated HCC.

Results Our findings demonstrated that bacteria and metabolites differed significantly between the durable clinical benefit (DCB) and non-durable clinical benefit (NDB) groups, whereas the differences were smaller for fungi. The overall diversity of bacteria and fungi before treatment was higher in the DCB group than in the NDB group, and the difference in diversity began to change with the use of immunotherapy after 6–8 weeks. We also explored the alterations of gut microbes in the DCB and NDB groups, established 18 bacterial species models as predictive biomarkers for predicting whether immunotherapy is of sustained benefit (area under the curve=75.63%), and screened two species of bacteria (*Actinomyces_sp_ICM47*, and *Senegalimassilia_anaerobia*) and one metabolite (galanthaminone) as prognostic biomarkers for predicting survival in patients with HCC treated with ICI.

Conclusions In this study, the status and characterization of the multi-kingdom microbiota, including gut bacteria, fungi, and their metabolites, were described by multiomics sequencing for the first time in patients with HCC treated with ICI. Our findings demonstrate the potential of bacterial taxa as predictive biomarkers of ICI clinical efficacy, and bacteria and their metabolites as prognostic biomarkers.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Gut bacteria may be associated with the efficacy of immunotherapy for hepatocellular carcinoma (HCC), but data are limited and the role of multi-kingdom gut microbiota is poorly reported.

WHAT THIS STUDY ADDS

⇒ The status and characterization of the multi-kingdom microbiota, including gut bacteria, fungi, and their metabolites, were described for the first time in immune checkpoint inhibitor-treated HCC. The bacteria and metabolites differed significantly between the durable clinical benefit and non-durable clinical benefit groups, whereas the differences were smaller for fungi.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ These findings show that multi-kingdom microbiota, including gut bacteria, fungi, and their metabolites, play an important role in efficacy, and some can be used as good prognostic or predictive markers in patients with HCC treated with immune checkpoint inhibitor.

INTRODUCTION

Advanced hepatocellular carcinoma (HCC) is an intractable global health concern associated with a poor prognosis, and it is the sixth leading cause of cancer-related mortality worldwide.[1] Recent noteworthy advancements in immune checkpoint inhibitor (ICI) therapies targeting the programmed death 1 (PD-1) and programmed death ligand 1 (PD-L1) axes have demonstrated clinical efficacy in treating advanced HCC, both as first-line and second-line treatments.[2] However, the response rates are heterogeneous and non-robust and a proportion of patients with advanced HCC fail to respond to this therapy or experience side effects.[2] Biomarkers such as PD-L1 expression, high tumor mutation burden, high microsatellite instability, microbiota, and metabolic profiles are used to select patients who are likely to show a positive response to ICI treatment in HCC and other cancers, and studies suggest that

J Immunother Cancer: first published as 10.1136/jitc-2023-008686 on 6 June 2024. Downloaded from http://jitc.bmj.com/ on June 6, 2024 by guest. Protected by copyright.

gut microbiome and metabolite properties are of great value.[3–7]

Recent studies have revealed that the gut microbiota mediates diverse responses to immunotherapy and may serve as a potential predictive biomarker.[8] Both preclinical and clinical research studies have provided growing evidence that gut microbiota and metabolites can modulate the oncogenesis of cancer and shape antitumor immune responses, affecting the efficacy and toxicity of immunotherapies, particularly ICIs.[8 9] The human gut harbors more than 103 microbes, including the multikingdom microbes, prokaryotes, viruses, protists, and fungi, which play vital roles in intestinal ecology and human health.[3 10] However, current research in the field of immunotherapy primarily focuses on the bacterial components of gut ecology.[3] The intricate associations between other microorganisms, particularly fungi or the "mycobiome", and ICI, remain unexplored. Despite constituting less than 0.1% of the gut flora, the mycobiome plays an indispensable role in the human gut microbiota and is tightly integrated into the bacterial community. Extensive evidence has shown its involvement in diverse disease pathogenesis and its profound influence on the host immune system, including antitumor responses.[11–14] Gut fungal and bacterial communities are intrinsically interconnected and likely impact each other in various ways and are intertwined with host metabolic pathways.[10 14] Growing evidence supports a close relationship between gut microbiota and various metabolic pathways, ultimately modulating the efficacy of ICIs through the regulation of gut bacterial functions and metabolites across different tumor types. For instance, microbiota-derived short-chain fatty acids (SCFAs) and bile acids (BA) are key mediators of this process.[3 15]

Previous studies, including ours, have provided insights into the association between specific gut bacteria and metabolites, and the efficacy of anti-PD-1 therapy in HCC. However, the findings vary significantly.[4–7] Therefore, it remains imperative to further investigate the characteristics of the gut microbiota in advanced HCC and to elucidate the potential roles of the gut microbiota and metabolites in predicting responses to ICI treatment. In this study, we performed a comprehensive analysis of metagenomic, ITS2, and metabolomic data sets to assess the characteristics of multi-kingdom microbiota, including the gut microbiome, mycobiome, and metabolome in ICI-treated HCC (online supplemental figure S1 and table S1).

METHODS

Study cohort and fecal sample collection

We prospectively included 188 patients with advanced HCC from three registered clinical trials (NCT03892577, NCT04010071, and NCT03895970) conducted at Peking Union Medical College Hospital (PUMCH), from November 2018 to April 2022. The patients included in the study met the following criteria: pathological or clinical diagnosis of HCC; treatment with a PD-1/PD-L1 inhibitor, with or without targeted therapy; and other criteria, including adequate function of major organs, an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0–2, and radiologically-evaluable lesions. Patients who had used other experimental drugs within 4 weeks prior to the start of the study, received antibiotics before or during treatment, underwent invasive biliary tract procedures, had organ transplants, or had autoimmune diseases were excluded. A total of 108 patients were excluded for these reasons and 80 patients were enrolled in this study (online supplemental figures S1 and S2).

The PD-1/PD-L1 inhibitor was administered intravenously every 3 weeks at the indicated dose for each drug. The treatment regimen was terminated when disease progression or intolerable toxicity was observed. Clinical response was assessed every 6–8 weeks according to Response Evaluation Criteria in Solid Tumours (RECIST) V.1.1 criteria.[16] Patients with efficacy assessed as continuous partial response (PR), complete response (CR), or stable disease (SD) ≥6 months, were defined as having achieved durable clinical benefit (DCB), while disease progression (PD) or continuous SD <6 months was defined as a non-durable clinical benefit (NDB).[17] Immune-related adverse events (irAEs) were assessed and monitored for each patient from the start of ICI treatment to the final follow-up time. IrAEs were diagnosed according to the National Comprehensive Cancer Network guidelines on ICI-related toxicities.[18] All irAEs were co-confirmed by at least two or more oncologist experts and excluded the possibility of other adverse events caused by targeted therapy. Fecal samples were dynamically collected at PUMCH within 2 weeks before the first treatment and before every second cycle of PD-1/PD-L1 inhibitor administration. All fresh fecal samples were stored in sterile containers and immediately stored in a −80°C refrigerator.

Metagenomic sequencing and bioinformatic analysis

Genomic DNA was extracted using a Feces Genomic DNA Purification Kit (BIOER, Binjiang, China). DNA concentration and purity were measured simultaneously using a Qubit V.3.0 (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and NanoDrop One (Thermo Fisher Scientific), respectively. Sequencing libraries were generated using NEB Next Ultra DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, Massachusetts, USA) following the manufacturer's recommendations and index codes were added. The library was sequenced on an Illumina NovaSeq 6000 platform (Illumina, San Diego, California, USA) and 150 bp paired-end reads were generated. Raw data were processed using fastp (V.0.19.7) to acquire clean data for subsequent analysis.[19] Trimmed reads aligned to the Homo sapien genome assembly hg37[20] were removed using KneadData integrated Bowtie (https://huttenhower.sph.harvard.edu/kneaddata/) to obtain metagenomic DNA sequences.

J Immunother Cancer: first published as 10.1136/jitc-2023-008686 on 6 June 2024. Downloaded from http://jitc.bmj.com/ on June 6, 2024 by guest. Protected by copyright.

The MetaPhlAn tool (V.4.0.3) was used to quantitatively profile the taxonomic composition of the metagenome,[21] whereas HUMAnN (V.3.0.0. alpha.4) was used to estimate the microbial metabolic and functional pathways.[22] Functional pathway analysis included metabolic pathway (MetaCyc) and Kyoto Encyclopedia of Genes and Genomes (KEGG) functions. Alpha diversity was used to evaluate the complexity of the species diversity for each sample using the Shannon index. Beta diversity calculations were applied to analyze the diversity of samples for species complexity using principal coordinate analysis (PCoA). The p value for the analysis of similarities was obtained using a permutation test. Linear discriminant analysis effect size was determined using the non-parametric factorial Kruskal-Wallis rank sum test and linear discriminant analysis (LDA) to determine the differential taxa between groups. The parameters were set as follows: the default logarithmic LDA score was 2.0 and the default p value was 0.05. Fivefold cross-validation was performed using the least absolute shrinkage and selection operator (LASSO) regression model (R V.4.2.0, glmnet package) using the 25-species which differed significantly between the DCB group (n=25) and NDB group (n=24) with a p value<0.05 in the training cohort. 18 species with minimum errors were chosen as the optimal set. The probability of the test cohort, including the 17 DCB and 14 NDB groups, was calculated using this set of species, and a receiver operating characteristic curve was drawn (R V.4.2.0, pROC package).

Metabolome sequencing and bioinformatic analysis

Fecal samples were analyzed using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) system. LC-MS/MS analyses were performed using a UHPLC system (Vanquish, Thermo Fisher Scientific) with a UPLC BEH (2.1 mm × 100 mm, 1.7 µm) amide column coupled to Q Exactive HFX mass spectrometer (Orbitrap MS, Thermo Fisher Scientific). Metabolites were detected in both the negative and positive ion models. Raw data were converted to the mzXML format using ProteoWizard,[23] and an internal standard was used for data normalization. Principal component analysis was performed using SIMCA software. To further investigate the differences between the groups, orthogonal partial least squares-discriminant analysis (OPLS-DA) modeling was performed, and the quality of the model was tested using sevenfold cross-validation. Student's t-test p<0.05) was used to screen differential metabolites, and the variable importance in the projection of the first principal component of the OPLS-DA model was greater than 1. KEGG (http://www.genome.jp/kegg/) and PubChem (https://pubchem.ncbi.nlm.nih.gov/) were used to annotate the metabolites.

ITS2 data analysis

The DNA was extracted using a DNA extraction kit for the corresponding sample. The concentration and purity were measured using a NanoDrop One (Thermo Fisher Scientific). The ITS2 hypervariable regions of the fungal ITS rRNA gene were amplified with the specific primers ITS3F: GCATCGATGAAGAACGCAGC and ITS4R: TCCTCCGCTTATTGATATGC using a Bio-Rad S1000 PCR system (Bio-Rad Laboratories, Hercules, California, USA).[24] Raw data were demultiplexed using the MiSeq Controller Software (Illumina). QIIME2 (V.2020.2) was used for downstream analysis.[25] Demultiplexed ITS2 sequences were denoised and grouped into amplicon sequence variants (ASVs) using DADA2.[26] ASV features present in only one sample were excluded from all cohorts. Individual ASVs were taxonomically classified based on the UNITE (V.8.2) database using the VSEARCH tool wrapped in QIIME2. Alpha diversity was estimated using the Shannon index at the operational taxonomic unit (OTU) level. PCoA was performed based on the unweighted UniFrac distance. FunGuild was employed for the functional prediction of fungi.[27]

Statistical analysis and correlation network

Differences in clinical characteristics were evaluated using the Pearson's χ^2 test or Fisher's exact test. Permutational multivariate analysis of variance (permutations=9,999, R V.4.2.0, vegan package) was performed to investigate the effect of each clinical characteristic based on the Bray-Curtis distance matrix of the species abundance profiles of the samples. A canonical correlations analysis (CCA) (R V.4.2.0, vegan package) was used to assess the effects of each clinical characteristic on the species abundance profile of the samples. Spearman's correlation coefficients between differentially enriched features were determined. Patients were categorized into high-ambudance and low-abundance groups based on the mean values of the relative abundances of different taxa. Survival-related taxa can be categorized as either beneficial for survival (high-abundance in DCB group, or low-abundance in NDB group) or not conducive to survival (low-abundance in DCB group, or high-abundance in NDB group). Finally, the survival benefits were categorized based on the combined expression of gut bacterial and metabolite profiles. A group exhibiting both benefits was classified as the good group, a group with no benefits was categorized as the poor group, and a group displaying one benefit and one lack of benefit was classified as the moderate group.[7] Survival curves were estimated using the Kaplan-Meier method and compared using the log-rank test. The follow-up time was estimated using the reverse Kaplan-Meier method. Progression-free survival (PFS) was defined as the time from the initiation of PD-1/PD-L1 inhibitor treatment to tumor progression or death. Overall survival (OS) was defined as the total time from the initiation of PD-1/PD-L1 inhibitor treatment until death from any cause. Univariate and multivariate analyses were performed using a Cox proportional hazards model. Unless otherwise stated, all statistical analyses were performed using R software (V.4.2.0), and p values<0.05 were considered statistically significant.

J Immunother Cancer: first published as 10.1136/jitc-2023-008686 on 6 June 2024. Downloaded from http://jitc.bmj.com/ on June 6, 2024 by guest. Protected by copyright.

RESULTS

Characteristics of study cohort

80 patients with HCC (mean (SD) age, 57.4 (10.4) years; 72 (90.0%) men) were enrolled in this study (online supplemental figure S2). The study was followed-up until August 2023, and the median follow-up time was 27.7 (95% CI: 23.5 to 33.4) months. Patient characteristics of the training and test cohorts are shown in online supplemental tables S2 and S3, respectively. Hepatitis, including chronic hepatitis B and C, was not significantly different between the DCB and NDB groups in both train and test cohorts. 25 (49.0%) patients in the train cohort developed an irAE, and there were only four Grade ≥3 irAEs, of which three were dermatological irAE and one was pneumonitis irAE (online supplemental table S2). All patients received ICI plus molecular targeted therapy, with the highest use of ICI for toripalimab (N=36, 45.0%) and camrelizumab (N=31, 38.7%), and targeted drugs for lenvatinib (N=62, 77.5%) (online supplemental table S4).

Diversity and composition of gut microbiome and mycobiome

At baseline, there were no significant differences in the alpha diversity of bacteria (species level) or fungi (OTU level) between the DCB and NDB groups (online supplemental figure S3A,D). Beta diversity in the DCB group was significantly higher in the DCB group than that in the NDB group for both bacteria (species level) and fungi (OTU level) (online supplemental figure S3B,E). The overall difference between the DCB and NDB groups was significant at the bacterial level, whereas the difference at the fungal level was relatively insignificant (online supplemental figure S3C,F). At the bacterial species level, no significant changes in alpha diversity were observed following ICI treatment (online supplemental figure S4A). However, after 6–8 weeks (point 2), the difference in bacterial beta diversity (species level) gradually disappeared between the two groups, suggesting that immunotherapy had a greater effect on the distribution of intestinal bacteria (online supplemental figure S4B). At the fungal level, there was an overall decreasing trend and then a gradual recovery of alpha diversity with the use of ICI. Differences between the DCB and NDB groups appeared after 6–8 weeks (point 2), after which they disappeared, and beta diversity was relatively stable (online supplemental figure S4C,D).

Based on multiomics data, 894 bacteria (based on species), 1,906 fungi (based on OTU), and 620 metabolites were detected (online supplemental figure S5A,E and table S5). The relative abundances of the DCB and NDB groups were compared at different phylogenetic levels to investigate the relationship between the composition of gut bacteria and fungi and ICI treatment. At the phylum level, the predominant bacterial (*Firmicutes* and *Bacteroidetes*) and fungal compositions (*k_Fungi; p_Ascomycota* and *k_Fungi; p_Basidiomycota*) were consistent in both the DCB and NDB groups (online supplemental figure S5B,F). At the species level, the bacteria with the highest percentages in the DCB and NDB groups were *s_Escherichia_coli* and *s_Faecalibacterium_prausnitzii*, respectively, and the fungi with the highest percentages in both groups were *k_Fungi; s_Candida_albicans* (online supplemental figure S5D,H). The gut microbiome and fungal composition at the phylum, genus, and species levels in the DCB and NDB groups are shown in online supplemental figure S5. After immunotherapy, in both the DCB and NDB groups, the composition of bacteria and fungi was relatively stable at dynamic points at the phylum level, whereas at the species level, the composition and percentage of bacteria and fungi were relatively more variable at dynamic points (online supplemental figure S6).

Significantly different gut bacteria, fungi, and metabolites in ICI-treated HCC

We identified 25 enriched species, 18 enriched fungi, and 37 enriched metabolites in the DCB and NDB groups (figure 1A,B; online supplemental table S5). We observed that certain gut bacteria or fungi, which were enriched in the DCB group, had a lower frequency of detection or were even undetectable in the NDB group, such as *s_Phascolarctobacterium_faecium* (76.0% in DCB and 37.5% in NDB), *s_Candidatus_Avimonas_narfia* (20.0% in DCB and not detected in NDB), and *k_Fungi; g_Cladosporium* (27.3% in NDB and 4.3% in DCB). Similarly, certain gut bacteria or fungi enriched in the NDB group were detected less frequently or even undetectable in the DCB group, such as *s_Actinomyces_sp_ICM47* (75.0% in NDB and 48.0% in DCB), *s_Senegalimassilia_anaerobia* (25.0% in NDB and 4.0% in DCB), *s_Faecalibacillus_faecis* (16.7% in NDB and not detected in DCB) and *k_Fungi; g_Trichoderma* (34.8% in NDB and 9.1% in DCB) (figure 1A; online supplemental figure S7A,B). Most of the differentially expressed metabolites were enriched in the DCB group (figure 1B; online supplemental figure S8). Furthermore, a phylogenetic tree was constructed based on the abundance of bacteria, which was mainly distributed in *Candidatus_Saccharibacteria* in the DCB group and *Firmicutes* in the NDB group (figure 1C). Based on the fungi, the phylogenetic tree demonstrated that *k_Fungi; p_Ascomycota* was distributed in the DCB group, and *k_Fungi; p_Basidiomycota* in the NDB group (online supplemental figure S7D). The distribution differences of bacteria were different between clinical assessment responses, PR, SD, and PD, with PD predominantly *Actinobacteria* at the phylum level and *Actinomyces_sp_ICM47*, *Senegalimassilia_anaerobia*, and *Faecalibacillus_faecis* at the species level (figure 1D; online supplemental figure S7C).

To gain insight into the potential interplay between multi-kingdom taxa and their potential roles in the response to HCC immunotherapy, we performed alterations in the multi-kingdom co-abundance network analysis based on the abundance of differential bacterial species, fungi, and metabolites. Generally, the ecological network of patients in the NDB group (75 taxa and 2,775 associations) was more complex than that of patients in the DCB group (69 taxa and 2,346 associations). Apart from

J Immunother Cancer: first published as 10.1136/jitc-2023-008686 on 6 June 2024. Downloaded from http://jitc.bmj.com/ on June 6, 2024 by guest. Protected by copyright.

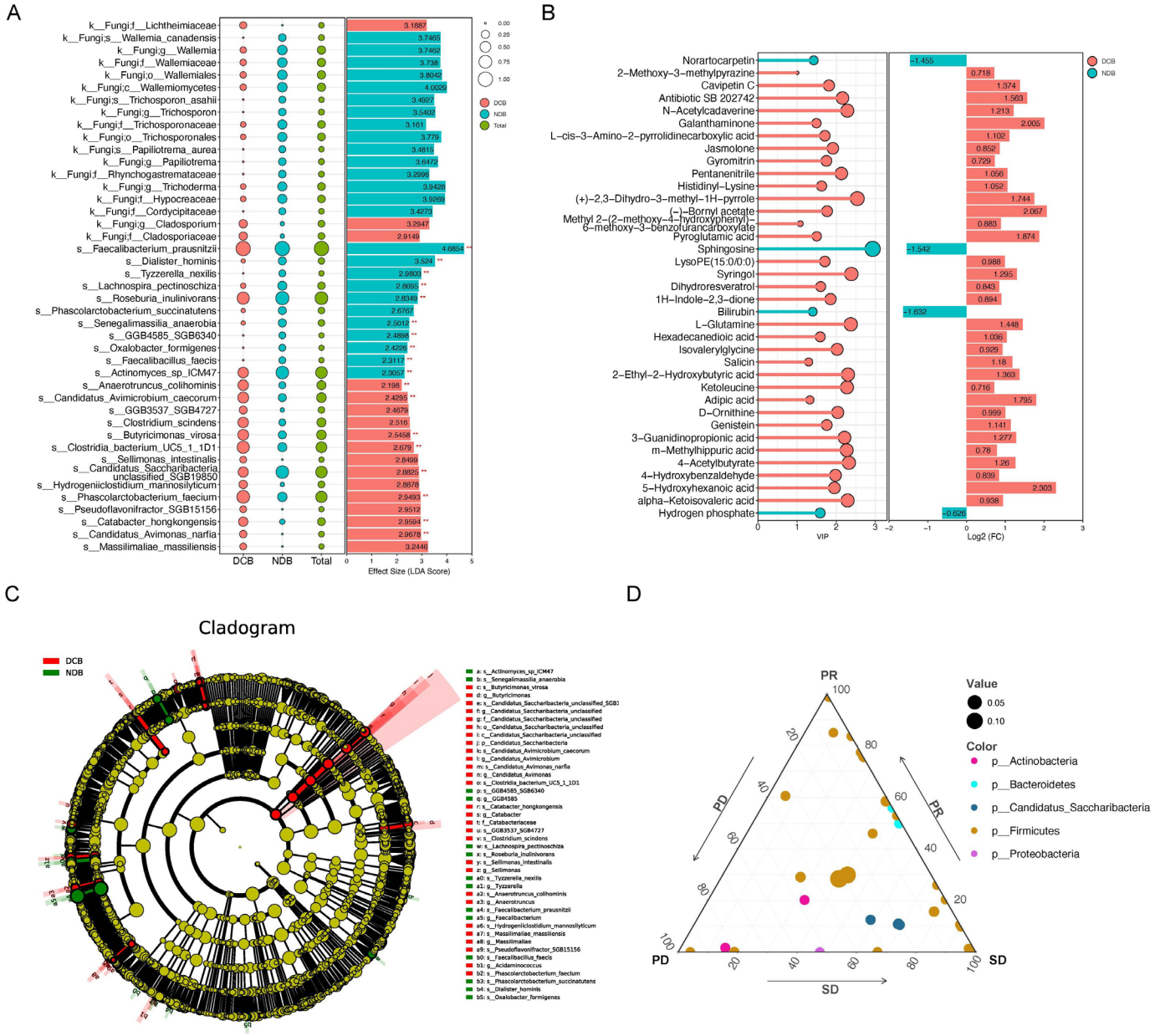


Figure 1 Significantly different gut bacteria, fungi, and metabolites in immune checkpoint inhibitor-treated hepatocellular carcinoma. (A) Significantly different abundant gut bacteria (species level) and fungi, and detection frequency in the DCB and NDB groups using LEfSe (LDA>2.0, p<0.05). **Represents the species incorporated in the predictive model. (B) Significantly different metabolites and variable importance in the projection between DCB and NDB groups. (C) Taxonomic cladogram from LEfSe showed different bacteria enriched in the DCB and NDB groups (LDA>2.0, p<0.05). (D) Ternary phase diagram for the prominent significantly different abundant species. The different colored dots show the phylum corresponding to species. DCB durable clinical benefit; LDA, linear discriminant analysis; LEfSe, Linear discriminant analysis effect size; NDB, non-durable clinical benefit; PD, disease progression; PR, partial response; SD, stable disease.

strong correlations between intrakingdom taxa, we found substantial associations between interkingdom species, especially between bacteria and metabolites, in both the DCB and NDB groups (online supplemental table S6). After adjusting for association coefficients (r>0.4), we found a higher number of associations in the DCB group than in the NDB group, suggesting that moderately associated taxa (such as bacteria and metabolites) may play a greater role in the immune-beneficial population (figure 2). In particular, emerging interkingdom interactions were discovered in the DCB group; for example, correlations between the differential bacterial species, such as *s_Butyricimonas_virosa* and *s_Candidatus_Avimonas_narfia*, and metabolites, such as syringol, sphingosine, and L-glutamine (figure 2). The low number of fungi highly associated with other multi-kingdom taxa in the DCB and NDB groups suggests that fungi play a relatively stable role throughout immunotherapy.

J Immunother Cancer: first published as 10.1136/jitc-2023-008686 on 6 June 2024. Downloaded from http://jitc.bmj.com/ on June 6, 2024 by guest. Protected by copyright.

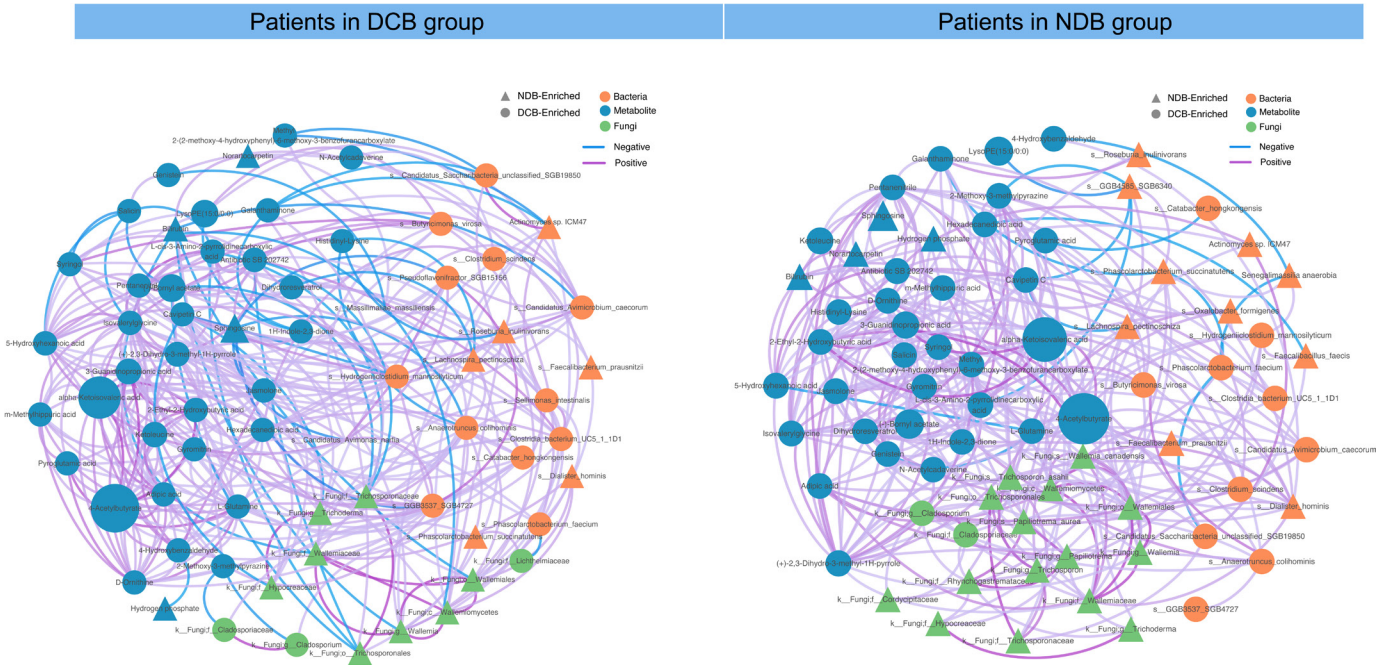


Figure 2 Coabundance correlations among multiomics taxa in patients with DCB and NDB groups. Moderate coabundance networks in DCB and NDB groups, with absolute correlations above 0.4 and with a significance cut-off of p value<0.05. The color of the line represents the size of the correlation coefficient, lighter means a smaller correlation coefficient and darker means a larger correlation coefficient. DCB, durable clinical benefit; NDB, non-durable clinical benefit.

Microbial functional alterations in ICI-treated HCC

Owing to the great inter-individual heterogeneity of the microbiota, it seems possible that different strains in different individuals may use common pathways to trigger similar pathological changes. Therefore, functional annotation by multiomics was used to explore some of the possible variations in micro-organisms in HCC immunotherapy and to provide possible research strategies. Nine KEGG pathways, 74 KEGG Orthology (KO) genes, and 18 metacyclic pathways for the metagenome and 35 KEGG pathways (based on the strength of the correlation, 15 of which were closely related to metabolic pathways) for LC-MS/MS were analyzed (figure 3A–C; online supplemental tables S5,S7,S8). Based on the level of KEGG pathways for metagenomic sequencing, alpha diversity did not differ between the two groups; however, beta diversity was significantly higher in the DCB group than in the NDB group (online supplemental figure S9A,B). Alpha diversity was relatively stable with the use of ICI, while whole beta diversity first showed a declining trend and then a gradual recovery (online supplemental figure S4E,F). Based on the KEGG pathway database and metabolite annotation analysis, KO00250 (alanine, aspartate, and glutamate metabolism), KO00910 (nitrogen metabolism), and KO00750 (vitamin B6 metabolism) were the pathways of interest common to both bacteria and metabolites, with KO00250 being the most important pathway for both bacteria and metabolites (figure 3B,C). Both KO00250 and KO00910 were detected in the NDB group (figure 3D). Bacteria (KO genes) and metabolites interacted through the KO00250 pathway to modulate the response of patients with HCC to immunotherapy, which may be an important way for microbes to regulate immune mechanisms. The metabolite L-glutamine played an important role in three of these pathways (online supplemental figure S9C). Functional annotation and prediction of fungi using FUNGuild revealed that there was no difference between the DCB and NDB groups based on trophic mode, trait, and guild classifications, except for differences in growth classification annotated as secotioid and gasteroid (online supplemental figure S10 and table S9).

Multi-kingdom intercorrelation and correlation with KEGG pathways

To further explore the ecological associations of bacteria, fungi, and metabolites, we analyzed the intracorrelations and intercorrelations among the 25 species, 18 fungi, and 37 metabolites, as well as their associations with nine KEGG pathways (figure 4). Overall, there were significantly more intracorrelations than intercorrelations, and intercorrelations were more frequent for bacterial-metabolite associations, whereas fungal-metabolite and bacterial associations were relatively rare. Regarding the correlation between multi-kingdom micro-organisms and KEGG pathways, *s_Faecalibacterium_prausnitzii* in bacteria was significantly associated with all nine KEGG pathways. In addition, KO00250 was positively correlated with *s_Phascolarctobacteriumsuccinatutens*, *s_Roseburia_inulinivorans*, *k_Fungi; g_Papiliotrema*, and *k_Fungi; f_Rhynchogastremataceae*. KO00910 was negatively correlated with *s_Catabacter_hongkongensis*, *s_Massilimaliae_massiliensis*, and *s_Sellimonas_intestinalis* and positively correlated with *k_Fungi; g_Trichosporon*, and *k_Fungi; s_Papiliotrema_aurea*.

J Immunother Cancer: first published as 10.1136/jitc-2023-008686 on 6 June 2024. Downloaded from http://jitc.bmj.com/ on June 6, 2024 by guest. Protected by copyright.

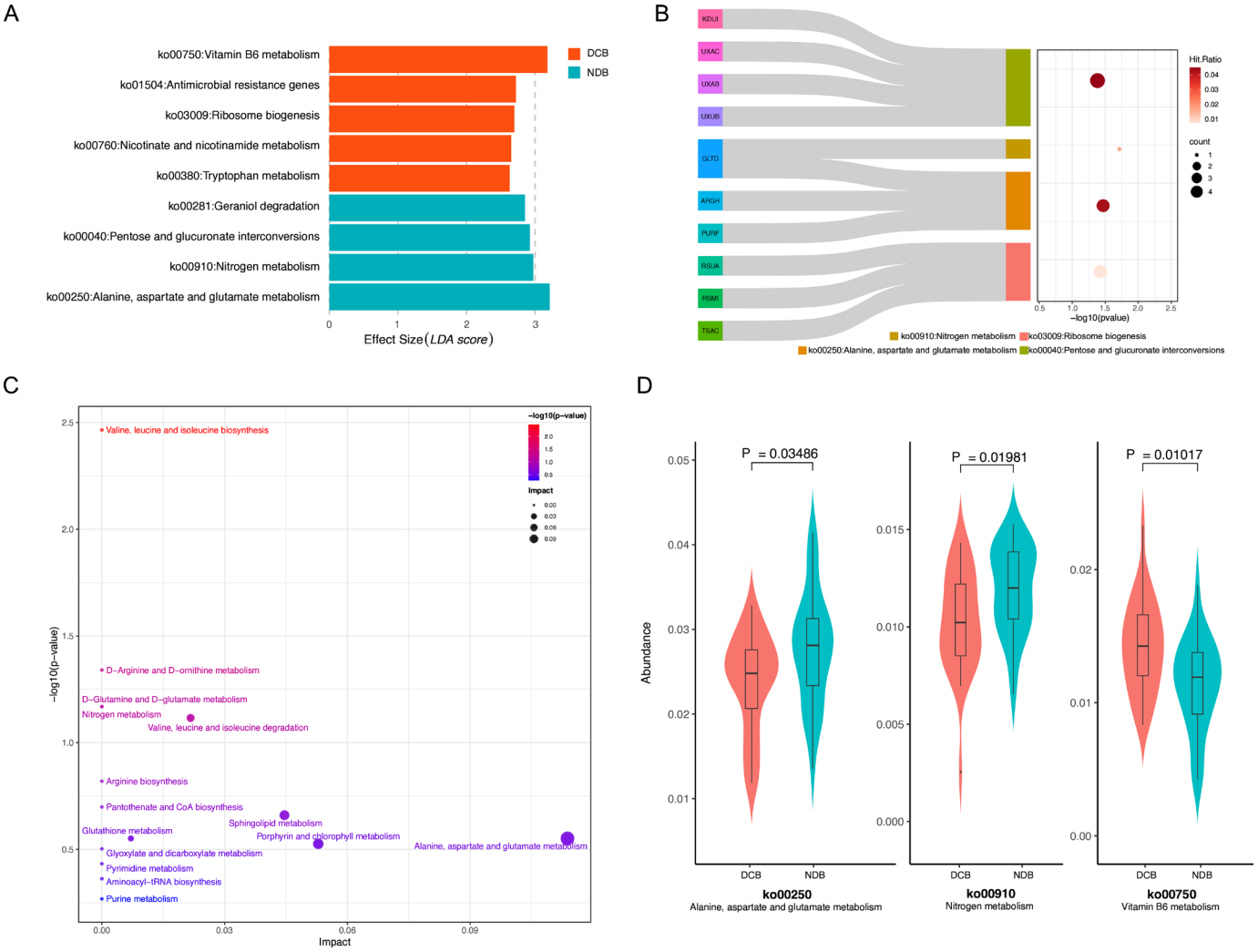


Figure 3 Functional annotation for metagenomic and metabolomic analysis. (A) Differential KEGG pathways for metagenomic sequencing. (B) Sankey bubble plot shows differences in the KEGG pathway, KO gene, and KO gene's contribution to the KEGG pathway. (C) Enrichment metabolic pathways for differential metabolite annotation. (D) Relative abundance comparison of KO00250, KO00910, and KO00750 pathways between the DCB and NDB groups. DCB, durable clinical benefit; KEGG, Kyoto Encyclopedia of Genes and Genomes; KO, KEGG Orthology; LDA, linear discriminant analysis; NDB, non-durable clinical benefit.

Metabolites and fungi showed a relatively weaker association with KEGG, whereas bacteria showed a stronger association with KEGG and were mostly positively correlated with metabolites expressed or enriched in the DCB group. The overall multi-kingdom microbial correlations suggest that bacteria are more closely associated with metabolites and KEGG pathways, whereas fungi have relatively fewer associations, suggesting that bacteria may play a greater role in HCC immunotherapy and influence KEGG pathways through multiple metabolites.

Predictive model and the association between gut microbiome, microbe-derived metabolites, and survival

To investigate potential biomarkers for predicting response status, we developed a LASSO regression model based on the species in the DCB and NDB groups. In the training set, 18 key microbial biomarkers were identified and a LASSO classifier was constructed with an area under the curve (AUC) value of 100% (figure 1A; online supplemental figure S7A). It could also predict the response status in the test set, with an AUC value of 75.63% (95% CI: 58.34% to 92.93%; figure 5A). These results suggest that the model of 18 bacterial associations is a good predictor and differentiator of whether patients with HCC will derive clinical benefit from immunotherapy.

To further explore the microbial taxa and metabolites associated with survival, we stratified patients with HCC in the training set into high-abundance and low-abundance groups based on the mean abundance of these differentially enriched taxa in the DCB and NDB groups. Patients with enriched *s_Actinomyces_sp_ICM47* had significantly worse PFS (median PFS: 7.45 vs 12.0 months, p=0.013) and OS (median OS: 17.3 vs NA months, p=0.021; figure 5B). *s_Senegalimassilia_anaerobia* was also associated with worse PFS (median PFS: 6.37 vs 12.0 months, p=0.025) and OS (median OS: 14.4 vs NA months, p=0.043; figure 5B). A survival benefit was observed in patients with enriched

J Immunother Cancer: first published as 10.1136/jitc-2023-008686 on 6 June 2024. Downloaded from http://jitc.bmj.com/ on June 6, 2024 by guest. Protected by copyright.

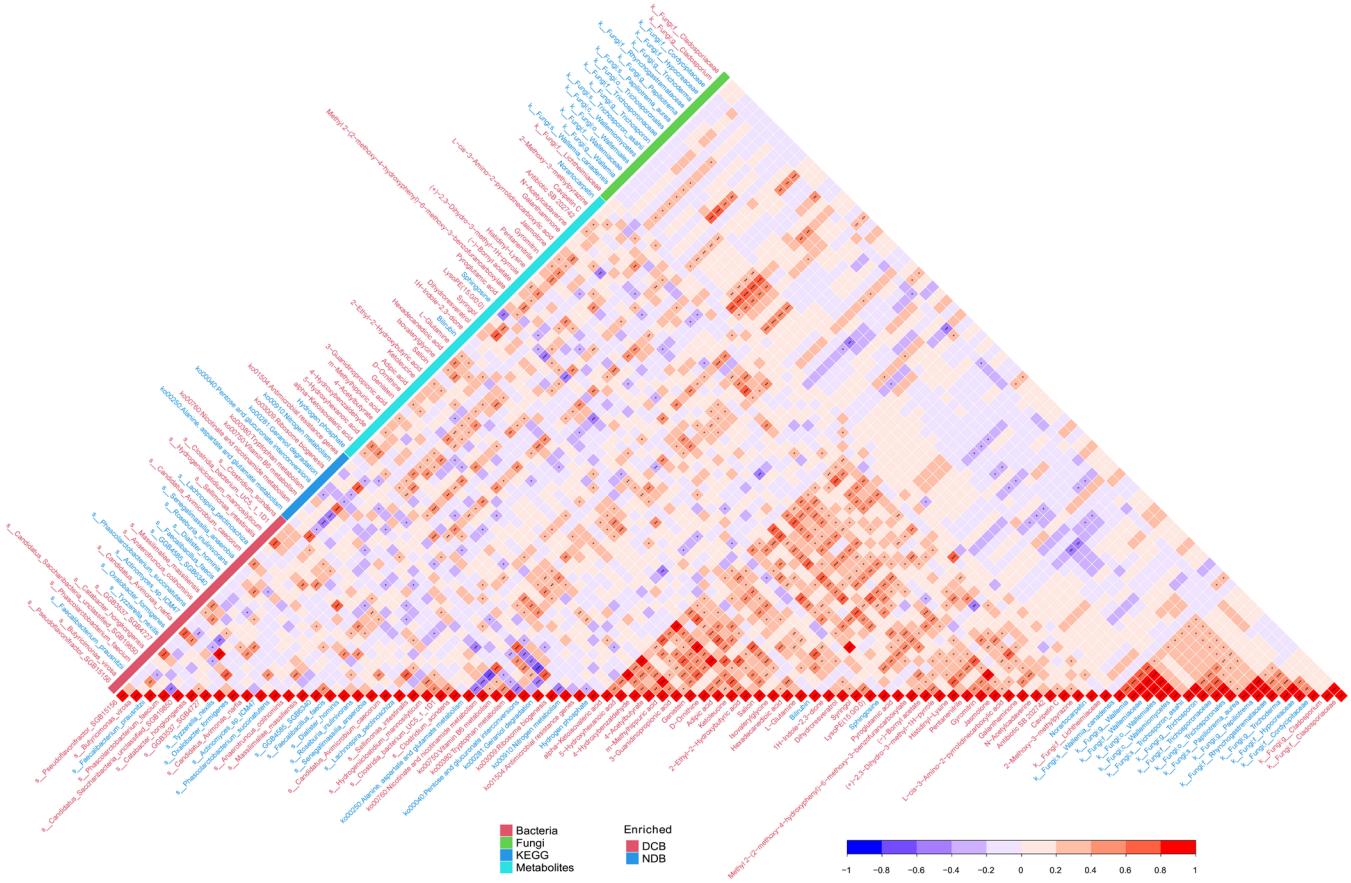


Figure 4 Multi-kingdom intercorrelation analysis among bacteria, fungi, metabolites, and KEGG pathways. DCB, durable clinical benefit; KEGG, Kyoto Encyclopedia of Genes and Genomes; NDB, non-durable clinical benefit.

galanthaminone (median PFS: 15.18 vs 9.33 months; p=0.029; median OS: NA vs 24.2 months, p=0.028; figure 5B). Patients with HCC were further categorized into good, moderate, and poor survival groups based on whether the bacteria or metabolites were simultaneously beneficial for survival. The results of univariate and multivariate analyses suggested that age (<60 years), ECOG PS (score 1), and maximum tumor diameter (≥5 cm) were risk factors, while *s_Actinomyces_sp_ICM47* or *s_Senegalimassilia_anaerobia* combined with galanthaminone (good group) was a positive factor for OS in patients with HCC (online supplemental table S10). Furthermore, combined bacterial and metabolite survival analyses revealed that low expression of *s_Actinomyces_sp_ICM47* or *s_Senegalimassilia_anaerobia* combined with galanthaminone predicted better survival (figure 5C,D). Our findings suggest that the enrichment of specific taxa or metabolites in gut microbes may enhance the clinical response to anti-PD-1 therapy and prolong survival, thereby predicting survival benefits in patients with HCC receiving immunotherapy. The combination of bacteria and metabolites predicted even higher survival, suggesting that the co-presence of these highly enriched bacteria or metabolites may potentiate the effects of immunotherapy in patients via some mechanism. An analysis of the patients, grouped into good, moderate, and poor survival categories based on the combination of *Actinomyces_sp_ICM47* and galanthaminone, did not reveal any statistically significant differences in the incidence of overall irAEs or organ-specific irAEs. Similarly, when the patients were grouped based on *Senegalimassilia_anaerobia* and galanthaminone, comparable results were observed (online supplemental table S11).

Gut bacteria, fungi, and metabolites are influenced by clinical factors

Gut microbiology, including bacteria, fungi, and metabolites, is a complex, dynamically balanced system influenced by multiple factors. To clarify the possible influence of clinical phenotypes on gut microbes, we performed a correlation analysis of differential bacteria, fungi, metabolites, and KEGG pathways with clinical phenotypes and explored the influence of clinical phenotypes on the distribution of bacteria and metabolites using CCA analysis. Association analyses suggested that differentially expressed bacteria, such as *s_Clostridium_scindens* and *s_Clostridia_bacterium_UC5_1_1D1*, were strongly associated with clinical phenotypes such as total bilirubin (Tbil) and BA. In contrast, bacteria predicted to be survival associated, such as *s_Actinomyces_sp_ICM47* and *s_Senegalimassilia_anaerobia*, were less influenced by clinical factors (figure 6). Tumor number and intrahepatic metastasis

J Immunother Cancer: first published as 10.1136/jitc-2023-008686 on 6 June 2024. Downloaded from http://jitc.bmj.com/ on June 6, 2024 by guest. Protected by copyright.

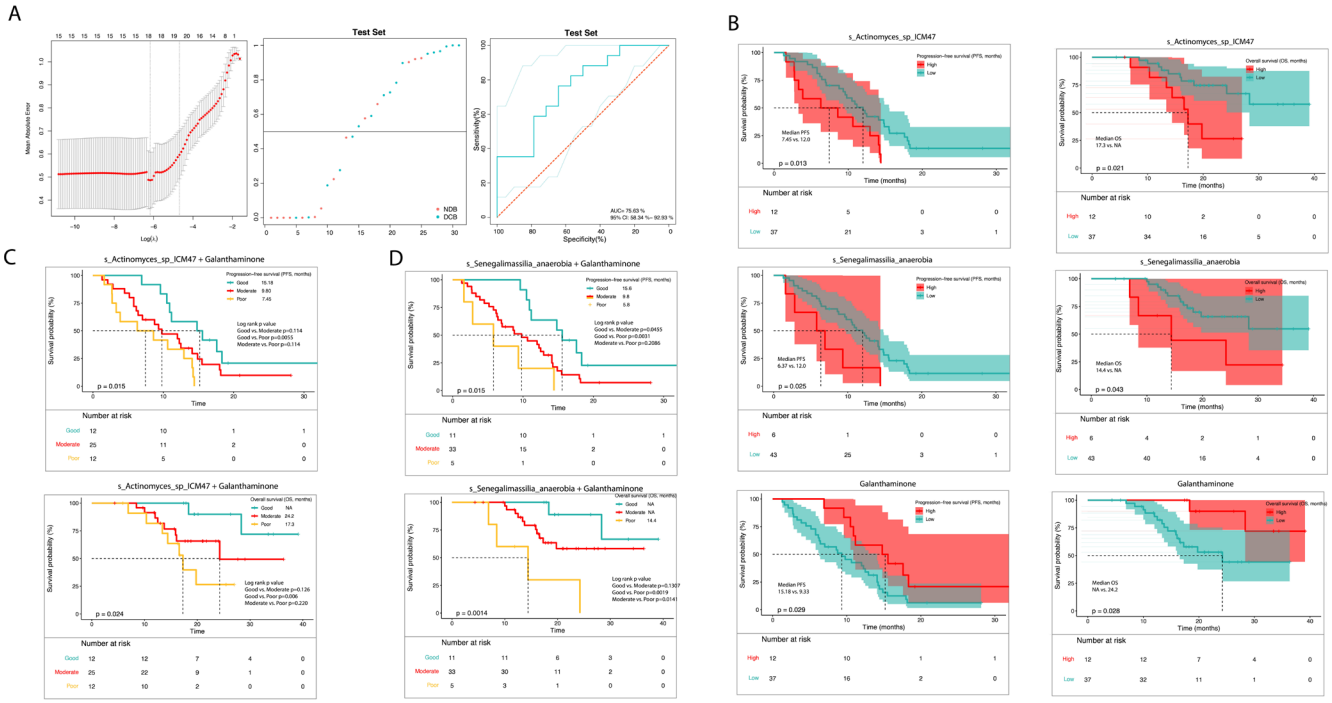


Figure 5 Predictive model and the association between gut microbiome, microbe-derived metabolites and survival. (A) Receiver operating characteristic curve of least absolute shrinkage and selection operator regression model for the test set. (B) Progression-free survival (PFS) and overall survival (OS) depended on the relative abundance of *s_Actinomyces_sp_ICM47*, *s_Senegalimassilia_anaerobia*, and galanthaminone. PFS and OS of *s_Actinomyces_sp_ICM47* combined with galanthaminone (C) and *s_Senegalimassilia_anaerobia* combined with galanthaminone (D). Good signature: coexistence of depleted *s_Actinomyces_sp_ICM47*/*s_Senegalimassilia_anaerobia* and enriched galanthaminone. Poor signature: coexistence of enriched *s_Actinomyces_sp_ICM47*/*s_Senegalimassilia_anaerobia* and depleted galanthaminone. Moderate signature: coexistence of depleted both or enriched both of gut microbiome and metabolites.

(IHM) were strongly associated with differential metabolites, whereas the survival-related metabolite, galanthaminone, was not affected by clinical factors (figure 6). Most of the differential fungi and KEGG pathways were less influenced by the clinical phenotype, whereas the KO00250 pathway was not significantly associated with the clinical phenotype, revealing the relative stability of the fungi and the importance of the role played by KO00250 pathway throughout immunotherapy (figure 6). The results of the CCA analysis suggested that the overall effect of the clinical phenotype on bacterial distribution was not significant, whereas it was relatively significant for the overall distribution of metabolites between the DCB and NDB groups (figure 7A,B,D,E). Multiple tumor-associated factors, such as aspartate aminotransferase, gamma glutamyl transferase (GGT), alanine aminotransferase, size, and vascular invasion significantly influenced the distribution of gut bacteria, such as *s_Candidatus_Avimonas_narfia*, whereas GGT, carbohydrate antigen 19–9, IHM, tumor number, and TBil significantly influenced the distribution of metabolites, such as galanthaminone, and sphingosine (figure 7B,C,E,F). The effect of clinical factors on the heterogeneity of gut microbial enrichment in the DCB and NDB groups suggests that the clinical response and survival benefit of ICI treatment depend on the diversity of microbes and differentially enriched taxonomic communities throughout the gut.

DISCUSSION

Immunotherapy has provided new hope for treating advanced HCC. The stability of gut micro-organisms plays a regulatory role in many diseases and states in the human body. In this study, the status and characterization of multi-kingdom microbiota, including gut bacteria, fungi, and their metabolites, in patients with HCC treated with ICI were described by multiomics sequencing for the first time. We also explored the alterations in gut microbes in the DCB and NDB groups, established 18 species of bacterial models as predictive biomarkers for predicting whether immunotherapy is of sustained benefit, and screened 2 bacterial species and 1 metabolite as prognostic biomarkers for predicting survival in patients with HCC treated with ICI. These results provide a foundation for future efforts to maximize the benefits of immunotherapy in patients with advanced HCC by determining and modifying gut microbial status.

Several studies, including those on HCC, have confirmed that bacterial diversity is correlated with the efficacy of tumor immunotherapy; however, the results

J Immunother Cancer: first published as 10.1136/jitc-2023-008686 on 6 June 2024. Downloaded from http://jitc.bmj.com/ on June 6, 2024 by guest. Protected by copyright.



Figure 6 Correlations between gut bacteria, fungi, metabolites, and KEGG pathways with clinical factors. Spearman's rank correlation analysis with two-tailed p values. *p<0.05; **p<0.01; ***p<0.001. DCB, durable clinical benefit; KEGG, Kyoto Encyclopedia of Genes and Genomes; NDB, non-durable clinical benefit.

are not entirely consistent.[3 5 28] In our study, alpha diversity based on bacteria and fungi at baseline did not differ between the DCB and NDB groups; however, beta diversity was higher in the DCB group than in the NDB group (significant bacteria-based and non-significant fungal-based differences). Bacteria-based differences in beta diversity disappeared after 6–8 weeks, whereas fungi-based dynamic alpha diversity showed an overall declining trend followed by recovery. The time point before the third course of immunotherapy (around 6–8 weeks after initiation of immunotherapy) is a watershed point in terms of the greater impact on bacterial and fungal diversity, and it is possible that this point is the cut-off point at which differences in the ability of patients with HCC to have a sustained benefit from immunotherapy begin to emerge. In future studies, more attention should be paid to changes in diversity after 6–8 weeks of immunotherapy, in addition to baseline gut bacterial and fungal diversity.

Studies have suggested that gut bacteria are good predictive biomarkers for tumor immunotherapy.[29 30] Fungi may be relatively more stable across disease states than bacteria, but can exert their influence on diseases through interactions with bacteria.[10 24 31–33] A cohort study of four types of tumors (gastrointestinal cancer, melanoma, non-small cell lung cancer, and renal cell carcinoma) suggested that fungal and fungal-bacterial combinations are good predictors of efficacy in tumor immunotherapy.[32] In our study, based on the fact that the 18 key microbes were a good class of taxa for predicting the efficacy of HCC immunotherapy, fungal differences were relatively less pronounced than those of bacteria in the DCB and NDB groups. Two bacteria, a metabolite, and a bacteria-metabolite combination, were prognostic predictors of HCC using immunotherapy; however, no suitable markers were screened at the fungal level. Based on the above findings, bacteria are good predictive and prognostic markers for tumors; however, more studies are needed to confirm whether fungi can be used as stable markers.

The bacteria and metabolites differed significantly between the DCB and NDB groups, whereas the differences were smaller for fungi. *Faecalibacterium prausnitzii* was mainly enriched in the NDB group and had the highest abundance and LDA scores among the species in the DCB and NDB groups. *F. prausnitzii* has been consistently reported as one of the main butyrate producers in the intestine.[34] Butyrate plays a crucial role in the gut physiology and host well-being. Interestingly, higher basal fecal levels of butyric acid have also been associated with decreased survival.[35] *F. prausnitzii* may reduce the efficacy of butyric acid immunotherapy; however, the exact mechanism remains unknown. In our study, both *Actinomyces*

J Immunother Cancer: first published as 10.1136/jitc-2023-008686 on 6 June 2024. Downloaded from http://jitc.bmj.com/ on June 6, 2024 by guest. Protected by copyright.

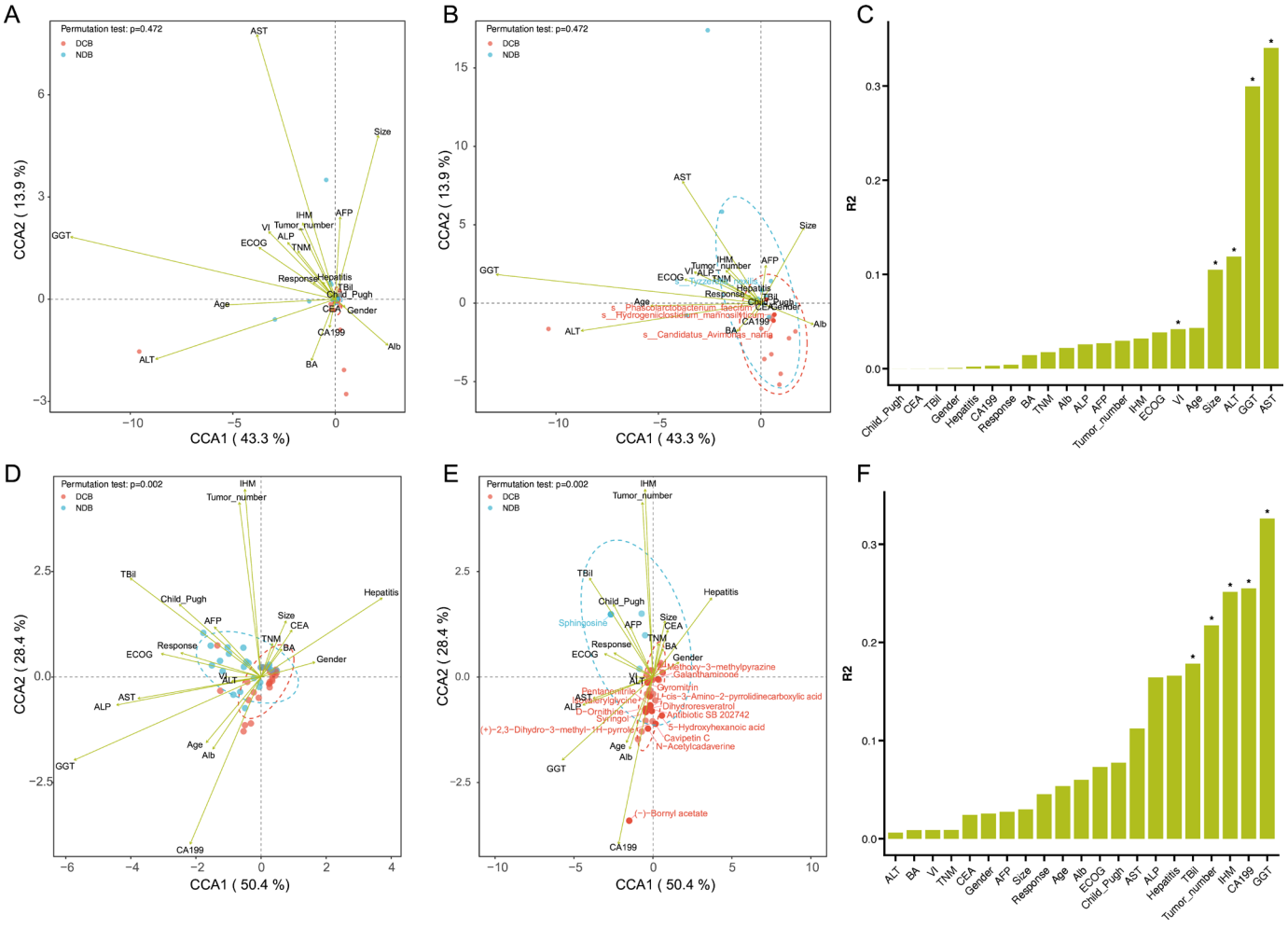


Figure 7 The distribution of gut microbiome and microbe-derived metabolites was affected by clinical factors. Canonical correspondence analysis (CCA) with permutation test showed the clinical factors associated with the distribution and contribution for gut microbiome (A) and microbe-derived metabolites (B) of patients with HCC in DCB and NDB groups. *p<0.05. ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BA, bile acids; CA19-9, carbohydrate antigen 19–9; CEA, carcinoembryonic antigen; DCB, durable clinical benefit; ECOG, Eastern Cooperative Oncology Group; GGT, gamma glutamyl transferase; IHM, intrahepatic metastasis; NDB, non-durable clinical benefit; TBil, total bilirubin; TNM, tumor, node, metastasis; VI, vascular invasion.

and *Senegalimassilia* were potential prognostic markers that were negatively associated with immunotherapy. *Actinomyces* were enriched in non-responders to advanced melanoma treated with immunotherapy, a non-response-associated module that linked *Actinomyces* to butyric acid (and derivatives) and cyclohexanecarboxylic acid ethyl ester, as well as to higher levels of neutrophils.[35] *Actinomyces* is more abundant in the high-risk group with diffuse large B-cell lymphoma[36] and lung cancer.[37] The presence of *Senegalimassilia*, which was detected almost exclusively in the NDB group in the current study (25% in NDB and 4% in DCB), may have a poorer prognosis, and a larger cohort is needed to confirm its feasibility and stability. There are fewer studies on *Senegalimassilia* and the results are inconsistent across different tumors. For example, Yin *et al*[38] found that *Senegalimassilia* is a risk factor for bladder cancer, while Jiang *et al*[39] reported that *Senegalimassilia* is indeed a protective factor for pancreatic cancer. No mechanistic studies of *Senegalimassilia* in tumorigenesis or tumor immunotherapy have been reported. The cornerstone of ICI treatment for tumors lies in the augmentation of T cell-mediated immune cytotoxicity by targeting coinhibitory molecules such as PD-1/PD-L1, thereby preventing immune evasion by tumor cells. The composition of gut bacteria and their metabolic products exert a significant influence on the efficacy of immunotherapy by modulating various types of T cells, either enhancing or inhibiting the therapeutic response.[40] For instance, specific bacteria, including *Lactobacillus rhamnosus*, have been shown to enhance the antitumor activity of PD-1 immunotherapy through the cyclic GMP-AMP synthase (cGAS)/stimulator of IFN genes (STING) signaling pathway, leading to the activation of interferon-α/β signaling and cytotoxic CD8+T cells.[41 42] SCFAs have been implicated in limiting the antitumor effects of cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) blockade by alleviating the suppressive activity of regulatory T cells. Moreover, a higher concentration of butyrate has been

J Immunother Cancer: first published as 10.1136/jitc-2023-008686 on 6 June 2024. Downloaded from http://jitc.bmj.com/ on June 6, 2024 by guest. Protected by copyright.

found to decrease the anticancer activity of ipilimumab by inhibiting the accumulation of CD4+T cells involved in the response.[43] Additionally, gut microbes play a role in nutrient metabolism and produce metabolites that can influence the body's immune response, ultimately impacting the effectiveness of ICIs.[40]

The mechanisms of action of bacteria, fungi, and metabolites in tumor immunotherapy may be multifaceted.[44 45] In our study, the KO00250 and KO00910 pathways were mainly upregulated in the NDB group, which may be associated with negative immunotherapeutic effects, and the metabolite L-glutamine may play an important role. Glutamine is an important non-essential amino acid. In tumor cells, "glutamine addiction" is evident. Studies have shown that an increase in glutamine levels promotes purine metabolism to support cancer cell growth and metabolism.[46 47] Additionally, altered glutamine metabolism contributes to a hypoxic, acidic, nutrient-depleted tumor microenvironment (TME), which is detrimental to the antitumor immune response.[47] In the KO00250 pathway, the metabolic conversion of glutamate to glutamine was relatively high in the NDB group. This also suggests that some immunotherapy-negative bacteria may indirectly reduce the efficacy of immunotherapy through the KEGG 250 pathway by directly affecting the expression levels of the metabolites, glutamate and glutamine. However, there was one contradictory result, where L-glutamine showed relatively higher expression in the overall differences in the DCB group. It has also been shown that glutamine metabolism in the TME promotes tumor growth but impairs the antitumor activity of immune cells.[48–50] These conflicting outcomes require further validation and confirmation in future studies.

Unlike other predictive and prognostic biomarkers of immunotherapy, the gut microbiota not only serves as a biomarker but also represents an important therapeutic target. In our study, we observed that *Actinomyces* and *Senegalimassilia* were predominantly enriched in the NDB group of patients with HCC treated with ICI, indicating their association with poor survival. Therefore, targeting and reducing the abundance of these specific gut bacteria may hold promise for improving the efficacy of ICI. Several interventions such as fecal transplantation, oral antibiotics, and dietary modifications can be employed to achieve the reduction of these detrimental bacteria.[40 51 52] Although clinical trials have explored the modulation of gut bacteria to enhance immunotherapy efficacy in liver cancer,[53] lung cancer,[54] and melanoma,[55] there is currently a paucity of research focusing on *Actinomyces* and *Senegalimassilia*. Further clinical trials are warranted to validate this therapeutic approach. This research gap provides an avenue for future investigations into potentiating immunotherapy for HCC. Alternatively, modulation of the immunotherapeutic response can be achieved by targeting intestinal metabolites or key molecules downstream in metabolic pathways using screened small molecule compounds. In our study, galanthaminone emerged as a protective factor associated with improved survival in patients with HCC undergoing ICI. Consequently, upregulating galanthaminone and related crucial molecules may hold therapeutic potential. However, in correlation analysis, galanthaminone did not show significant associations with any of the nine significantly different KEGG pathways. Therefore, further studies are required to elucidate the mechanism by which galanthaminone influences HCC immunotherapy, facilitating the development of more precise therapeutic strategies. In addition, targeting the glutamine metabolic pathway, in combination with immunotherapy, is also a novel therapeutic strategy based on the findings of this study.

Several studies have suggested a positive correlation between the occurrence of irAEs and survival, with the gut bacteria exhibiting predictive potential for irAE occurrence.[56 57] However, in the present study, there were no significant differences observed in the overall and organ-specific irAEs between the good, moderate, and poor groups of patients categorized based on *Actinomyces*/*Senegalimassilia* and galanthaminone. Several potential reasons can be considered for these findings. First, the moderate group had a higher percentage of patients, indicating a need for further differentiation in combination with other biomarkers to better understand its characteristics. Additionally, it is possible that severe irAEs are more closely associated with survival outcomes. However, due to the relatively small sample size and the low proportion of severe irAE cases (only four cases), our study was unable to thoroughly elucidate the correlation between severe irAEs and survival. Consequently, future studies with larger sample sizes are warranted to provide more robust evidence, enabling clearer prediction of survival subgroups based on specific bacteria or metabolites. Furthermore, the importance of severe irAEs should be emphasized and thoroughly investigated in future research.

Our study had the following limitations. First, the sample size was small, and a larger external cohort is needed to validate the predictive model and prognostic biomarkers. In addition, because of the sample size, the model construction involved only bacteria and did not incorporate fungi and metabolites. Thus, it was not possible to build a model for the combination of multiple groups of micro-organisms. In the future, cohorts with large sample sizes are needed to construct multiomics microbial joint prediction models to predict the efficacy of HCC immunotherapy. Similarly, large sample size cohort studies can compensate for the shortcomings in dynamic point analyses in this study. Second, there is currently a dearth of comprehensive research elucidating the mechanisms underlying the differential effects of gut microbes, including *Actinomyces* and *Senegalimassilia*. However, given the vast array of gut microbes types, this study offers valuable insights that may guide future investigations in a more focused and precise direction. Third, the majority of patients included in this study had HCC in combination with hepatitis, while a smaller proportion had non-hepatitis HCC. As a result, the generalizability

J Immunother Cancer: first published as 10.1136/jitc-2023-008686 on 6 June 2024. Downloaded from http://jitc.bmj.com/ on June 6, 2024 by guest. Protected by copyright.

of the findings may be limited to specific etiologies of HCC. To better understand the role of hepatitis as a contributing factor and the underlying mechanisms, it is recommended to increase the sample size of patients with non-hepatitis etiologies such as alcoholic liver disease or autoimmune liver disease in future studies. Finally, gut microbes also include viruses and archaea, and further studies are needed to confirm whether these other microbes play an important reciprocal role in modulating HCC immunotherapy.

CONCLUSIONS

These findings show that multi-kingdom microbiota, including gut bacteria, fungi, and their metabolites, play an important role in efficacy, and some can be used as good prognostic or predictive markers in patients with HCC treated with ICI.

Author affiliations
[1]Department of Liver Surgery, State Key Laboratory of Complex Severe and Rare Diseases, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College (CAMS & PUMC), Beijing, China
[2]Department of General Surgery Center, Beijing Youan Hospital, Clinical Center for Liver Cancer, Capital Medical University, Beijing, China
[3]Microbiome Research Center, Moon (Guangzhou) Biotech Ltd, Guangzhou, China
[4]Department of Pulmonary and Critical Care Medicine, State Key Laboratory of Complex Severe and Rare Diseases, Chinese Academy of Medical Sciences & Peking Union Medical College (CAMS & PUMC), Beijing, China

Acknowledgements We thank Guangdong Magigene Biotechnology Co., Ltd (Guangzhou, China) for their excellent technical support in obtaining raw data for metagenomic and metabolome sequencing.

Contributors HZ, XJ, HW, and XY designed the study. CZhu, CZha, SW, ZX, DZ, ZL, LZ, JC, YL, ZP, and CN performed the experiment. HZ, XY, and XS provided supervision and funding. JC and XY provided key materials. CZhu, CZha, and ZL completed data analysis. CZhu, CZha, SW, and ZX finished drawing and figures modification. CZhu, CZha, and SW wrote and finalized the manuscript. HZ, XJ, HW, and XY have assessed and verified the data. All authors read and approved the final version of the manuscript. Guarantor: HZ.

Funding This work was supported by the National High Level Hospital Clinical Research Funding (2022-PUMCH-B-128), CAMS Innovation Fund for Medical Sciences (CIFMS) (2022-I2M-C&T-A-003) (2021-I2M-1-061) (2021-I2M-1-003), CSCO-hengrui Cancer Research Fund (Y-HR2019-0239) (Y-HR2020MS-0415) (Y-HR2020QN-0414), CSCO-MSD Cancer Research Fund (Y-MSDZD2021-0213) and National Ten-thousand Talent Program.

Competing interests None declared.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants and was approved by Institutional Review Board and Ethics Committee of Peking Union Medical College Hospital (PUMCH, IRB NO. JS-2296). Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request. Additional data from the analyses presented in this paper are available in the Supplementary material. The raw data sets used and analyzed during the current study are available from the corresponding author on reasonable request.

Supplemental material This content has been supplied by the author(s). It has not been vetted by BMJ Publishing Group Limited (BMJ) and may not have been peer-reviewed. Any opinions or recommendations discussed are solely those of the author(s) and are not endorsed by BMJ. BMJ disclaims all liability and responsibility arising from any reliance placed on the content. Where the content includes any translated material, BMJ does not warrant the accuracy and reliability of the translations (including but not limited to local regulations, clinical guidelines, terminology, drug names and drug dosages), and is not responsible for any error and/or omissions arising from translation and adaptation or otherwise.

Open access This is an open access article distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited, appropriate credit is given, any changes made indicated, and the use is non-commercial. See http://creativecommons.org/licenses/by-nc/4.0/.

ORCID iD
Haitao Zhao http://orcid.org/0000-0002-3444-8044

REFERENCES

1 Siegel RL, Miller KD, Wagle NS, *et al*. Cancer statistics, 2023. *CA Cancer J Clin* 2023;73:17–48.
2 Foerster F, Gairing SJ, Ilyas SI, *et al*. Emerging immunotherapy for HCC: a guide for hepatologists. *Hepatology* 2022;75:1604–26.
3 Jin Y, Dong H, Xia L, *et al*. The diversity of gut microbiome is associated with favorable responses to anti-programmed death 1 immunotherapy in Chinese patients with NSCLC. *J Thorac Oncol* 2019;14:1378–89.
4 Mao J, Wang D, Long J, *et al*. Gut microbiome is associated with the clinical response to anti-PD-1 based immunotherapy in hepatobiliary cancers. *J Immunother Cancer* 2021;9:e003334.
5 Wu H, Zheng X, Pan T, *et al*. Dynamic microbiome and metabolome analyses reveal the interaction between gut microbiota and anti-PD-1 based immunotherapy in hepatocellular carcinoma. *Int J Cancer* 2022;151:1321–34.
6 Shen Y-C, Lee P-C, Kuo Y-L, *et al*. An exploratory study for the association of gut microbiome with efficacy of immune checkpoint inhibitor in patients with hepatocellular carcinoma. *J Hepatocell Carcinoma* 2021;8:809–22.
7 Lee P-C, Wu C-J, Hung Y-W, *et al*. Gut microbiota and metabolites associate with outcomes of immune checkpoint inhibitor–treated unresectable hepatocellular carcinoma. *J Immunother Cancer* 2022;10:e004779.
8 Lu Y, Yuan X, Wang M, *et al*. Gut microbiota influence immunotherapy responses: mechanisms and therapeutic strategies. *J Hematol Oncol* 2022;15:47.
9 Andrews MC, Vasanthakumar A. Gut Microbiota – a double-edged sword in cancer immunotherapy. *Trends Cancer* 2023;9:3–5.
10 Shuai M, Fu Y, Zhong H-L, *et al*. Mapping the human gut mycobiome in middle-aged and elderly adults: multiomics insights and implications for host metabolic health. *Gut* 2022;71:1812–20.
11 Zhang F, Aschenbrenner D, Yoo JY, *et al*. The gut mycobiome in health, disease, and clinical applications in association with the gut bacterial microbiome assembly. *Lancet Microbe* 2022;3:e969–83.
12 Lai S, Yan Y, Pu Y, *et al*. Enterotypes of the human gut mycobiome. *Microbiome* 2023;11:179.
13 Shiao SL, Kershaw KM, Limon JJ, *et al*. Commensal bacteria and fungi differentially regulate tumor responses to radiation therapy. *Cancer Cell* 2021;39:1202–13.
14 Doron I, Leonardi I, Li XV, *et al*. Human gut mycobiota tune immunity via CARD9-dependent induction of anti-fungal IgG antibodies. *Cell* 2021;184:1017–31.
15 Zhu X, Li K, Liu G, *et al*. Microbial metabolite butyrate promotes anti-PD-1 antitumor efficacy by modulating T cell receptor signaling of cytotoxic CD8 T cell. *Gut Microbes* 2023;15:2249143.
16 Schwartz LH, Seymour L, Litière S, *et al*. RECIST 1.1 – Standardisation and disease-specific adaptations: perspectives from the RECIST working group. *Eur J Cancer* 2016;62:138–45.
17 Rizvi NA, Hellmann MD, Snyder A, *et al*. Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non–small cell lung cancer. *Science* 2015;348:124–8.
18 Thompson JA, Schneider BJ, Brahmer J, *et al*. NCCN guidelines insights: management of Immunotherapy-related toxicities. version 1.2020. *J Natl Compr Canc Netw* 2020;18:230–41.
19 Chen S, Zhou Y, Chen Y, *et al*. Fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* 2018;34:i884–90.
20 Rosenbloom KR, Armstrong J, Barber GP, *et al*. The UCSC genome browser database: 2015 update. *Nucleic Acids Res* 2015;43:D670–81.
21 Truong DT, Franzosa EA, Tickle TL, *et al*. MetaPh1An2 for enhanced metagenomic taxonomic profiling. *Nat Methods* 2015;12:902–3.
22 Franzosa EA, McIver LJ, Rahnavard G, *et al*. Species-level functional profiling of metagenomes and metatranscriptomes. *Nat Methods* 2018;15:962–8.

J Immunother Cancer: first published as 10.1136/jitc-2023-008686 on 6 June 2024. Downloaded from http://jitc.bmj.com/ on June 6, 2024 by guest. Protected by copyright.

23 Kessner D, Chambers M, Burke R, *et al*. Proteowizard: open source software for rapid proteomics tools development. *Bioinformatics* 2008;24:2534–6.
24 Zuo T, Wong SH, Cheung CP, *et al*. Gut fungal dysbiosis correlates with reduced efficacy of fecal microbiota transplantation in clostridium difficile infection. *Nat Commun* 2018;9:3663.
25 Bolyen E, Rideout JR, Dillon MR, *et al*. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol* 2019;37:852–7.
26 Callahan BJ, McMurdie PJ, Rosen MJ, *et al*. DADA2: high-resolution sample inference from Illumina Amplicon data. *Nat Methods* 2016;13:581–3.
27 Nguyen NH, Song Z, Bates ST, *et al*. Funguild: an open annotation tool for parsing fungal community datasets by ecological guild. *Fungal Ecology* 2016;20:241–8.
28 Gopalakrishnan V, Spencer CN, Nezi L, *et al*. Gut microbiome modulates response to anti-PD-1 immunotherapy in melanoma patients. *Science* 2018;359:97–103.
29 Greten TF, Villanueva A, Korangy F, *et al*. Biomarkers for immunotherapy of hepatocellular carcinoma. *Nat Rev Clin Oncol* 2023;20:780–98.
30 Thomas AM, Fidelle M, Routy B, *et al*. Gut oncomicrobiome signatures (GOMS) as next-generation biomarkers for cancer immunotherapy. *Nat Rev Clin Oncol* 2023;20:583–603.
31 Liu N-N, Jiao N, Tan J-C, *et al*. Multi-kingdom microbiota analyses identify bacterial–fungal interactions and biomarkers of colorectal cancer across cohorts. *Nat Microbiol* 2022;7:238–50.
32 Huang X, Hu M, Sun T, *et al*. Multi-kingdom gut microbiota analyses define bacterial-fungal interplay and microbial markers of Pan-cancer immunotherapy across cohorts. *Cell Host Microbe* 2023;31:1930–43.
33 Kaźmierczak-Siedlecka K, Dvořák A, Folwarski M, *et al*. Fungal gut microbiota dysbiosis and its role in colorectal, oral, and pancreatic carcinogenesis. *Cancers (Basel)* 2020;12:1326.
34 Lopez-Siles M, Duncan SH, Garcia-Gil LJ, *et al*. Faecalibacterium Prausnitzii: from microbiology to diagnostics and prognostics. *ISME J* 2017;11:841–52.
35 Vandoni G, D'Amico F, Fabbrini M, *et al*. Gut microbiota, metabolome, and body composition signatures of response to therapy in patients with advanced melanoma. *Int J Mol Sci* 2023;24:11611.
36 Zhang Y, Han S, Xiao X, *et al*. Integration analysis of tumor metagenome and peripheral immunity data of diffuse large-B cell lymphoma. *Front Immunol* 2023;14:1146861.
37 Zhao F, An R, Wang L, *et al*. Specific gut microbiome and serum metabolome changes in lung cancer patients. *Front Cell Infect Microbiol* 2021;11:725284.
38 Yin Z, Liu B, Feng S, *et al*. A large genetic causal analysis of the gut microbiota and urological cancers: a bidirectional mendelian randomization study. *Nutrients* 2023;15:4086.
39 Jiang Z, Mou Y, Wang H, *et al*. Causal effect between gut microbiota and pancreatic cancer: a two-sample mendelian randomization study. *BMC Cancer* 2023;23:1091.
40 Zhao L-Y, Mei J-X, Yu G, *et al*. Role of the gut microbiota in anticancer therapy: from molecular mechanisms to clinical applications. *Signal Transduct Target Ther* 2023;8:201.
41 Si W, Liang H, Bugno J, *et al*. *Lactobacillus Rhamnosus* GG induces cGAS/STING- dependent type I interferon and improves response to immune checkpoint blockade. *Gut* 2022;71:521–33.
42 Sivan A, Corrales L, Hubert N, *et al*. Commensal bifidobacterium promotes antitumor immunity and facilitates anti-PD-L1 efficacy. *Science* 2015;350:1084–9.
43 Coutzac C, Jouniaux J-M, Paci A, *et al*. Systemic short chain fatty acids limit antitumor effect of CTLA-4 blockade in hosts with cancer. *Nat Commun* 2020;11:2168.
44 Kim CH. Immune regulation by microbiome metabolites. *Immunology* 2018;154:220–9.
45 Hayase E, Jenq RR. Role of the intestinal microbiome and microbial-derived metabolites in immune checkpoint blockade immunotherapy of cancer. *Genome Med* 2021;13:107.
46 Huang R, Wang H, Hong J, *et al*. Targeting glutamine metabolic reprogramming of SLC7A5 enhances the efficacy of anti-PD-1 in triple-negative breast cancer. *Front Immunol* 2023;14:1251643.
47 Wang Y, Zhu X, Wang K, *et al*. Cell metabolomics study on synergistic anti-hepatocellular carcinoma effect of Aidi injection combined with doxorubicin. *Biomedical Chromatography* 2022;36:e5451.
48 An R, Yu H, Wang Y, *et al*. Integrative analysis of plasma metabolomics and proteomics reveals the metabolic landscape of breast cancer. *Cancer Metab* 2022;10:13.
49 Yuan Q, Deng D, Pan C, *et al*. Integration of transcriptomics, proteomics, and metabolomics data to reveal Her2-associated metabolic heterogeneity in gastric cancer with response to immunotherapy and neoadjuvant chemotherapy. *Front Immunol* 2022;13:951137.
50 Wang S, Li M, Yan L, *et al*. Metabolomics study reveals systematic metabolic dysregulation and early detection markers associated with incident pancreatic cancer. *Int J Cancer* 2022;150:1091–100.
51 Zhang M, Liu J, Xia Q. Role of gut microbiome in cancer immunotherapy: from predictive biomarker to therapeutic target. *Exp Hematol Oncol* 2023;12:84.
52 Blake SJ, Wolf Y, Boursi B, *et al*. Role of the microbiota in response to and recovery from cancer therapy. *Nat Rev Immunol* 2024;24:308–25.
53 Fessas P, Naeem M, Pinter M, *et al*. Early antibiotic exposure is not detrimental to therapeutic effect from immunotherapy in hepatocellular carcinoma. *Liver Cancer* 2021;10:583–92.
54 Takada K, Shimokawa M, Takamori S, *et al*. Clinical impact of probiotics on the efficacy of anti-PD-1 monotherapy in patients with nonsmall cell lung cancer: a multicenter retrospective survival analysis study with inverse probability of treatment weighting. *Int J Cancer* 2021;149:473–82.
55 Matson V, Fessler J, Bao R, *et al*. The commensal microbiome is associated with anti-PD-1 efficacy in metastatic melanoma patients. *Science* 2018;359:104–8.
56 Ng KYY, Tan SH, Tan JJE, *et al*. Impact of immune-related adverse events on efficacy of immune checkpoint inhibitors in patients with advanced hepatocellular carcinoma. *Liver Cancer* 2022;11:9–21.
57 Hu M, Lin X, Sun T, *et al*. Gut microbiome for predicting immune checkpoint blockade-associated adverse events. *Genome Med* 2024;16:16.

J Immunother Cancer: first published as 10.1136/jitc-2023-008686 on 6 June 2024. Downloaded from http://jitc.bmj.com/ on June 6, 2024 by guest. Protected by copyright.