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Protocol: Faecal microbiota transfer in liver cancer to overcome resistance to atezolizumab/bevacizumab – a multicentre, randomised, placebo-controlled, double-blind phase II trial (the FLORA trial)

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To cite: Rauber C, Roberti MP, Vehreschild MJGT, *et al*. Protocol: Faecal microbiota transfer in liver cancer to overcome resistance to atezolizumab/bevacizumab – a multicentre, randomised, placebo-controlled, double-blind phase II trial (the FLORA trial). *BMJ Open* 2025;**15**:e097802. doi:10.1136/bmjopen-2024-097802

► Prepublication history and additional supplemental material for this paper are available online. To view these files, please visit the journal online (https://doi.org/10.1136/bmjopen-2024-097802).

Received 10 December 2024
Accepted 12 August 2025

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ABSTRACT

Introduction Combined vascular endothelial growth factor/programmed death-ligand 1 blockade through atezolizumab/bevacizumab (A/B) is the current standard of care in advanced hepatocellular carcinoma (HCC). A/B substantially improved objective response rates compared with tyrosine kinase inhibitor sorafenib; however, a majority of patients will still not respond to A/B. Strong scientific rationale and emerging clinical data suggest that faecal microbiota transfer (FMT) may improve antitumour immune response on PD-(L)1 blockade. Early trials in melanoma with FMT and reinduction of immune checkpoint blockade (ICI) therapy in patients with anti-PD-1-refractory metastatic melanoma were reported in 2021 and demonstrated reinstatement of response to ICI therapy in many patients. Due to anatomical vicinity and the physiological relevance of the gut-liver axis, we hypothesise HCC to be a particularly attractive cancer entity to further assess a potential benefit of FMT in combination with ICI towards increased antitumour immunity. Additionally, HCC often occurs in patients with liver cirrhosis, where liver function is prognostically relevant. There is evidence that FMT may increase hepatic function and therefore could positively affect outcome in this patient population.

Methods and analysis This prospective, multicentre, randomised, placebo-controlled, double-blind phase II clinical trial has been designed to assess immunogenicity and safety of FMT via INTESTIFIX 001 combined with A/B in advanced HCC in comparison to A/B with placebo. Primary endpoints are measured as tumour CD8+ T cell infiltration after 2 cycles of treatment with vancomycin, A/B+INTESTIFIX 001 in comparison to vancomycin-placebo, A/B+INTESTIFIX 001-placebo and safety of the therapeutic combination in advanced HCC. INTESTIFIX 001 is an encapsulated FMT preparation by healthy donors with a high alpha-diversity in their gut microbiome for oral administration, manufactured by the Cologne Microbiota Bank (CMB). Sample size was calculated to achieve a specific expected accuracy for the primary immunological endpoint. 48 subjects will be randomised to reach a goal of 42 usable measurements in the modified intention-to-treat set. Subjects will be randomised in a 2:1 ratio to A/B or placebo (28 A/B, 14 placebo).

Ethics and dissemination The study was approved by ethics committee review and the German Federal Ministry

STRENGTHS AND LIMITATIONS OF THIS STUDY

⇒ Using faecal microbiota transfer from healthy donors produced under Good Manufacturing Practices (GMP) complies with high-quality standards, yields superior microbiological safety and can be easily scalable to clinical practice.
⇒ The trial is performed as a multicentric, double-blind, randomised controlled trial (RCT) ensuring a high level of scientific validity and minimising bias.
⇒ Tissue CD8-T-cell infiltration has been shown to be a useful surrogate marker for immune checkpoint blockade response across various tumour entities, including pre-existing CD8-T-cell infiltration in hepatocellular carcinoma and response to atezolizumab/bevacizumab.
⇒ The study intervention consists of a combination of vancomycin followed by faecal microbiota transfer; the study design does not allow determination of whether vancomycin or faecal microbiota transplantation alone affects endpoints.
⇒ The study is not designed with sufficient statistical power to assess significant differences in clinical and radiologic endpoints, such as overall survival or progression-free survival.

of Drugs and Medical Devices. The trial is registered under EU CT no. 2023-506887-15-00. The outcome of the study will be disseminated via peer-reviewed publications and at international conferences.

Trial registration number NCT05690048.

INTRODUCTION

Strong scientific rationale and emerging clinical data suggest that faecal microbiota transfer (FMT) may improve antitumour immune response to the combined vascular endothelial growth factor (VEGF)/programmed death-ligand 1 (PD-L1) blockade through atezolizumab/bevacizumab (A/B) in advanced hepatocellular carcinoma (HCC).[1 2] Several early clinical trials testing FMT and reinduction of immune checkpoint blockade (ICI) therapy in patients with anti-PD-1-refractory metastatic melanoma demonstrated reinstatement of response to ICI therapy in some patients.[3–5] The combined blockade of VEGF and PD-L1 through the administration of A/B currently represents the standard of care for patients with advanced HCC.[1] While A/B significantly enhances objective response rates in comparison to tyrosine kinase inhibitor sorafenib, a substantial proportion of patients do not exhibit a partial or complete response to this treatment regimen.[6] Previous FMT studies predominantly employed treatment beyond progression strategies, assessing the potential for (re)sensitisation to ICI.[3 4] It is crucial to note that the absence of an active comparator group and blinding in these studies introduces a notable source of uncertainty and potential bias in the results.[3–5] It remains plausible that the ICI response observed may have been influenced by treatment beyond progression, irrespective of FMT, as reported for several tumour entities, including HCC.[7–9] Additionally, studies by Baruch *et al* and Davar *et al* used FMTs from so-called 'elite donors', ie, donors with malignant melanoma selected for their good initial response to immunotherapy, a practice that significantly limits the pool of suitable donors and poses regulatory challenges due to malignancy of the donor thereby complicating the feasibility of implementing add-on FMT treatment in oncological settings.[3 4 10 11] Encouragingly, a recent study by Routy *et al* demonstrated that FMT from healthy donors also effectively improved ICI response rates in melanoma, as compared with historical controls.[5] Due to anatomical vicinity and the physiological relevance of the gut-liver axis, we hypothesise HCC to be a particularly attractive cancer entity to further assess a potential benefit of FMT in combination with ICI towards increased antitumour immunity. This so-called gut-liver axis has been consistently shown to contribute to HCC development and progression.[12] Additionally, HCC often occurs in patients with liver cirrhosis, where liver function is prognostically relevant. There is evidence that FMT may increase hepatic function and therefore could positively affect outcome in this patient population. Delineating the mechanisms of the gut microbiota-mediated immunomodulation is an active area of research and requires understanding of the communication pathways between the gut microbiota and the immune system.[13 14] FMT, as used in the Faecal Microbiota Transfer in Liver Cancer to Overcome Resistance to Atezolizumab/Bevacizumab (FLORA) trial, is only one of many strategies for manipulating the gut microbiome. As indicated, certain isolated species have been validated to have a causal link on preclinical models with supporting mechanisms of action. However, clinical data on several indications, including refractory *Clostridium difficile* colitis, ulcerative colitis and other gastrointestinal conditions in which gut microbiota manipulation has been tested, demonstrated FMT to be more effective than isolated/mixed probiotic species.[15 16] One of the consensual findings among studies is that a high alpha diversity, ie, a broad range of bacteria species, is associated with good outcomes.[17–20] A high alpha diversity can typically be found in young and healthy donors, with minimal exposure to antibiotics.[21–23]

The trial's associated exploratory research will provide first functional evidence of relevant biochemical and cellular mechanisms by which the gut microbiota regulates host antitumour immunity in HCC. As the identity of functionally relevant bacteria (both positive and negative) is getting better understood, the ideal selection of FMT donor for a given patient will be facilitated.

METHODS AND ANALYSIS

Study design

FLORA is a multicentre, randomised, placebo-controlled, double-blind phase II trial conducted at 9 university hospitals in Germany within the National Center for Tumor diseases (NCT) framework to investigate the safety and immunogenicity of vancomycin and FMT product INTESTIFIX 001 in combination with A/B in subject from ≥18 years of age with the indication of systemic treatment of HCC. The study will recruit 48 patients with advanced, non-resectable hepatocellular carcinoma. The trial is designed in a parallel-group design with two arms randomised in a 2:1 ratio (32 patients INTESTIFIX 001, 16 patients placebo) (figure 1).

Objectives

Primary endpoint

The primary objectives are to assess immunogenicity and safety of vancomycin, A/B+INTESTIFIX 001 in comparison to vancomycin-placebo, A/B+INTESTIFIX 001-placebo in patients with advanced HCC.

The *primary immunogenicity endpoint* is the tumour CD8+ T cell infiltration at d40 (up to 2 cycles of treatment with A/B), used regardless of emergency antibiotics, treatment discontinuation or use of probiotics. The *primary safety endpoints* are incidence and severity of adverse events (AEs) and serious adverse events (SAEs) until safety follow-up visit (d105).

Secondary endpoints

Secondary objectives of the main trial are to assess an antitumoural effect of the therapeutic combination

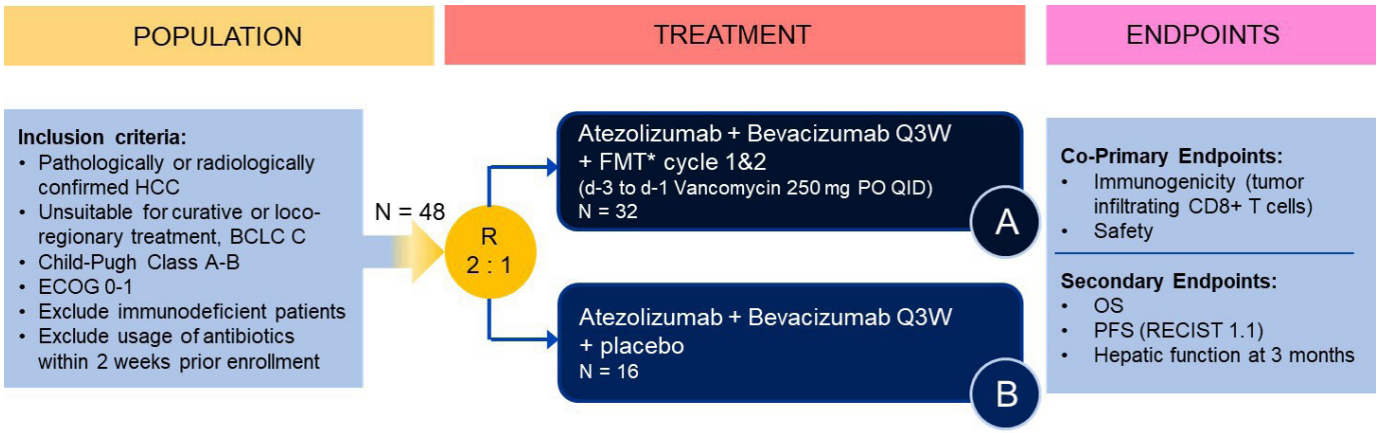

Figure 1 Trial design. The study will recruit 48 patients with advanced, non-resectable hepatocellular carcinoma. The trial is designed in a parallel-group design with two arms randomised in a 2:1 ratio, intervention:control. FMT, faecal microbiota transfer; HCC, hepatocellular carcinoma; OS, overall survival; PFS, progression-free survival.

with standard endpoints in oncology, such as in terms of radiological response to treatment, to evaluate an effect on overall survival, as well as an influence on subjective patient well-being through patient-reported outcomes. Additionally, changes in hepatic function will be assessed. The specific secondary endpoints are:

1. Overall survival (OS).
2. Progression-free survival (PFS).
3. Disease control (DC).
4. Objective response (OR).
5. Duration of response (DoR).
6. Alpha-fetoprotein serological response rate.
7. Hepatic function.

Inclusion/exclusion criteria

Key inclusion criteria:

- Age 18 years or older.
- Confirmed imaging or histological diagnosis of unresectable HCC, Barcelona Clinic Liver Cancer (BCLC) stadium C.
- Eastern Cooperative Oncology Group (ECOG) performance status of 0–1.

Key exclusion criteria:

- Advanced liver cirrhosis (Child-Pugh Score C).
- Condition of immunodeficiency (eg, HIV, immunosuppressants).
- Usage of antibiotics within 2 weeks prior to enrolment.

Detailed inclusion/exclusion criteria are listed in online supplemental material 1.

Sample size estimation

Sample size was calculated to achieve a specific expected accuracy for the primary immunological endpoint. Assuming normality and an SD of 165, a sample size of 42 would lead to a 95% CI for the difference in means between the groups with a width of around 218. This is deemed to be sufficient to decide whether investigation in further studies would be warranted. To account for missing endpoints, for example, because on-treatment biopsy at day 40 was contraindicated, subjects might decide to reject on-treatment biopsy; a drop-out/loss to follow-up rate of 12.5% is assumed. Therefore, 48 subjects would need to be randomised to reach our goal of 42 usable measurements in the modified intention-to-treat set. As subjects are blinded to their treatment allocation, the risk of bias due to drop-outs is expected to be minor.

Recruitment and randomisation

Eligible patients are approached by investigators for their interest in trial participation. All screened subjects receive a screening number. Screening failures are defined as subjects who consent to participate in the clinical trial but are subsequently not included. As the decision to include patients is based on routine data collected prior to informed consent, screening failures are unlikely. At baseline, subjects will be allocated to the treatment and placebo group in 2:1 ratio at random, using a good clinical practice (GCP)-compliant web-based tool (www.randomizer.at). Randomisation will be performed using a permuted block design with differing block lengths. The block lengths will be concealed from investigators to minimise predictability of subject assignment. Since no centre differences are to be expected, the randomisation is not stratified.

Blinding

Study medication with investigational medicinal product (IMP) (vancomycin/placebo, INTESTIFIX 001/placebo) is blinded to subjects and site personnel. For all trial personnel, including biometricians, patient treatment with IMP shall remain blinded from the time of randomisation until final database lock. If it is medically imperative to know what clinical trial medication the subject is receiving, the investigator or authorised person should break the blind of the respective subject. Breaking the blind will be performed using the online randomisation tool (Randomizer).

Faecal microbiota transfer (FMT) preparation

INTESTIFIX 001 is an encapsulated FMT preparation for oral administration manufactured by the Cologne Microbiota Bank (CMB). The basis for the production of INTESTIFIX 001 is a suspension derived from a stool donation of healthy donors. From 50 g of stool, 30 capsules are prepared. For this purpose, 50 g of the stool donation and 250 mL of saline solution (0.9% NaCl) are homogenised and filtered. Based on the ingredients stool, saline solution (0.9 %) and glycerol (100 %), a drug is manufactured via filtration, various centrifugation steps and finally encapsulation. The capsules are storable at −80°C for 12 months. Before, during and after the actual production, various steps serve to ensure safety and quality control. Donor screening represents the first safety step (online supplemental material 2). In order to account for relevant incubation periods of transmissible infections, a 6-week donation pause takes place after a 6-week donation period. This is followed by a rescreening of the donor blood (online supplemental material 2). Quality control includes examination of fresh stool from each stool donation for potentially infectious pathogens and 16S microbiome analysis to determine diversity (specification: Shannon Diversity Index >2, Richness >30 genera). The viability of each product suspension is also determined. In-process controls are performed during manufacturing (eg, visual inspections, weight control). Only if these controls do not reveal any abnormalities and the rescreening of donor stool and blood remains without findings, the FMT products are approved by the Qualified Person. Immediately after its production, INTESTIFIX 001 is stored in ultra-low temperature freezers at −70°C to −90°C. In order to assure an uninterrupted cold chain, shipment from the manufacturer to the study sites has to be conducted on dry ice according to a validated transport protocol with temperature log. A temporary interim storage at −20°C was validated for 2 weeks. Shelf life has been validated to 1 year after storage at −70°C to −90°C. If INTESTIFIX 001 has not been used after 1 year of storage at −70°C to −90°C, the product has to be discarded according to the specifications in the study protocol. A recruitment of 4 donors for the FMT production for the FLORA trial is planned, but more donors may be needed if initial donors are no longer available later in the study. Each subject in the intervention group will receive the two FMTs from the same donor. No pooled FMT samples will be used.

Intervention

After randomisation and clinical assessment, the trial starts with a vancomycin or 'Vancomycin' placebo pretreatment from day 3 to day 1, followed by the treatment starting on day 0 with INTESTIFIX 001 FMT capsules or INTESTIFIX 001 placebo capsules and A/B (figures 1 and 2). A/B will be administered every 3 weeks within the current standard of care and according to the protocol of the IMbrave150 trial.

The treatment schedule is depicted in detail in figure 2. The schedule of assessments is depicted in online supplemental material 3.

Investigational Medicinal Product (IMP) and placebo:

1. INTESTIFIX 001 FMT capsules or INTESTIFIX 001 placebo capsules will be swallowed by the patient within 10 to 30 min of removal from the freezer under supervision of trained study personnel.
2. Vancomycin 250 mg capsules or 'Vancomycin' placebo capsules (subjects will be instructed by the site to document the self-administration of vancomycin/placebo at home in a diary).

Auxiliary medicinal products (AxMP):

1. Atezolizumab (Tecentriq).
2. Bevacizumab.

Intervention group (Arm A)

Vancomycin 250 mg per os (p.o.) is given four times daily from day 3 to day 1. Then, atezolizumab 1200 mg intravenous+bevacizumab 15 mg/kg body weight (bw) intravenous+15 INTESTIFIX 001 FMT capsules p.o. will be administered on day 0 and day 21, then atezolizumab

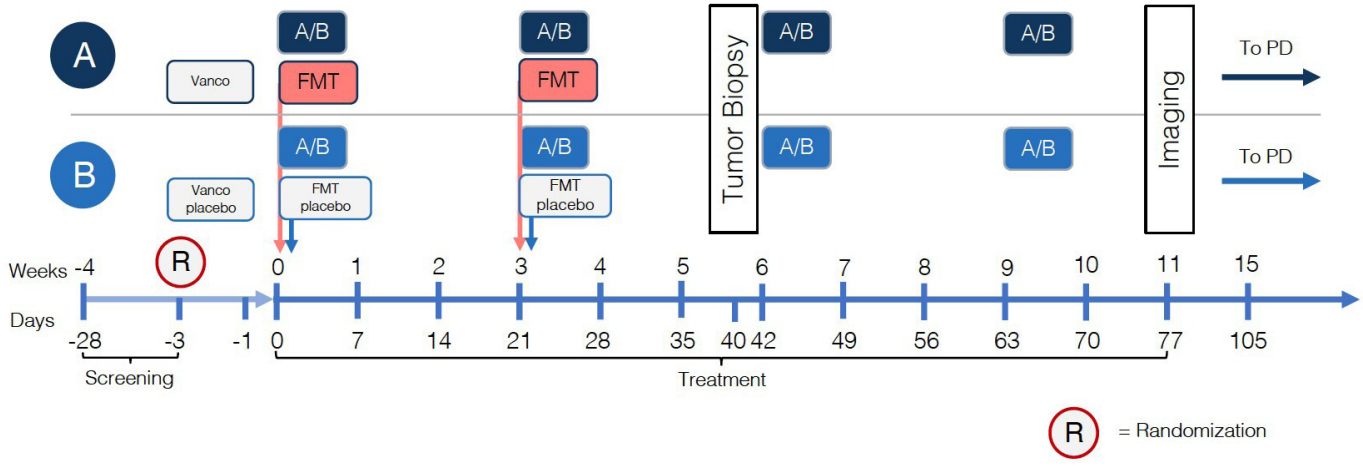

Figure 2 Treatment schedule. The trial begins with a pretreatment phase from day 3 to day 1 using either vancomycin or matching placebo. On day 0, participants receive FMT capsules or placebo capsules in combination with treatment A/B. Treatment A/B is administered every 3 weeks in accordance with the current standard of care. A/B, atezolizumab/bevacizumab; FMT, faecal microbiota transfer.

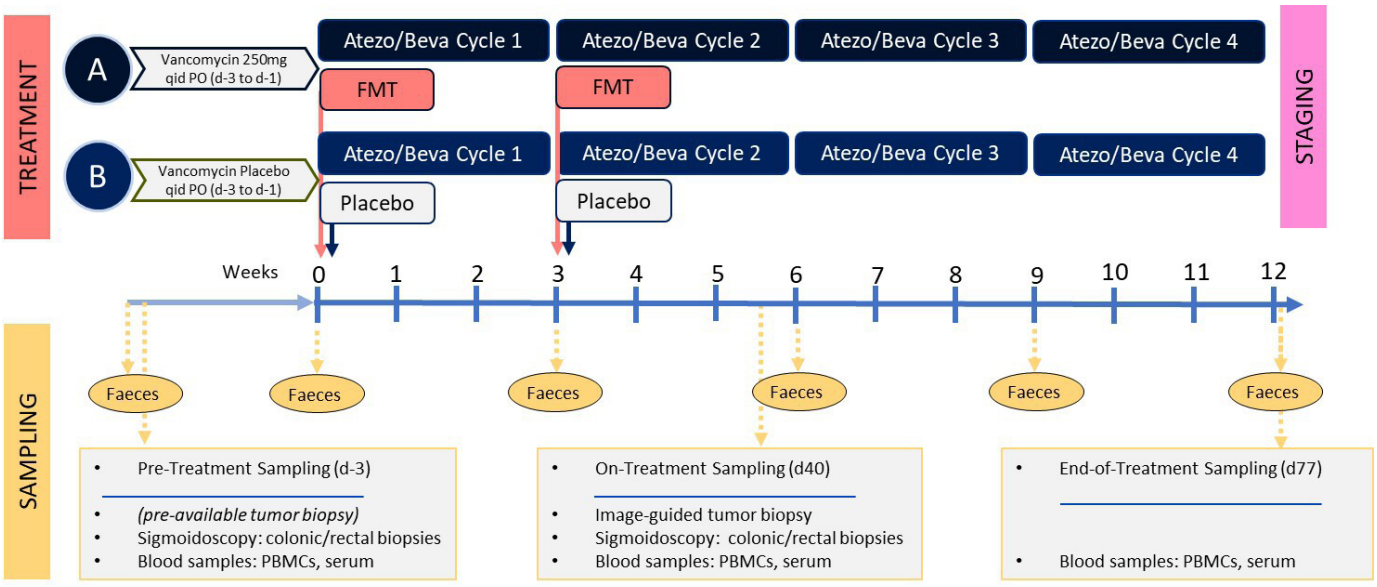

Figure 3 Sampling schedule. Faecal samples will be collected prior to treatment initiation and at each cycle of immunotherapy throughout the trial period. Pretreatment tumour tissue will be obtained from archival material, and an additional tumour sample will be collected during treatment. Intestinal tissue and blood samples will be collected at baseline (pretreatment) and during treatment, with the on-treatment collection occurring after the second cycle of immunotherapy. For blood samples, an additional collection will be performed at the end of treatment. FMT, faecal microbiota transfer.

1200 mg intravenous+bevacizumab 15 mg/kg bw intravenous alone will be continued on days 42 and 63 (figures 1 and 2).

Control group (Arm B)

Vancomycin-placebo p.o. is given four times daily from day 3 to day 1. Then, atezolizumab 1200 mg intravenous+bevacizumab 15 mg/kg bw intravenous+15 INTESTIFIX 001-placebo capsules p.o. will be administered on day 0 and day 21, then atezolizumab 1200 mg intravenous+bevacizumab 15 mg/kg bw intravenous alone will be continued on days 42 and 63 (figures 1 and 2).

Sampling

Pretreatment (d-7 to d-3) sampling consists of colon biopsies by sigmoidoscopy, blood and faecal samples (figure 3). Analysis of liver tumour biopsy will be included, if previously performed during diagnostic work-up, but is not required. On-treatment sampling (d35-41) consists of a percutaneous tumour biopsy and colon biopsies by sigmoidoscopy and blood samples (figure 3). Additional faecal samples will be collected by the participants at home after every treatment visit and dispatched by mail (figure 3). Routine laboratory work-up will be conducted at every treatment visit. Routine radiologic staging including blood and faecal sampling is scheduled after 4 cycles of A/B for d77±3 (figures 2 and 3). Subjects will be asked for consent to use biological specimens and clinical data for biobanking ("Secondary Use of Pseudonymized Study Data for Medical Research in the NCT Network"). Measures are in place to comply with the applicable rules for collection, biobanking and future use of biological samples and clinical data. In particular, sample and data usage have to be in accordance with the separate biobanking informed consent. Samples collected in this trial may be stored for up to 30 years (or according to local regulations) for additional research. Samples will be used to understand factors of the host (immune system, microbiome) in the context of systemic treatment of HCC. The research may begin at any time during the trial or the post-trial storage period.

Follow-up

Participants alive at the end of the study will undergo a 3-monthly survival follow-up for a minimum of 12 months after starting INTESTIFIX 001. Treatment choice after the end of the study is at the investigator's discretion (figure 2).

Discontinuation and early termination

Any subject can withdraw from the treatment (whole trial or parts, for example, withdrawal of consent to tumour biopsy) or the clinical trial verbally or in writing at any time without personal disadvantages and without having to give a reason. Further standard of care treatment will not be affected by this decision. The investigator can also discontinue the trial treatment after considering the risk-to-benefit ratio, if he/she no longer considers the treatment justifiable. In all subjects who discontinue the trial treatment, the end of treatment visit should be completed whenever possible.

Audits and inspections

Representatives of the sponsor may visit the trial site at any time during or after completion of the trial to audit compliance with applicable regulatory requirements and sponsor policies. Similarly, officials of the responsible authorities may carry out inspections either as part

of a national GCP compliance programme or to review the trial results in support of a regulatory submission. Both audits and inspections will require access to all trial records and source documents. The investigator and site personnel must be available for consultation during site audits/inspections. An audit of the vendor of INTESTIFIX 001 has been initiated by the sponsor.

Safety

Safety will be observed as from the first administration of study medication until 4 weeks after end of treatment. The end of safety follow-up determines the End of Study for the individual subject. All subjects who have reportable adverse events, whether considered associated with the use of the clinical trial medication or not, will be monitored to determine the outcome.

Patient and public involvement

Patients representatives are involved in planning and conduct of the study and will be involved in the reporting and dissemination plans of this research.

Data protection, collection and management

The data obtained in the course of the clinical trial will be treated pursuant to the EU General Data Protection Regulation (GDPR) and national regulatory requirements for example, Bundesdatenschutzgesetz (BDSG). To ensure confidentiality of records and personal data, only pseudonymised data will be transferred to the sponsor by using a subject identification number instead of the subject's name. In order to meet regulatory requirements (Guidance for Computerized Systems Used in Clinical Trials, International Conference on Harmonisation, GCP 2001/20/CE), eCRF design, data monitoring and database extractions will be performed with the REDCap secure web application. A data monitoring committee will not be established for the following reasons: used IMPs are generally considered safe, have been approved or are in clinical practice already for other indications; treatment with IMPs covers a short time period; and no interim analysis is planned for this trial.

Ethics and dissemination

The study was approved by ethics committee review and the German Federal Ministry of Drugs and Medical Devices. The trial is registered under EU CT no. 2023-506887-15-00. The responsible ethics committee was the "Ethik-Kommission an der Medizinischen Fakultät der Eberhard-Karls-Universität und am Universitätsklinikum Tübingen, Gartenstraße 47, 72074 Tübingen, Germany": The ethics protocol was processed under the reference number B_01099. Findings from this study will be published in peer-reviewed journals and presented at relevant scientific conferences. Involvement of professional writers is not planned. We will make data supporting the results available upon reasonable request, in accordance with BMJ Open's data sharing policy.

Statistical analysis

Description of the primary endpoint analysis and population

The primary immunological endpoint will be analysed for all patients for whom the endpoint could be observed, grouped as randomised (modified intention-to-treat set). Patients whose endpoint could not be measured due to contraindications to/refusal of biopsy, or death will be omitted from the immunological analysis. These events are considered independent of the intervention and hence, the omission is not expected to introduce bias. Per-group means and SDs of absolute lymphocyte counts and of changes to baseline will be provided. 95% CIs of the between-group difference of changes to baseline will be calculated.

The primary safety endpoint will be analysed for all patients with valid informed consent who received the trial medication at least once, grouped as treated. Different AE-related summary variables (total number of AEs, AEs per subject, time to first AE, …) will be described using absolute and relative frequencies/means and SDs and CIs. The description will be categorised by grade, relatedness to study treatment, outcome and change in medication.

Sensitivity and subgroup analyses

As a sensitivity analysis of potential centre-specific effects, a mixed linear model of the immunological endpoint will be fit with fixed effects group and baseline CD8+ T cell count and random effect centre. Furthermore, the primary analysis will be repeated for the subgroup of subjects who did not require antibiotics.

Secondary endpoints

Depending on the type of endpoint, absolute and relative frequencies/means and SDs/medians and quantiles will be provided per group together with CIs and descriptive p values.

The planned analyses will be described in full detail in a statistical analysis plan which will be finalised at the latest before database closure.

Study timeline

Study start occurred on June 18, 2025. The recruitment period is planned to last for 3 years, with a 12-month follow-up period for the last patient enrolled. As a result, the anticipated completion date for the study is 17 June 2029, at the latest.

Implications

The FLORA trial aims to build on existing knowledge derived from studies, demonstrating that FMT can potentially enhance responsiveness to ICI in melanoma and apply it to advanced HCC.[3–5] The FLORA trial will adopt a double-blind randomised controlled trial design, which is the current gold standard for clinical testing. This approach will significantly enhance the quality of evidence obtained compared with previous studies, which lacked an active comparator and blinding. To address concerns regarding the limited

and inconsistent availability of elite donor FMT, we will employ a Good Manufacturing Practices (GMP)-certified FMT compound, INTESTIFIX 001. This choice of IMP is intended to ensure greater feasibility in routine clinical settings and to mitigate the inherent risk of infection associated with FMT. One potential limitation of the study is the focus on CD8+ T cell infiltration within tumour tissue following treatment with INTESTIFIX 001+A/B vs placebo+A/B. CD8+ T cell infiltration is a well-established histological feature that correlates with ICI responsiveness, displaying a high degree of biological plausibility.[24 25] Intratumoural pretreatment CD8+ T cell density was associated with better clinical outcomes in clinical trials for immunotherapy in HCC, including A/B (GO30140 phase 1b and IMbrave150 phase 3).[26–28] In addition to serving as a surrogate marker for response, CD8+ T cell infiltration in tumour tissue after treatment initiation may offer valuable mechanistic insights.[29]

In summary, we anticipate that the FLORA trial will yield high-quality clinical and translational-mechanistic insights regarding the potential of add-on FMT treatment to enhance ICI efficacy in advanced HCC.

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Acknowledgements We would like to thank Tobias Krämer, Nina Roetz and Christiane Miesel-Gröschel from KKS Heidelberg for their support in writing the protocol.

Contributors CR, MPR and MTD drafted the manuscript and accounted for all aspects of the work, contributing equally to the study. AS and LDS contributed to design of the study and planned outcome measures. MJGTV and AT contributed to the study design in the context of FMT intervention. All authors contributed editorially to the manuscript and read and approved of the submitted version. CR is the guarantor.

Funding This work was supported by the German NCT Proof-of-concept (PoC) Trial Program and the German NCT Overarching Clinical Translational Trial (OCT2) Program.

Competing interests AK receives grants from Bayrische Forschungsstiftung, Bayrisches Zentrum für Krebsforschung, payment for honoraria lectures from Janssen-Cilag, Fujifilm, AbbVie, Boston-Scientific, AastraZeneca, MSD Sharp, Roche, Bayer Vital, support for attending meetings from Janssen-Cilag, AbbVie, Lilly. CR honoraria lectures for BMS, support for attending meetings from AbbVie. CS advisory board member for AstraZeneca, BMS, Incyte, MSD, Roche, Servier, Taiho and honoraria lectures for Astrazeneca, Servier. DJ consulting fees from CureVac AG, VAXIMM AG, F. Hoffmann-La Roche Ltd, Oncolyticcs Biotech, Genmab A-S, Definiens, OncoOne Research&Development Research GmbH, honoraria lectures for SEK Kliniken Heilbronn, Terrapin, BMS, Touch Medical Media, ForTra GmbH, BCPPC, Roche Pharma, Georg Thieme verlag, MSD, BMS, Gruppe 4 Filmproduktion, Norwegian Cancer Society, Astra Zeneca, payment for expert testimony for Else-Kröner-Fresenius-Stiftung, Wilhelm-Sander-Stiftung, NordForsk, Schering Stiftung, support for attending meetings for Amgen, Oryx, Roche, Parexel, BMS, Berlin Institute of health, Aey Congresse GmbH, bioevents congress, Oncogenics InC, Falconfon Corp, participation on advisory boards for CureVac, Deiniens, F. Hoffmann-La Roche Ltd, Genmab, Life Science Inkubator GmbH, VAXIMM AG, OncoOne research, Oncolytics, leadership or fiduciary role for BMS Stiftung Immunonkologie. LJ honoraria lectures for Boston Scientific, AstraZeneca, Roche; participation data safety Bboard Boston scientific, AstraZenecam Roche. MTD honoraria lectures for Roche, travel support from AstraZeneca, Falk Foundation, participation data safety boards for AstraZeneca, Eisai. MJGTV receives grants form MSD, Heel, BioNTech, Roche, SD Biosensor, Tillots Pharma, consulting fees from Ferring, Tillots Pharma, Bioaster, honoraria lectures for Akademie für Ärztliche Fort- und Weiterbildung, Akadmie für Infektionsmedizin, Astra Zeneca, bioMerieux, DGI, EUMEDICA, European Society of Neurogastroenterology, Ferring, FomF GmbH, Förderkreis Malteser, Frankfurter Bürger Universität, GILEAD, GSK, Helios Kliniken, Hessisches Landessozialgericht, Janssen Cilag GmbH, Jörg Eikerle Beratung, Klinikum Leverkusen, Lahn-Dill Kliniken, Landesärztekammer Hessen, LMU Kliniken, Med. Gesellschaft Bad Homburg, MSD, Pfizer, St. Vincent Hospital, Tillotts, Falk Foundation. PM receives consulting fees from AstraZeneca, honoraria lectures for Falk, AstraZeneca, Roche. TE honoraria lectures from AstraZeneca, Roche, participation on data monitoring boards for AstraZeneca, BMS. All other authors declared no conflict of interest.

Patient and public involvement Patients and/or the public were involved in the design, or conduct, or reporting, or dissemination plans of this research. Refer to the Methods section for further details.

Patient consent for publication Not applicable.

Provenance and peer review Not commissioned; externally peer reviewed.

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