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ORIGINAL ARTICLE

First-in-class Microbial Ecosystem Therapeutic 4 (MET4) in combination with immune checkpoint inhibitors in patients with advanced solid tumors (MET4-IO trial)

A. Spreafico[1,2*], A. A. Heirali[3], D. V. Araujo[1,4], T. J. Tan[1,5], M. Oliva[1,6,7], P. H. H. Schneeberger[3,8,9], B. Chen[10], M. K. Wong[11], L.-A. Stayner[1], A. R. Hansen[1], S. D. Saibil[1], B. X. Wang[2], K. Cochrane[12], K. Sherriff[12], E. Allen-Vercoe[12], W. Xu[10], L. L. Siu[1,2] & B. Coburn[3*]

[1]Division of Medical Oncology and Hematology, Princess Margaret Cancer Centre, University Health Network, Toronto; [2]Tumor Immunotherapy Program, Princess Margaret Cancer Centre, University Health Network, Toronto; [3]Toronto General Hospital Research Institute, University Health Network, Toronto, Canada; [4]Department of Medical Oncology, Hospital de Base, Sao Paulo, Brazil; [5]Division of Medical Oncology, National Cancer Centre Singapore, Singapore, Singapore; [6]Department of Medical Oncology, Institut Catala d' Oncologia, L'Hospitalet de Llobregat, Barcelona; [7]Universitat de Barcelona, Barcelona, Spain; [8]Swiss Tropical and Public Health Institute, Department of Medical Parasitology and Infection Biology, Allschwil; [9]University of Basel, Basel, Switzerland; [10]Biostatistics Department, Princess Margaret Cancer Centre, University Health Network, Toronto; [11]Department of Immunology, University of Toronto, Toronto; [12]Nubiyota LLP, Guelph, Canada

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Background: The intestinal microbiome has been associated with response to immune checkpoint inhibitors (ICIs) in humans and causally implicated in ICI responsiveness in animal models. Two recent human trials demonstrated that fecal microbiota transplant (FMT) from ICI responders can rescue ICI responses in refractory melanoma, but FMT has specific limitations to scaled use.

Patients and methods: We conducted an early-phase clinical trial of a cultivated, orally delivered 30-species microbial consortium (Microbial Ecosystem Therapeutic 4, MET4) designed for co-administration with ICIs as an alternative to FMT and assessed safety, tolerability and ecological responses in patients with advanced solid tumors.

Results: The trial achieved its primary safety and tolerability outcomes. There were no statistically significant differences in the primary ecological outcomes; however, differences in MET4 species relative abundance were evident after randomization that varied by patient and species. Increases in the relative abundance of several MET4 taxa, including *Enterococcus* and *Bifidobacterium*, taxa previously associated with ICI responsiveness, were observed and MET4 engraftment was associated with decreases in plasma and stool primary bile acids.

Conclusions: This trial is the first report of the use of a microbial consortium as an alternative to FMT in advanced cancer patients receiving ICI and the results justify the further development of microbial consortia as a therapeutic co-intervention for ICI treatment in cancer.

Key words: intestinal microbiome, first in class microbial ecosystem therapeutic 4, immune checkpoint inhibitors, advanced solid tumors

INTRODUCTION

The composition of the human intestinal microbiome is implicated in response to immune checkpoint inhibitor (ICI) treatment in cancer,[1-3] and consequently a target for therapeutic augmentation.[4] Fecal microbiota transplantation (FMT) is now under investigation as a co-therapy designed to augment ICI responses in multiple trials registered on ClinicalTrials.gov, including two trials with published results demonstrating rescue of ICI non-response with FMT in melanoma,[5,6] indicating a broad interest in this new modality. However, FMT has practical limitations affecting its generalizability, safety and appropriateness for use at scale.[7] Microbial consortia (multi-species mixtures of cultivated microbes) represent an intermediate approach intended to balance the ecological and functional complexity of FMT and the practical advantages of cultivated microbes, and have been successfully used as alternatives to FMT for other indications such as *Clostridioides*

*Correspondence to: Dr Anna Spreafico, Division of Medical Oncology & Hematology, Princess Margaret Cancer Centre, University Health Network, 700 University Avenue, Suite 7-621, Toronto, ON M5G 1Z5, Canada. Tel: +416-946-4501; ext: 5083

E-mail: anna.spreafico@uhn.ca (A. Spreafico).

*Dr Bryan Coburn, Division of Infectious Diseases, University Health Network, 101 College St, 10-358, Toronto, ON M5G 1L7, Canada. Tel: +416-634-7457

E-mail: Bryan.Coburn@uhn.ca (B. Coburn).

difficile infection,[8,9] including in a phase III trial in which efficacy similar to FMT was reported.[10]

Microbial Ecosystem Therapeutic 4 (MET4) is an orally delivered defined mixture of pure live cultures of intestinal bacteria isolated from the stool of a healthy donor, purified and grown in conditions modeling those of the human distal gut.[8] MET4 is composed of 30 phylogenetically and functionally diverse bacterial species including taxa previously associated with ICI responsiveness in published reports (Supplementary Table S1, available at https://doi.org/10.1016/j.annonc.2023.02.011). MET4 is cultured *in vitro* and each strain is individually characterized genotypically and phenotypically, including for antimicrobial susceptibilities. MET4-IO is a single-center investigator-initiated clinical trial designed to evaluate the safety, tolerability and engraftment of MET4 in patients with advanced solid tumors receiving ICI. This study included a safety cohort (group A) and two additional cohorts of ICI-naïve (group B) or pre-exposed (group C) patients, randomized to receive either standard-of-care ICI alone or in combination with MET4 (NCT03686202).

PATIENTS AND METHODS

Patient population

Adult patients with advanced solid malignancies with an Eastern Cooperative Oncology Group performance status of 0-2, able to swallow and receiving (groups A and C) or planned to receive (group B) standard-of-care anti-programmed cell death protein 1 (PD-1) monotherapy or anti-PD-1 plus anti-cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) combination immunotherapy were included in the study. Multiple tumor types were enrolled. Additional eligibility criteria included measurable disease by computed tomography or magnetic resonance imaging as per RECIST v1.1 and willingness to undergo serial collection of blood and stool samples. Gastrointestinal disorders likely to interfere with absorption and prior treatment with immune checkpoint blockade in group B were key exclusion criteria (full protocol in Supplementary Material, available at https://doi.org/10.1016/j.annonc.2023.02.011).

Study design and treatment

This single-center, open-label, investigator-initiated study initially included three cohorts of patients (groups A, B and C). In group A (safety cohort), MET4 was added to standard-of-care anti-PD-1 antibody until unacceptable toxicity or progression. Upon completion of group A, groups B and C were opened to enrollment. In group B, eligible subjects with advanced solid tumors naïve to ICI were randomized in a 3 : 1 ratio to receive MET4 in combination with ICI (experimental arm) or ICI alone (control arm) with a run-in period of ICI therapy (one cycle). In group B, patients could be treated beyond progression provided they had a clinical benefit without clinical deterioration and did not have substantial adverse effects, as assessed by the investigator. In group C, eligible subjects with advanced solid tumors already on treatment with standard-of-care ICI with first unconfirmed progression on evaluation scans, clinically stable and suitable to be treated beyond progression as per investigator's assessment were randomized in a 1 : 1 ratio to receive MET4 in addition to ICI inhibitor (experimental arm) or continue with ICI alone (control arm) (Supplementary Figure S1A, available at https://doi.org/10.1016/j.annonc.2023.02.011). The protocol was amended to include group D, designed to evaluate MET4 in high-risk melanoma patients on adjuvant immunotherapy. Results of group D will be reported separately once accrual is completed.

In groups A, B and C, MET4 capsules were administered orally with an initial loading dose of 20 capsules (2-10 $\times$ 10^{10} colony-forming units) over 2 days, followed by a maintenance dose of 3 capsules (6-30 $\times$ 10^{9} colony-forming units) continuous daily dosing for a total of 1 year or until unacceptable toxicity, progression of disease or discontinuation of treatment for any cause. Standard-of-care ICI was dependent on tumor type and included single-agent nivolumab (480 mg flat dose q4w), or pembrolizumab (200 mg flat dose q3w), or nivolumab (360 mg flat dose q3w) in combination with ipilimumab (at either 3 mg/kg q3w or 1 mg/kg q3w for up to four infusions) followed by maintenance nivolumab at 480 mg flat dose q4w as per standard of care, until unacceptable toxicity, disease progression, completion of therapy based on approval indication or discontinuation for any cause.

Sample collection

In groups A and C, stool samples were collected before initiation of MET4 (screening visit: T0); day 10-16 from initiation of MET4 (T1); week 3-4 (window: $+2$ weeks) from MET4 initiation (T2); week 24 (window: ± 2 weeks) from MET4 initiation (T3); and 1-2 weeks post-end of treatment (EOT) (T4). Blood samples were collected at T0, T2 and T3, based on the same timepoint definitions.

In group B, stool samples were collected before initiation of ICI (T -1); week 3-4 post-ICI, before initiation of MET4 (window: $+2$ weeks) (T0); day 10-16 from initiation of MET4 (T1); week 3-4 (window: $+2$ weeks) from MET4 initiation (T2), week 24 (window: ± 2 weeks) from MET4 initiation (T3); and 1-2 weeks post-EOT (T4). Blood samples were collected at baseline T -1, T0, T2 and T3, based on the same timepoint definitions. Study design and timeline of sample collection are summarized in Supplementary Figure S1B, available at https://doi.org/10.1016/j.annonc.2023.02.011.

Outcome measures

Primary endpoints included cumulative relative abundance of MET4 taxa at T1, changes in relative abundance of MET4 taxa between T0 and T1 and treatment-related adverse events (AEs) assessed by the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE) v5.0. For the ecological co-primary endpoint, patients were considered assessable if stool samples were obtained at T0 and T1 (a total of two stool samples in groups A and C and three stool samples in group B).

Secondary endpoints included cumulative relative abundances of MET4 taxa at T2-T4, changes in relative abundance of MET4 taxa between baseline (T0) and post-randomization timepoints and bacterial taxonomic diversity between T0 and T2-T4. Exploratory outcome measures included overall response rate measured as per RECIST v1.1 and immune RECIST (iRECIST).

Study assessments

Response assessments were defined according to RECIST v1.1. Time of assessment was based on investigator evaluation and tumor type and typically occurred every 2-3 cycles of immunotherapy until disease progression or treatment discontinuation. Patients treated beyond progression were considered to have progressive disease at the time of the initial progression event, as assessed by the investigator, regardless of subsequent tumor response. Any patient who received at least one dose of MET4 was included in the assessment of safety. Patients were required to complete a study diary to assess appropriate dosing and study compliance. Reason for any missed doses of MET4 was recorded. AEs attributable to immunotherapy and MET4 were graded according to the NCI-CTCAE v5.0. Safety assessments were carried out continuously during treatment, and up to resolution or stabilization of the AEs, whichever occurred first.

Study oversight

The study protocol (Supplementary Material, available at https://doi.org/10.1016/j.annonc.2023.02.011) and all the related amendments were approved by the Institutional Review Ethics Board. The study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice as defined by the International Conference on Harmonization. Before enrollment, all patients provided written informed consent. Established bi-weekly safety calls occurred to provide oversight of safety. Data collection and monitoring were carried out throughout the study and after enrollment was completed. Monitoring of study conduct, including all AEs, was carried out by the Princess Margaret Cancer Centre Data Safety Monitoring Committee twice a year and as needed.

Microbiome analysis

DNA was extracted from the patients' frozen fecal material using the Quick-DNA Fecal/Soil Microbe Kits (Zymo Research, Irvine, CA) and normalized by stool weight. Library generation and next generation sequencing were done at MR DNA Molecular Research (Shallowater, TX). The 16S ribosomal RNA gene V4 variable region was amplified with PCR using primers 515F (GTGYCAGCMGCCGCGGTTA) and 806R (GGACTACNVGGGTWTCTAAT), with the barcode on the forward primer, and HotStarTaq Plus Master Mix Kit (Qiagen, Germantown, MD). PCR consisted of 30 cycles of 94°C for 3 min, then 30-35 cycles of 94°C for 30 s, 53°C for 40 s and 72°C for 60 s, and a final elongation step at 72°C for 5 min. After amplification, PCR products were resolved by electrophoresis on a 2% agarose gel to determine amplification and relative band intensity. Multiple samples were pooled in equal proportions, on the basis of their molecular weight and DNA concentrations and purified with calibrated AMPure XP beads (Beckman Coulter, Brea, CA). Pooled and purified PCR product was used to prepare an Illumina (San Diego, CA) Nextera DNA library. Sequencing was done by MR DNA using an Illumina MiSeq with version 3 reagents and generating 300-bp paired-end reads. Reads in which >70% of bases had a Phred score of 30 or more were retained and trimmed using DADA2 (v1.14.1). Taxonomy was assigned with a native implementation of the naïve Bayesian classifier method and trained with the Silva database (v132). Amplicon sequence variants were assigned and collated to the closest related taxon using NCBI BLAST.

Targeted metabolomics

Plasma and stool samples were sent to The Metabolomics Innovation Centre (TMIC) (Edmonton, Alberta, Canada) for targeted metabolomic profiling using liquid chromatography-tandem mass spectrometry as described in the Supplementary Methods, available at https://doi.org/10.1016/j.annonc.2023.02.011. Samples were profiled for panels of bile acids (BAs) and short-chain fatty acids (SCFAs). Analytes were included in statistical analyses if they were detectable in at least 40% of samples.

Statistical analysis

Frequencies of immune-related AEs (irAEs) between MET4 recipients and controls, and single versus combination therapy ICI were compared by chi-square test. Ecological outcomes (MET4 relative abundance, change from baseline, number of taxa >1%, Shannon diversity and observed operational taxonomic units) were compared between MET4 recipients and controls as continuous variables with unpaired *t*-tests or analysis of variance (ANOVA) with post-tests. For alpha diversity metrics, samples were rarefied to a sequencing depth of 35 501 reads (the lowest depth among all the samples included in the analysis). Rarefied and unrarefied analyses were carried out and compared. Fold change in relative abundance between baseline samples (pre-MET4) and post-MET4/control exposure timepoints was generated by dividing post-treatment relative abundance by the baseline relative abundance and log transforming the resulting fold change, and then using one-sample *t*-tests to compare the distribution of these values to a 'no change' reference value of 0. Volcano plots for changes in relative abundance in taxa after randomization between MET4 recipients and controls were generated by using MaAsLin2 with study participant included as a random effect. Compositional differences in Bray—Curtis dissimilarity were plotted on principal coordinate analysis plots and compared by permutational multivariate analysis of variance (PERMANOVA).

Concentrations of metabolites were compared across sampling timepoints for MET4-treated and control randomized individuals using ANOVA with log10 transformation when appropriate. Log2-fold change (L2FC) in

metabolite concentration was calculated by dividing T2 (post-MET4) metabolite concentrations by T0 (baseline, pre-MET4) metabolite concentration and log2 transforming the data. Patients were defined as ecological responders (EcoRs) if they had at least five MET4 taxa increasing by at least log10 post-MET4 initiation. Differences between L2FC in metabolites were compared for MET4-treated patients in EcoRs and ecological non-responders (EcoNRs) by ANOVA.

All analyses were carried out in GraphPad Prism or R (San Diego, CA).

RESULTS

Study patient population

Between December 2018 and December 2020, 40 patients receiving standard-of-care monotherapy or combination ICI were enrolled. The trial profile, total population and assessable subjects are summarized in Supplementary Figure S1, available at https://doi.org/10.1016/j.annonc.2023.02.011. In an initial safety cohort (group A, $n = 6$), one subject was enrolled and received one cycle of anti-PD-1 antibody within the trial. However, due to rapid disease progression, the patient never started MET4 and was replaced, for a total of five assessable patients. In group B ($n = 30$), patients were randomized 3 : 1 to the experimental arm (ICI plus MET4, $n = 22$) or control arm (ICI, $n = 8$). Accrual in group C ($n = 4$) was discontinued before enrollment was complete, due to the limited number of pseudo-progression events in patients receiving ICI, clinical deterioration at the time of disease progression and alternative treatment opportunities as a preferred strategy by both patient and physician. Baseline demographics, disease characteristics and number of previous lines of therapy are presented in Supplementary Table S2, available at https://doi.org/10.1016/j.annonc.2023.02.011. Head and neck squamous cell carcinoma (HNSCC) ($n = 20$) and melanoma ($n = 16$) were the most common tumor types. All patients (cohorts A, B and C) received anti-PD-1 antibodies and 13 (33%) received anti-PD-1 and anti-CTLA-4 antibodies in combination. Twenty-six patients ($n = 5$ in group A, $n = 19$ in group B and $n = 2$ in group C) received at least one dose of oral MET4 in combination with ICI. Patient characteristics according to HNSCC and melanoma tumor types are summarized in Supplementary Tables S3 and S4, available at https://doi.org/10.1016/j.annonc.2023.02.011, respectively.

Median follow-up duration, defined as the time from enrollment (for group A) and randomization (for groups B and C) to data cut-off (22 May 2021) or last follow-up, whichever occurred first, was 164 days (range 41-858 days) in group A, 104 days (range12-666 days) in group B and 125.5 days (range 74-259 days) in group C. Median MET4 duration of treatment was 38 days (range 0-334 days) excluding missed doses. At the time of analysis at data cut-off, three patients remained in follow-up (one in group A and two in group B) and five patients (group B) remained on treatment, three of them being in the experimental arm with MET4.

MET4 was safe and tolerable in standard-of-care ICI recipients

In total, 39 patients received at least one cycle of ICI and were assessable for safety analysis. The ICI-related AEs observed in our patient population were consistent with the literature with a higher frequency of severe AEs in the patients receiving anti-PD-1 with anti-CTLA-4 antibody combination (10 grade 3-4 AEs in 13 patients, 77%) as compared to single-agent anti-PD-1 antibody (6 grade 3-4 AEs in 26 patients, 23%) (Figure 1A, Supplementary Table S5, available at https://doi.org/10.1016/j.annonc.2023.02.011). There were no statistically significant differences between the MET4 and control groups with respect to the number of irAEs of any grade or grade ≥ 3 only (Figure 1B). Of the 26 patients (5 in group A, 19 in group B and 2 in group C) who received at least one dose of MET4, 10 of them were treated with anti-PD-1/anti-CTLA-4 antibody combination. Within groups A, B and C, a total of 29 patients were assigned to receive MET4 either in combination with anti-PD-1 or anti-PD-1 and anti-CTLA-4. MET4-attributed AEs occurred in 17% (5/29) of patients, were mainly gastrointestinal, mild/moderate in severity (grade 1-2) and all resolved without sequelae. No MET4-related grade ≥ 3 AEs were observed in the study population.

Treatment outcomes in MET4 recipients and controls

RECIST v1.1 best treatment response in all groups is summarized in Supplementary Table S6, available at https://doi.org/10.1016/j.annonc.2023.02.011. In the entire cohort, there were 2 patients with complete responses (1 control, 1 MET4), 7 with partial responses (PRs) (all MET4 recipients), 9 with stable disease (SD) (3 control and 6 MET4 recipients) and 17 with progressive disease (5 control, 12 MET4 recipients). Four patients were not assessable for response assessment. RECIST treatment responses for patients assessable for ecological primary outcomes and by tumor types are summarized in Supplementary Tables S7 and S8, available at https://doi.org/10.1016/j.annonc.2023.02.011, and Figure 2 (for cohort B only, which included ICI-naïve patients). The overall RECIST response rate for MET4 recipients in cohort B was 35% (6/17) versus 14% (1/7) in controls (Fisher's exact $P = 0.37$). Clinical benefit (patients with PR or SD ≥ 6 months) was observed in 53% (9/17) of MET4 recipients as compared to 20% (1/5) of patients in the control arm, $P = 0.18$. The clinical benefit could not be assessed in two of the seven patients in the control arm due to inadequate follow-up (<6 months).

MET4 treatment increased the number and relative abundance of administered taxa in a subset of recipients, but not across all recipients

A total of 147 stool samples were sequenced [113 from MET4 recipients and 34 from subjects treated with ICI alone (control)], of which 92 were collected after exposure to MET4 or the control intervention post-randomization (including all time points). A total of 30 patients [5 in group A, 21 in group B (15 in the experimental arm and 6 in

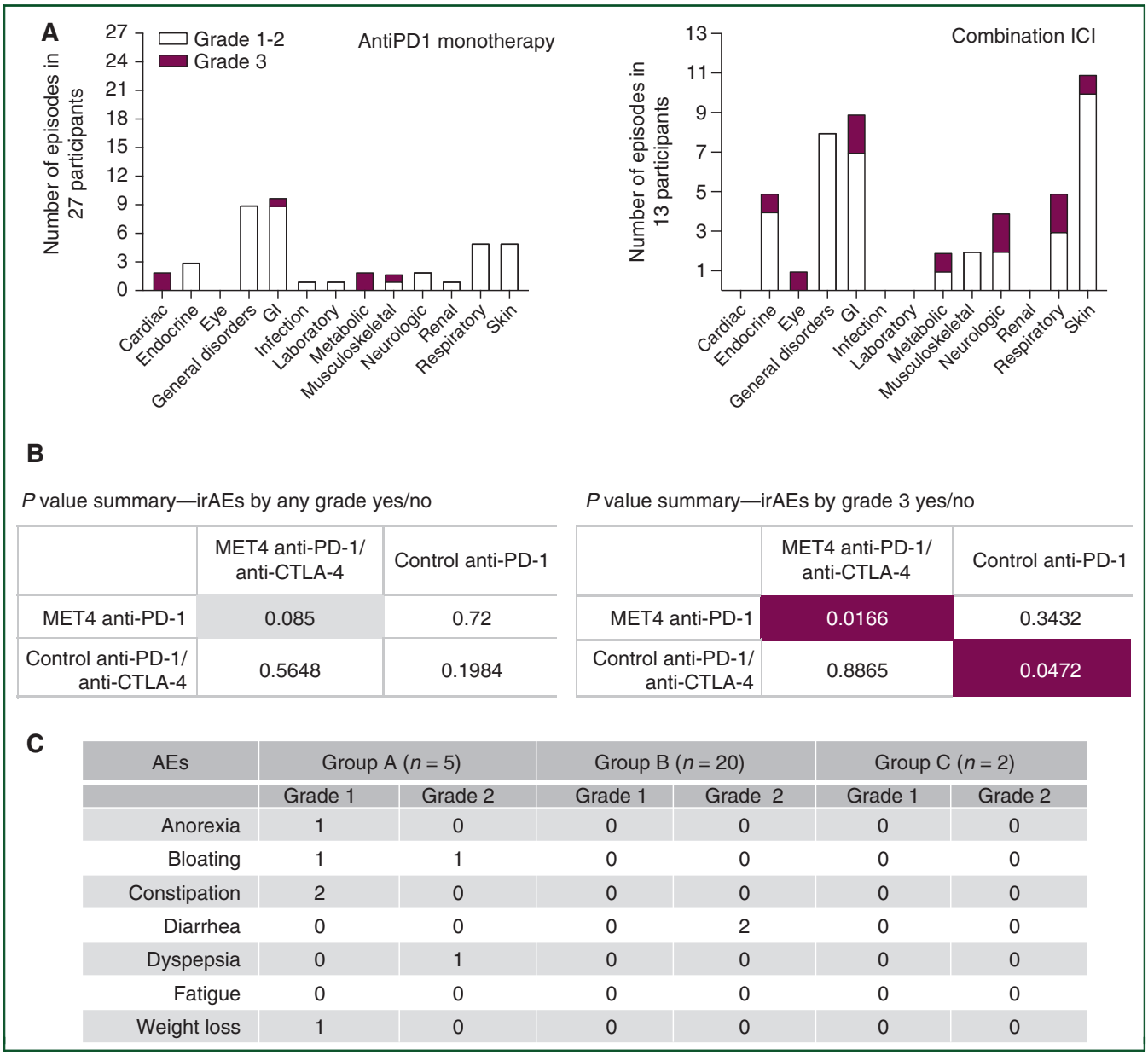

Figure 1. Immune-related and MET4-attributed AEs in the MET4-IO trial. (A) Type of irAE experienced by system for anti PD1 monotherapy and combination ICI recipients, colored by grade. Details of each system category are reported in Supplementary Table S5, available at https://doi.org/10.1016/j.annonc.2023.02.011. (B) Chi-square *P* values for comparison of irAEs between MET4 recipients and controls and combination versus single-agent ICI receipt for any AE (left) and grade 3 AEs only. (C) MET4-attributed AEs for MET4 recipients in groups A-C.

AEs, adverse events; CTLA-4, cytotoxic T-lymphocyte-associated antigen 4; GI, gastrointestinal; ICI, immune checkpoint inhibitor; irAE, immune-related adverse event; MET4, Microbial Ecosystem Therapeutic 4; PD-1, programmed cell death protein 1.

the control arm) and 4 in group C] were assessable for the ecological co-primary objective. Two patients in cohort B (B009 and B022) did not provide a stool sample at T1 window and were included only for safety/tolerability and ecological secondary outcomes. Two patients (B011 and B027) received only one dose of MET4 and were excluded from analysis of all ecological outcomes (Supplementary Figure S1, available at https://doi.org/10.1016/j.annonc.2023.02.011).

The trial ecological co-primary outcomes, the relative abundance of MET4 taxa at T1 (range day 10-16) and change in relative abundance of MET4 between T0 and T1 are shown in Figure 3A and B. The mean (±standard deviation) cumulative relative abundance of MET4 taxa on T1 in the MET4 group was 0.30 ± 0.13 versus 0.22 ± 0.11 in controls ($P = 0.098$). The mean change in relative abundance of MET4 taxa in the MET4 group was an increase of 0.033 ± 0.12 versus a decrease of 0.063 ± 0.10 in the control group ($P = 0.059$). Paired analysis of pre-/post-MET4 alpha diversity and cumulative relative abundance in the stool of MET4 recipients was not significantly different. There were no differences in the secondary ecological outcomes between MET4 recipients and controls (Figure 3C-F), including the cumulative relative abundance of MET4 taxa or change in cumulative relative abundance of MET4 taxa at later timepoints, or taxonomic Shannon

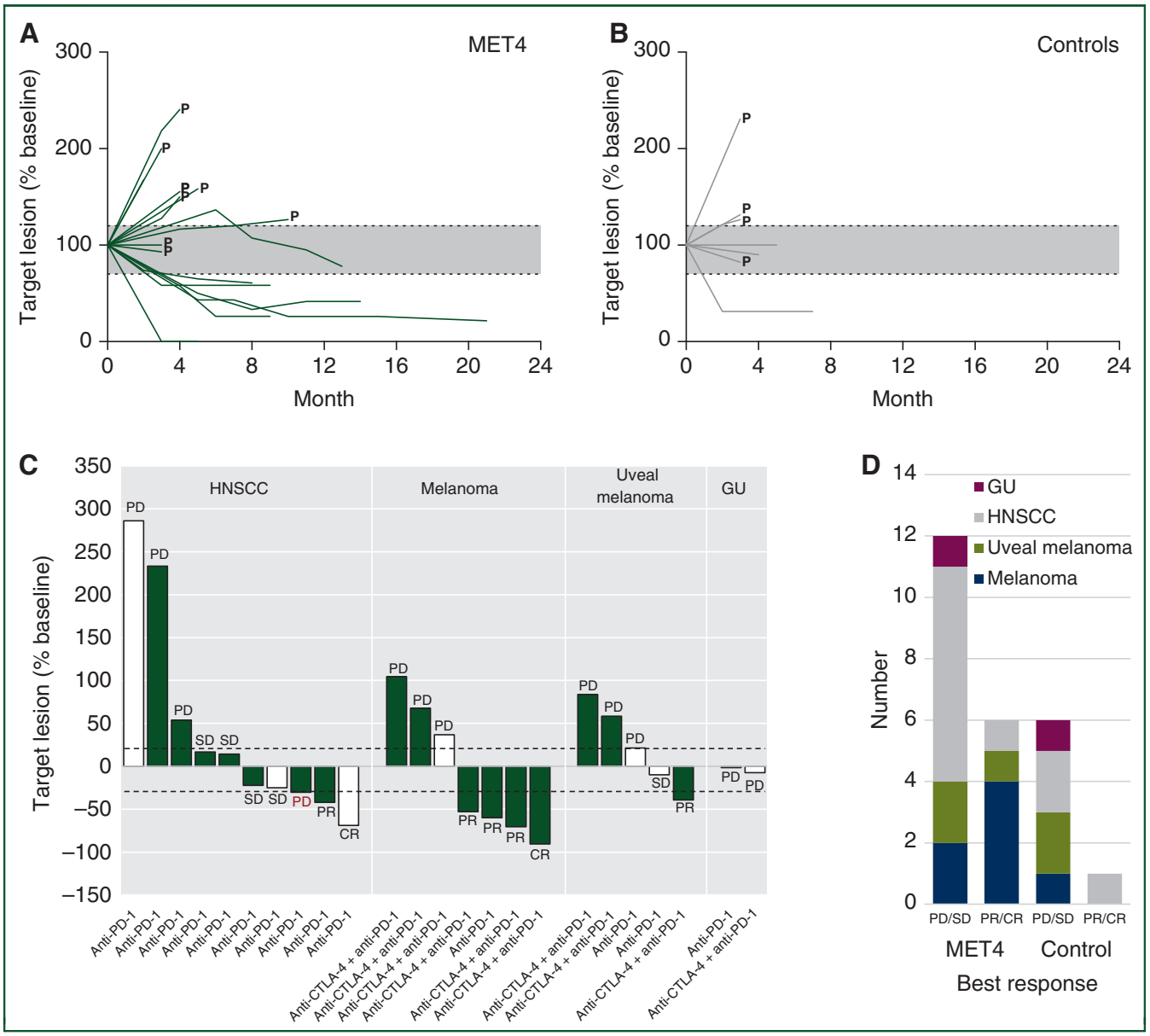

Figure 2. Therapeutic responses in study patients. (A) Patients' target lesion. Target lesion size as a percentage of baseline is shown in panels A (MET4 recipients) and B (controls, $n = 6$) for the study period for patients with evaluable tumors. *P* indicates unequivocal progression of a non-target lesion. (C) Waterfall plots indicate best response change in tumor size compared to baseline stratified by tumor type. RECIST response is indicated for each individual and bars are colored to indicate treatment allocation (green = MET4, white = control). Dashed lines indicate +20% or −30% growth for panels A-C. (D) Best RECIST response by tumor type and treatment allocation is indicated.

CR, complete response; CTLA-4, cytotoxic T-lymphocyte-associated antigen 4; GU, genitourinary cancer; HNSCC, head and neck squamous cell carcinoma; MET4, Microbial Ecosystem Therapeutic 4; PD, progressive disease; PD-1, programmed cell death protein 1; PR, partial response; SD, stable disease.

diversity (Figure 3E) or richness (observed taxa, Figure 3F) at any timepoint. Diversity indices were not different between rarefied and non-rarefied analyses (rarefied analysis presented). In an exploratory analysis, a greater number of MET4 taxa comprised >0.01 relative abundance in the MET4-treated group than in the control group at T1 (6.7 ± 2.8 versus 4.6 ± 1.9, $P = 0.035$, Supplementary Figure S2A, available at https://doi.org/10.1016/j.annonc.2023.02.011), and the number of MET4 taxa comprising at least 0.01 of the bacterial community following MET4 exposure was greater in MET4 recipients than in controls at T2 (6.7 ± 2.0 versus 4.4 ± 2.4, $P = 0.025$, Supplementary Figure S2B, available at https://doi.org/10.1016/j.annonc.2023.02.011). Fewer patients were assessable for these ecological measures at T3 and T4 and differences observed were not statistically significant.

Post-treatment changes in MET4 taxon relative abundance varied significantly by individual and taxon (Figures 4 and 5, Supplementary Figure S3, available at https://doi.org/10.1016/j.annonc.2023.02.011). In cohort B, 8 of 17 (47%) MET4 recipients had statistically significant increases in MET4 taxa in at least one post-treatment sample (defined as a one-sample *t*-test $P < 0.05$ compared to no change), while 3 (17.6%) had decreases in at least

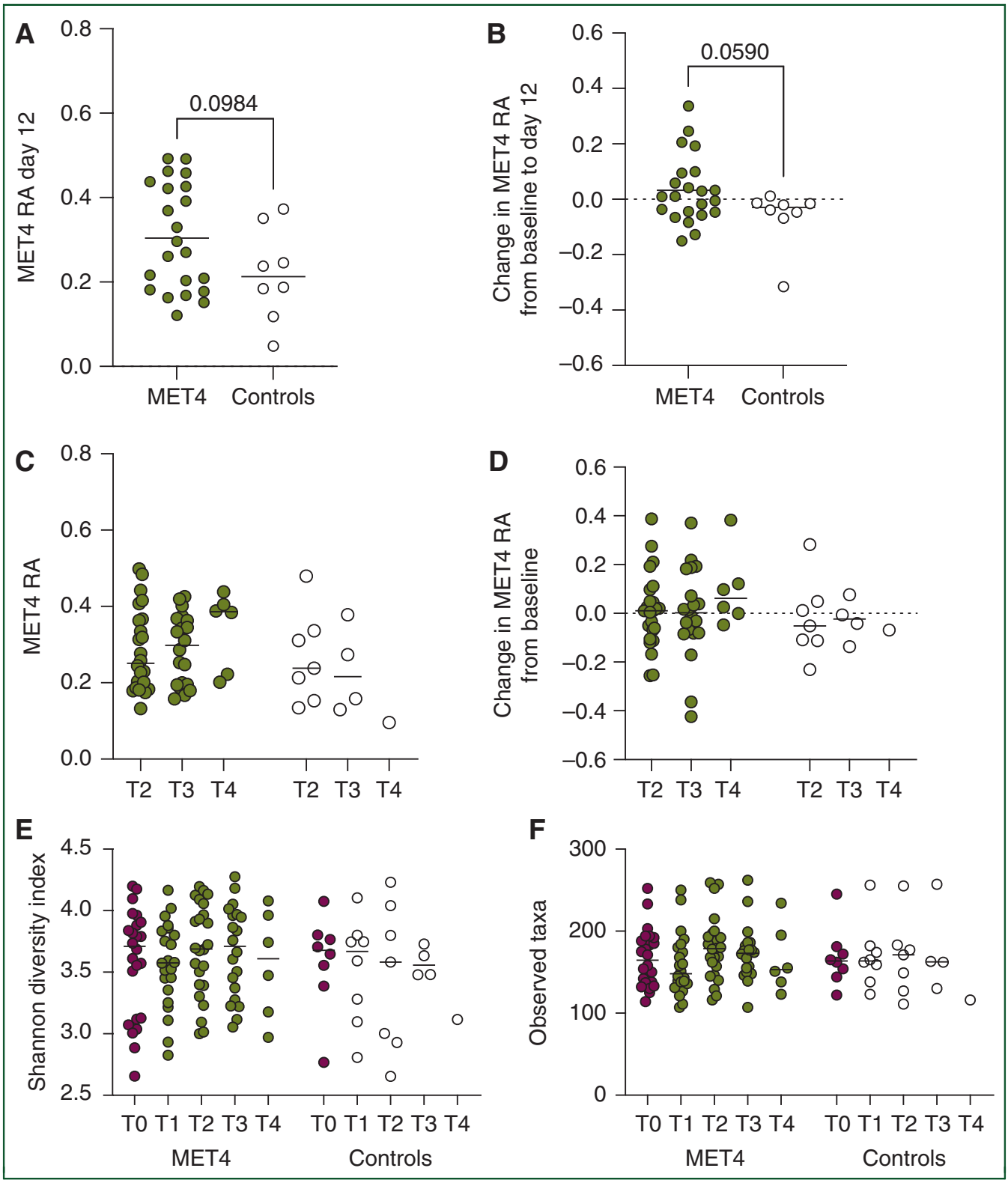

Figure 3. Ecological primary and secondary endpoints. Stool samples were collected at 3-4 weeks post-ICI/pre-MET4 (T0) and at four prespecified timepoints (day 12 post-MET4/T1, week 3-4 post-MET4/T2, week 24 post-MET4/T3 and at the end of therapy or 1 year/T4) after randomization to receive MET4 or standard-of-care ICI. 16S rRNA gene sequencing was used to determine: (A) cumulative RA of MET4 taxa and (B) change in cumulative MET4 RA. (C, D) Ecological outcomes at subsequent timepoints are shown. E, F The Shannon diversity index and observed taxa are shown for all timepoints for both groups and did not differ at any timepoint.
ICI, immune checkpoint inhibitor; MET4, Microbial Ecosystem Therapeutic 4; RA, relative abundance; rRNA, ribosomal RNA.

one sample, compared to none with increases and 3 (50%) with decreases in the controls. For cohort A, one patient had an increase and one patient had a decrease in MET4 taxa, and for cohort C, one MET4 recipient and two controls had a decrease. Notably, several individuals had >10-fold increases in multiple taxa, with increases in as many

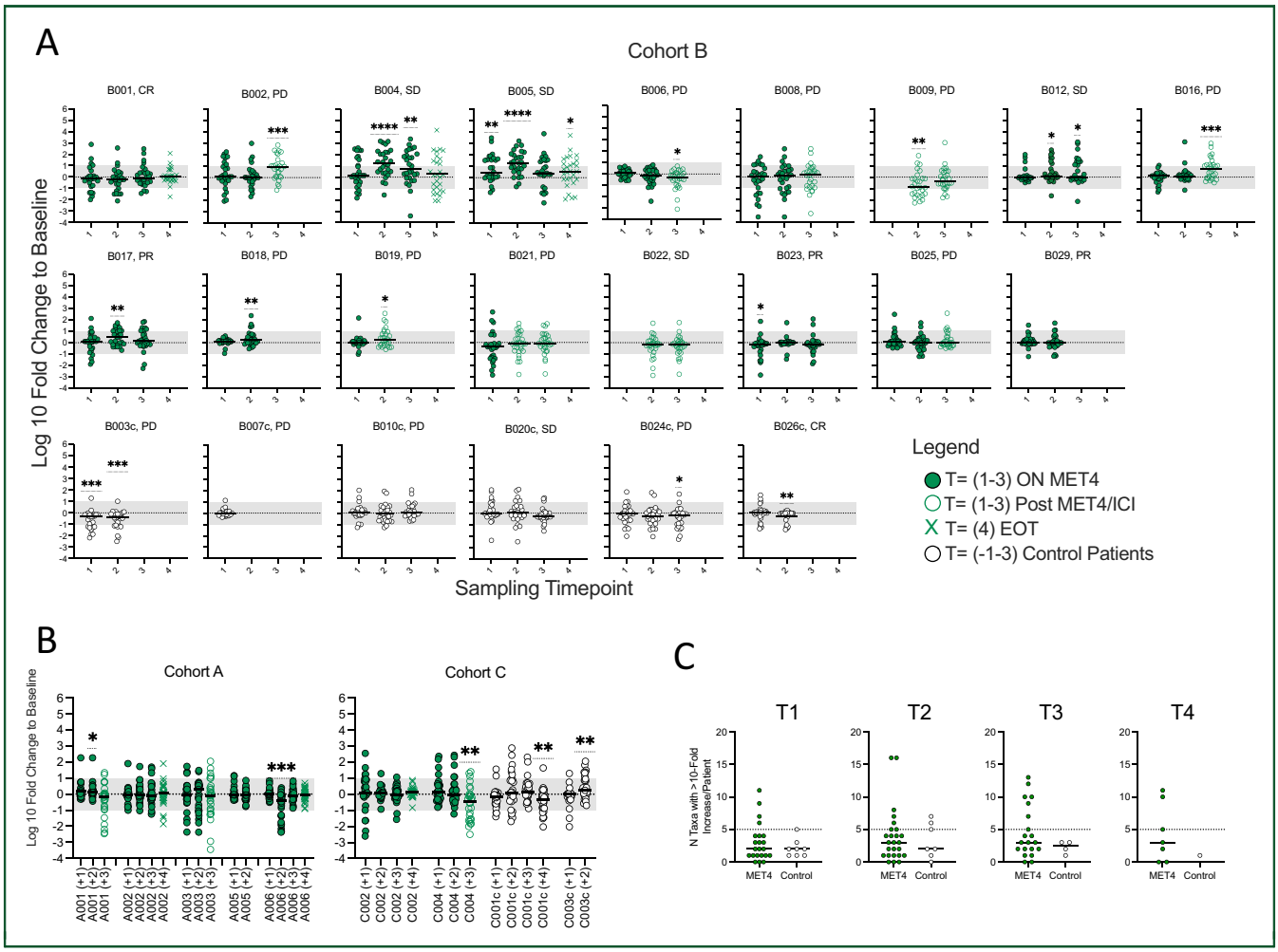

Figure 4. Changes in MET4 relative abundance by individual. Log fold change in relative abundance of MET4 taxa between baseline and subsequent timepoints is plotted for individual patients in groups B (A) and A/C (B). Each point represents the log fold change of a single MET4 taxon. One-sample *t*-tests were carried out to test whether taxonomic fold change was non-zero. Markers for the MET4 group are marked by whether the individual was on MET4 (filled circles), had discontinued MET4 >1 week before providing a stool sample (green empty circles) or was at the prespecified end of therapy (X). Controls are empty circles. (C) The number of taxa/patient with >10-fold increase in relative abundance compared to baseline at each timepoint. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, one-sample *t*-test.
CR, complete response; MET4, Microbial Ecosystem Therapeutic 4; PD, progressive disease; PR, partial response; SD, stable disease.

as 16 taxa seen at some timepoints. For example, patients B004 and B005 had >10-fold increases in the relative abundance of 9 and 11 MET4 taxa at T1, respectively, and 16 taxa each at T2. Changes in relative abundance varied by MET4 taxon, with significant increases in *Bifidobacterium*, *Enterococcus*, *Eubacterium eligens*, *Phascolarctobacterium succinatutens*, *Collinsella aerofaciens* and *Ruminococcus torques* in MET4 recipients after treatment, with a general decrease in MET4 taxa observed in controls (Figure 5A and B, Supplementary Figure S3, available at https://doi.org/10.1016/j.annonc.2023.02.011). Although inter-individual differences in ecological responses were evident, there were no generalizable differences in 16S community composition between MET4 recipients and controls, or pre-/post-MET4 treatment timepoints, and the strongest predictor of microbial community composition was trial participant (Supplementary Figures S4 and S5, available at https://doi.org/10.1016/j.annonc.2023.02.011).

Collectively, these data indicate that MET4 administration achieves measurable increases in MET4 taxa in a subset of MET4 recipients, but not controls, including increases in >5 MET4 taxa in 35% of MET4 recipients and significant increases in multiple MET4 genera, including several previously implicated in ICI responsiveness.

Baseline ecological and post-treatment metabolomic differences in MET4 ecological responders/non-responders

Amongst MET4 recipients, variable ecological responses were observed. We thus stratified MET4 recipients into those with and without an ecological response, defined as an increase of at least five MET4 taxa by at least 10-fold (a level which was associated with greater than median post-treatment MET4 relative abundance). We first assessed pre-MET4 treatment samples for predictors of ecological response/non-response. We did not observe statistically significant differences in baseline stool microbial diversity (Shannon diversity index, observed taxa, inverse Simpson) between EcoRs and EcoNRs (Supplementary Figure S6A-C, available at https://doi.org/10.1016/j.annonc.2023.02.011).

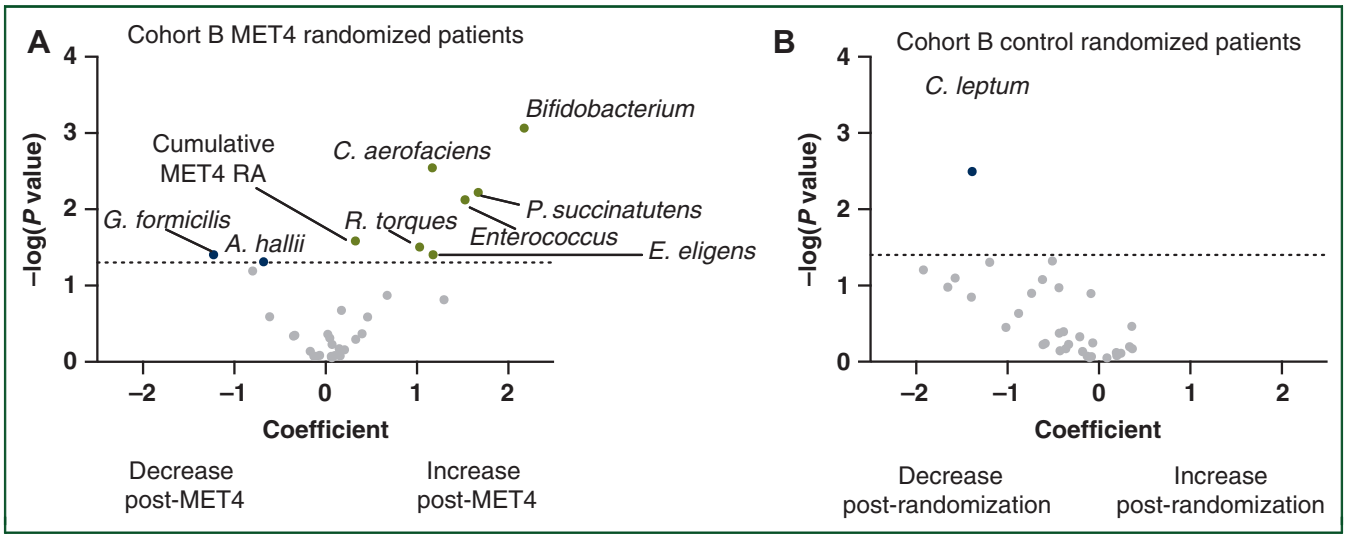

Figure 5. Volcano plots depicting differentially abundant MET4 taxa post-randomization compared to samples collected before randomization. (A) Increase (green) and decrease (blue) in MET4 taxa post-MET4 initiation. (B) Decrease in MET4 taxa post-alpha diversity randomization to the control arm. Grey dots include features that were not significantly different. MET4 taxa and alpha diversity metrics were log transformed and analyzed using MaAsLin2. Fixed effects included MET4 versus control randomization and pre- versus post-treatment; patient was set as a random effect to account for repeated measures. Benjamini—Hochberg correction was used to adjust for multiple tests with an FDR threshold of 0.25.
FDR, false discovery rate; MET4, Microbial Ecosystem Therapeutic 4.

Twenty-one of 28 MET4 taxa trended toward lower abundance at pre-MET4 initiation timepoints (T −1, T0) in EcoRs than in EcoNRs, 3 of which were significant before correction for multiple comparisons (Supplementary Figure S6D, available at https://doi.org/10.1016/j.annonc.2023.02.011). While not definitive in this limited dataset, pre-treatment colonization with endogenous MET4 taxa may inhibit MET4-induced ecological responses, or conversely that low MET4 species relative abundance and/or alpha diversity allows MET4 engraftment.

Recently, ICI responsiveness after FMT in patients with refractory melanoma was correlated with changes in microbial metabolites including increased transformation of primary to secondary BAs.[5] We therefore assessed the subset of cohort B patients in whom plasma samples were available at T0 ($n = 25$ samples) and T1 ($n = 25$ samples) and T2 ($n = 18$ samples) by targeted metabolomics. No significant differences were observed in plasma SCFAs and BAs between MET4 recipients and controls, or between MET4 recipients who had an ecological response and those without (Supplementary Figure S7, available at https://doi.org/10.1016/j.annonc.2023.02.011). There were no differences between timepoints or treatment groups in plasma BAs; however, three primary BAs decreased in individuals who had ecological engraftment (Supplementary Figure S8, available at https://doi.org/10.1016/j.annonc.2023.02.011), suggesting that engraftment may be associated with measurable changes in metabolites in plasma that have previously been associated with ICI response after FMT.[5] Stool SCFA and BA levels were similarly assessed. No significant differences in stool SCFA were observed across timepoints between treatment groups, or were there differences in change in SCFA levels between EcoRs, EcoNRs and controls (Supplementary Figure S9, available at https://doi.org/10.1016/j.annonc.2023.02.011). Similar to plasma, stool primary BAs did not differ between treatment groups across timepoints (Supplementary Figure S10A, available at https://doi.org/10.1016/j.annonc.2023.02.011), but decreases in primary BAs were noted in EcoRs, but not in EcoNRs or controls after treatment (Supplementary Figure S10B, available at https://doi.org/10.1016/j.annonc.2023.02.011), indicating that MET4-associated ecological response is associated with metabolic changes in both plasma and stool.

DISCUSSION

In this first-in-human trial of a cultivated microbial consortium administered as a co-therapy for ICI, we found that MET4 was well tolerated, with no high-grade AEs or worsening of ICI-associated irAEs, and that MET4 administration was associated with significant increases in therapeutic taxa in a subset of individuals. This engraftment was associated with peripheral metabolome changes recently associated with response to ICI after FMT.[5]

Interest in FMT as a microbiome-remediating strategy for both infectious and non-infectious diseases has increased significantly since FMT by duodenal infusion was shown to be effective for the treatment of recurrent *C. difficile* infection in a human interventional trial.[11] Multiple studies of FMT as a co-therapy for ICI are registered on ClinicalTrials.gov, including several phase II or phase I-II trials.[5,12-17] However, because safety, reproducibility and barriers to production at scale significantly limit the use of FMT, alternative strategies are needed. While single- or limited-strain probiotics are an alternative microbiome-targeting strategy, they have important caveats as a co-therapy to ICI. Firstly, probiotic effects on the composition of the microbiome do not reproduce the ecological effects of FMT in individuals with low microbial diversity and are

associated with decreased gut microbiome diversity compared to no treatment or FMT.[18] Secondly, in ICI recipients, limited complexity probiotic use may be associated with decreased ICI responsiveness, whereas dietary fiber, which promotes a complex and diverse microbiome, is associated with ICI response.[19] Thirdly, in cross-cohort analyses, no single species has emerged as uniformly ICI response-associated.[20] An important caveat to this observation is that the studies included were relatively small and are thus not definitive; however, it is also possible that 'narrow-spectrum' microbial therapies may not adequately reproduce the ecological and functional complexity of ICI response-associated microbiomes. Alternatives to FMT as an ICI co-therapy will ideally promote ecologically complex, multi-species responses in the recipient and be safe, tolerable and ecologically and physiologically significant in ICI recipients. There are a total of three clinical trials registered for evaluating microbial consortia as an ICI co-therapy,[21-23] and to our knowledge, our study is the first report of a microbial consortium used in combination with ICI in advanced cancer patients. A randomized phase I study of CBM588, a *Clostridium butyricum*-containing probiotic designed to promote Bifidobacteria,[24] in combination with anti-PD-1 and anti-CTLA-4 antibodies in ICI-naïve metastatic renal cell carcinoma patients, failed to meet its primary endpoint of a change in *Bifidobacterium* spp. at 12 weeks. Interestingly, statistically significant longer progression-free survival in the investigational treatment arm as compared to the control arm was observed in this small study. However, an imbalance in patients with poor international metastatic database consortium risk score was noted between the two arms. In contrast to this report, we evaluated a novel microbial consortium in which microbial species function in an ecologically complex manner.

In our trial, MET4 was tolerable and delivered safely in ICI recipients regardless of tumor type, indicating that this novel therapeutic approach may be feasible broadly in ICI recipients. We observed ecological response in a proportion of MET4 recipients, which included increases in multiple taxa that have been associated with ICI responses, such as *Enterococcus*, *Bifidobacterium* and *Phascolarctobacterium*, and we also observed changes in metabolites associated with ecological response. These results indicate the viability of microbial consortia as an ICI co-therapy. However, there are several important observations and limitations of this study and our findings are exploratory in nature. We are not adequately powered to assess the reasons for variability in ecological responsiveness in this small study. Notably unlike recent trials of FMT which enrolled ICI-resistant patients,[5,6] this study mainly included ICI-naïve patients receiving immunotherapy as standard of care; therefore, a response would be expected regardless of the addition of MET4. Populations with prior non-response or recent antimicrobial exposure may demonstrate different ecological responses to MET4. The association between ecological responsiveness and clinical response could not be assessed in this early-phase trial, especially given the heterogeneity of tumor types and variability of ICI regimens in the enrolled patients. We did not collect fresh tumor biopsies in this trial, therefore unable to assess the impact of MET4 administration or ecological responsiveness on the circulating or tumor immune phenotype. Finally, our sequencing approach was not able to distinguish between endogenous and exogenous MET4 strains. In spite of these limitations, we believe that the presence of engraftment in some MET4 recipients, changes in plasma and stool metabolite concentrations associated with engraftment and safety and tolerability of the intervention justify the pursuit of a larger trial of microbial consortia in ICI recipients with solid tumors. A pan-Canadian, randomized, placebo-controlled phase II trial in PD-L1-selected patients with recurrent/metastatic squamous cell cancer receiving anti-PD-1 antibody has been endorsed by the Canadian Cancer Trial Group. This study will evaluate the efficacy of MET4 as an adjunct to ICI (https://www.ctg.queensu.ca/public/head_neck/head-neck-disease-site) with comprehensive correlative predictive and pharmacodynamic biomarker evaluation of tumor, blood and stool samples.

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DATA SHARING

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request. All sequence data are availabile in GenBank/NCBI under the accession numbers PRJNA835435 and PRJNA819052.

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