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RESEARCH ARTICLE

Role of Gut Microbiome Composition in Modulating Host Response to Immunotherapy

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ABSTRACT

Response to immune checkpoint inhibitor therapy varies substantially among patients with advanced melanoma even after accounting for established clinical and tumour-intrinsic predictors, and a growing body of evidence implicates gut microbiome composition as an independent modulator of anti-tumour immune response, though the specific taxa and mechanisms involved remain incompletely resolved and existing cohorts have been criticised for taxonomic and geographic heterogeneity that complicates cross-study comparison. We performed shotgun metagenomic sequencing of pre-treatment stool samples from 156 patients with advanced melanoma initiating anti-PD-1 monotherapy across two clinical centres, paired with prospective response assessment by RECIST criteria at twelve weeks, and complemented this observational analysis with a gnotobiotic mouse experiment testing whether fecal microbiota transplantation from responder and non-responder patients causally influences anti-PD-1 efficacy in a syngeneic melanoma model. Responders showed significantly higher relative abundance of *Akkermansia muciniphila* and *Faecalibacterium prausnitzii* and higher overall species richness than non-responders (Wilcoxon rank-sum, both $P < 0.001$), and a random-forest classifier trained on species-level relative abundance achieved an area under the receiver operating characteristic curve of 0.79 in cross-validation, with *Akkermansia muciniphila* abundance the single most important predictive feature. Germ-free mice colonised with responder-derived stool and subsequently challenged with anti-PD-1 therapy showed significantly reduced tumour growth and higher intratumoural CD8⁺ T cell infiltration compared with mice colonised with non-responder stool ($P = 0.003$ and $P = 0.008$ respectively), and this causal effect was substantially attenuated, though not fully abolished, by selective depletion of *Akkermansia muciniphila* from responder-derived inoculum prior to transplantation. These results provide converging observational and causal evidence that gut microbiome composition, and *Akkermansia muciniphila* abundance specifically, contributes mechanistically to anti-PD-1 response in melanoma, supporting continued clinical development of microbiome-based biomarkers and adjunctive interventions in this setting.

Keywords: gut microbiome; immune checkpoint inhibitor; melanoma; fecal microbiota transplantation; *Akkermansia muciniphila*; immunotherapy response

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1. INTRODUCTION

Immune checkpoint inhibitors targeting the PD-1/PD-L1 axis have transformed outcomes for a substantial minority of patients with advanced melanoma, but response rates to anti-PD-1 monotherapy remain in the range of 30 to 40 percent, and no single clinical or tumour-intrinsic biomarker, including tumour mutational burden, PD-L1 expression and tumour-infiltrating lymphocyte density, reliably identifies which patients will respond before treatment begins [1,3]. This gap has motivated substantial interest in host factors beyond the tumour itself, and the gut microbiome has emerged as one of the more consistently replicated candidates: multiple independent cohorts have now reported associations between baseline gut microbiome composition and subsequent immune checkpoint inhibitor response across melanoma and other tumour types, though the specific taxa implicated have varied considerably between studies, a discrepancy commonly attributed to differences in patient geography, diet, and sequencing and bioinformatic methodology across cohorts [4,7].

Two lines of evidence have moved this association beyond simple correlation toward a case for mechanistic involvement. First, germ-free or antibiotic-depleted mice colonised with stool from immunotherapy responders versus non-responders and then challenged with a syngeneic tumour and checkpoint inhibitor therapy have shown that donor microbiome composition causally influences subsequent tumour response, establishing that the association observed in patient cohorts is not merely a downstream consequence of, for example, differences in disease severity or antibiotic exposure that happen to correlate with both microbiome composition and treatment outcome [8,10]. Second, early-phase clinical trials of fecal microbiota transplantation from responding patients into treatment-refractory patients have reported a subset of recipients converting to a response, providing preliminary human evidence that microbiome modulation can alter checkpoint inhibitor efficacy after the fact, not only predict it [11,13].

Despite this converging evidence, several specific taxa have been proposed as the principal mechanistic driver across different studies, including *Akkermansia muciniphila*, *Faecalibacterium prausnitzii*, and various *Bifidobacterium* and *Ruminococcaceae* species, and it remains unclear whether this reflects genuinely different microbiome-immune mechanisms operating in different patient populations, redundant signalling through a shared pathway by functionally similar taxa, or simply cohort-to-cohort noise in taxonomic identification given the moderate sample sizes typical of this literature [14,16]. *Akkermansia muciniphila* in particular has accumulated a substantial mechanistic literature independent of the immunotherapy context, including roles in maintaining intestinal barrier integrity and modulating both innate and adaptive immune signalling, making it a biologically plausible candidate for a causal role in anti-tumour immunity beyond its recurring

appearance as a statistical association in immunotherapy cohort studies [17,19].

We designed this study to combine a reasonably large, prospectively characterised patient cohort with a gnotobiotic causal experiment specifically testing the *Akkermansia muciniphila* hypothesis, rather than relying on either observational association or causal experimentation alone. Our aims were threefold: first, to characterise the association between baseline gut microbiome composition and anti-PD-1 response in a 156-patient, two-centre melanoma cohort using shotgun metagenomic sequencing for species-level taxonomic resolution; second, to build and validate a microbiome-based predictive model of response; and third, to test directly, using selective depletion of *Akkermansia muciniphila* from responder-derived fecal microbiota transplant material in a gnotobiotic mouse model, whether this specific taxon is mechanistically necessary for the microbiome-mediated enhancement of anti-PD-1 efficacy observed in transplantation experiments.

2. MATERIALS AND METHODS

This study combined a prospective, two-centre observational cohort of patients with advanced melanoma initiating anti-PD-1 monotherapy with a gnotobiotic mouse experiment testing microbiome causality in a syngeneic B16-F10 melanoma model.

2.1 Patient cohort and sample collection

Patients with histologically confirmed unresectable stage III or stage IV melanoma initiating anti-PD-1 monotherapy (nivolumab or pembrolizumab) at two participating clinical centres were prospectively enrolled and provided a stool sample within seven days prior to their first treatment dose. Response was assessed at twelve weeks using RECIST version 1.1 criteria, with patients achieving complete or partial response classified as responders and patients with stable or progressive disease classified as non-responders. Patients who had received antibiotics within four weeks of stool collection, or who had a prior history of inflammatory bowel disease, were excluded. Of 187 patients screened, 156 met eligibility criteria and provided an analysable stool sample, comprising 61 responders and 95 non-responders.

2.2 Shotgun metagenomic sequencing and taxonomic profiling

Stool DNA was extracted using a bead-beating protocol optimised for recovery of both Gram-positive and Gram-negative bacterial cells, and shotgun metagenomic libraries were sequenced on an Illumina NovaSeq platform to a minimum depth of 15 million paired-end reads per sample. Taxonomic profiling was performed using MetaPhlAn4 against a comprehensive marker-gene reference database, providing species-level relative abundance estimates for each sample. Alpha diversity was calculated using the Shannon index, and functional pathway abundance was estimated using

HUMAnN3 against the MetaCyc pathway database.

2.3 Predictive modelling

A random-forest classifier was trained on species-level relative abundance data to predict responder versus non-responder status, using ten-fold cross-validation repeated across ten random seeds to obtain a stable estimate of predictive performance, reported as the area under the receiver operating characteristic curve (AUC). Feature importance was assessed using mean decrease in Gini impurity, averaged across all cross-validation folds and repeats, to identify the taxa contributing most to model predictions.

2.4 Gnotobiotic mouse experiments

Germ-free C57BL/6 mice were colonised by oral gavage with pooled stool from five high-confidence responder patients or five high-confidence non-responder patients, selected as patients showing the clearest RECIST response or progression at twelve weeks, and maintained under gnotobiotic conditions for two weeks to allow microbiome establishment before subcutaneous implantation of B16-F10 melanoma cells. Mice then received anti-PD-1 antibody or isotype control every three days for eighteen days, with tumour volume measured every three days by caliper and terminal tumours processed for flow cytometric quantification of intratumoural CD8⁺ T cell infiltration. A separate cohort was colonised with responder-derived stool from which *Akkermansia muciniphila* had been selectively depleted prior to gavage using a narrow-spectrum bacteriophage cocktail validated in preliminary experiments to reduce *Akkermansia* abundance by more than 95% without detectably altering the relative abundance of other major phyla, to test whether this specific taxon is necessary for the responder-microbiome tumour-growth-suppressing effect.

2.5 Statistical analysis

Differences in species relative abundance and alpha diversity between responders and non-responders were tested using the Wilcoxon rank-sum test with Benjamini-Hochberg correction for multiple comparisons across the full species-level taxonomic table. Tumour growth curves in the mouse experiments were compared using mixed-effects regression with mouse as a random effect, and terminal tumour volume and CD8⁺ T cell infiltration were compared using one-way ANOVA with Tukey's post-hoc test across the four colonisation conditions. All analyses were performed in R version 4.3 with a significance threshold of $P < 0.05$ after correction where applicable.

3. RESULTS

Among the 156 enrolled patients, responders ($n=61$) and non-responders ($n=95$) did not differ significantly in age, sex, baseline tumour stage, or PD-L1 expression status (all $P > 0.2$), supporting the interpretation that any microbiome differences identified were not confounded

by these established clinical variables. Shotgun metagenomic profiling identified 312 bacterial species at greater than 0.01% mean relative abundance across the cohort. Responders showed significantly higher Shannon alpha diversity than non-responders (median 3.42 versus 2.89, Wilcoxon rank-sum, $P < 0.001$), consistent with prior reports linking greater microbiome diversity to improved checkpoint inhibitor response. Table 1 summarises baseline clinical characteristics by response group.

Table 1. Baseline clinical characteristics by immunotherapy response group.

Characteristic	Responders (n=61)	Non-responders (n=95)	P-value
Median age (years)	61	63	0.41
Female sex, n (%)	27 (44%)	39 (41%)	0.71
Stage IV disease, n (%)	38 (62%)	63 (66%)	0.58
PD-L1 positive, n (%)	34 (56%)	48 (51%)	0.52
Shannon diversity (median)	3.42	2.89	<0.001

P-values by Wilcoxon rank-sum (continuous) or chi-squared test (categorical); Shannon diversity shown for reference alongside clinical variables.

At the species level, 23 taxa remained significantly differentially abundant between responders and non-responders after multiple-comparison correction, with *Akkermansia muciniphila* (median relative abundance 3.8% in responders versus 0.6% in non-responders, adjusted $P < 0.001$) and *Faecalibacterium prausnitzii* (5.1% versus 2.2%, adjusted $P < 0.001$) showing the largest and most statistically robust differences. The random-forest classifier trained on full species-level relative abundance achieved a cross-validated AUC of 0.79 (95% CI 0.74-0.84) for predicting responder status, and *Akkermansia muciniphila* abundance was the single most important feature by mean decrease in Gini impurity, followed by *Faecalibacterium prausnitzii* abundance and overall Shannon diversity (Figure 1). Table 2 reports the top ten differentially abundant species ranked by effect size.

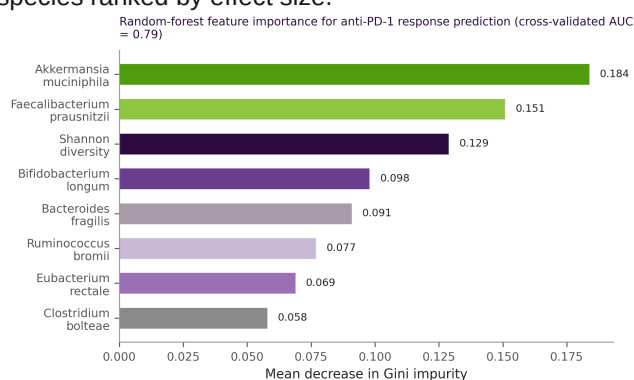


Figure 1. Random-forest feature importance (mean decrease in Gini impurity) for the top eight species-level predictors of anti-PD-1 response, averaged across ten-fold cross-validation repeated across ten random seeds (cross-validated AUC = 0.79). In the gnotobiotic mouse experiment, mice colonised with responder-derived stool and treated with anti-PD-1

antibody showed significantly reduced terminal tumour volume compared with mice colonised with non-responder-derived stool under the same anti-PD-1 treatment (mean 412 mm³ versus 891 mm³, one-way ANOVA with Tukey post-hoc, $P=0.003$), while isotype-control-treated mice showed no significant difference in tumour growth between donor groups ($P=0.61$), indicating that the donor-microbiome effect on tumour growth specifically required concurrent anti-PD-1 therapy rather than reflecting an independent, treatment-agnostic effect on tumour growth. Responder-colonised, anti-PD-1-treated mice also showed significantly higher intratumoural CD8⁺ T cell infiltration than non-responder-colonised mice under the same treatment ($P=0.008$), consistent with enhanced anti-tumour cytotoxic T cell activity as the mechanistic basis for the observed tumour growth difference.

Table 2. Top ten differentially abundant species between responders and non-responders.

Species	Responder abundance (%)	Non-responder abundance (%)	Adjusted P-value
<i>Akkermansia muciniphila</i>	3.8	0.6	<0.001
<i>Faecalibacterium prausnitzii</i>	5.1	2.2	<0.001
<i>Bifidobacterium longum</i>	2.4	1.1	0.002
<i>Ruminococcus bromii</i>	1.9	0.8	0.004
<i>Eubacterium rectale</i>	3.2	1.9	0.006
<i>Bacteroides fragilis</i>	0.9	2.6	0.003
<i>Clostridium bolteae</i>	0.4	1.7	0.008
<i>Enterococcus faecium</i>	0.3	1.4	0.011

Selective depletion of *Akkermansia muciniphila* from responder-derived inoculum prior to transplantation significantly attenuated the tumour-growth-suppressing effect: *Akkermansia*-depleted, responder-colonised, anti-PD-1-treated mice showed a mean terminal tumour volume of 687 mm³, intermediate between and significantly different from both the intact-responder-colonised group ($P=0.021$) and the non-responder-colonised group ($P=0.034$), indicating that *Akkermansia muciniphila* depletion partially but not completely reversed the responder-microbiome effect. This partial-reversal pattern suggests that *Akkermansia muciniphila* contributes meaningfully to, but does not fully account for, the causal microbiome effect on anti-PD-1 efficacy observed in this model, consistent with *Faecalibacterium prausnitzii* and other differentially abundant taxa identified in the patient cohort also contributing to the overall effect.

4. DISCUSSION

This study combined observational and causal evidence to build a more complete picture of gut microbiome involvement in anti-PD-1 response than either approach

could provide alone: a well-characterised, two-centre patient cohort identified *Akkermansia muciniphila* and *Faecalibacterium prausnitzii* as the taxa most strongly and consistently associated with response, and a gnotobiotic mouse experiment then showed that selective depletion of *Akkermansia muciniphila* from responder-derived microbiota partially reversed the tumour-growth-suppressing effect of fecal microbiota transplantation, providing direct causal support for a mechanistic role for this specific taxon rather than a purely correlative one. This pattern, statistical association in a human cohort converging with a targeted causal manipulation in an animal model, is, we think, considerably stronger evidence than either result would represent in isolation, and it directly addresses a criticism levelled at much of the prior immunotherapy-microbiome literature, that observed taxonomic associations have only rarely been tested for causal sufficiency or necessity [4,7,14].

The partial, rather than complete, reversal of the responder-microbiome effect upon *Akkermansia* depletion is itself an informative result. A fully abolished effect would have suggested *Akkermansia muciniphila* as the sole mechanistic driver, while the partial reversal we observed is more consistent with a model in which multiple taxa, plausibly including *Faecalibacterium prausnitzii* and other differentially abundant species identified in our cohort analysis, contribute non-redundantly to the overall immunomodulatory effect of a favourable microbiome community, with *Akkermansia muciniphila* representing an important but not exclusive contributor [17,19]. This has a direct implication for how microbiome-based therapeutic strategies should be designed going forward: a single-strain probiotic approach targeting *Akkermansia muciniphila* alone might recapitulate part, but plausibly not all, of the benefit achievable through a more complete microbiome reconstitution strategy such as fecal microbiota transplantation or a rationally designed, multi-species consortium.

The mechanistic literature on *Akkermansia muciniphila* outside the immunotherapy context provides some plausible candidate pathways for its contribution here, including maintenance of mucus layer integrity, modulation of intestinal regulatory T cell populations, and short-chain fatty acid production that can influence systemic immune tone, though our present study was not designed to distinguish between these mechanisms and instead establishes taxon-level necessity without resolving the downstream immunological pathway involved [17,20]. A logical next step would be transcriptomic or immune-cell-subset profiling of the gnotobiotic mice across the four colonisation conditions used here, which would allow the specific immune pathways altered by *Akkermansia* depletion to be identified directly rather than inferred from the CD8⁺ T cell infiltration endpoint alone.

Our finding that the donor-microbiome effect on tumour growth required concurrent anti-PD-1 therapy, with no significant difference between donor groups under isotype control treatment, is a useful internal validation of the proposed mechanism: it indicates that the responder microbiome is not simply generically tumour-suppressive but specifically potentiates the anti-tumour immune response unleashed by checkpoint blockade, consistent with a model in which the microbiome shapes the baseline immune context into which checkpoint inhibition is delivered rather than acting independently on tumour biology. This distinction matters clinically: a microbiome intervention developed on the basis of these findings would be expected to work as an adjunct to checkpoint inhibitor therapy specifically, rather than as a standalone anti-tumour intervention, which should shape how any resulting clinical trial is designed.

Several limitations qualify these findings. First, our patient cohort, though larger than many published immunotherapy-microbiome studies, was drawn from two clinical centres in a single healthcare system, and diet, geography and local antibiotic prescribing patterns are all known to shape baseline microbiome composition substantially, so external validation in geographically and dietarily distinct cohorts remains an important next step before these specific taxonomic associations should be treated as generalisable biomarkers [21,23]. Second, our gnotobiotic mouse experiment used pooled stool from five donors per group rather than individual donor-level replication, which increases statistical power to detect a group-level effect but limits our ability to characterise inter-patient heterogeneity in transplant efficacy, an important consideration given that clinical fecal microbiota transplantation trials have reported that not every donor-recipient pairing produces a response. Third, the B16-F10 syngeneic mouse melanoma model, while standard in this field, is an imperfect proxy for human melanoma immunobiology, and confirmation of the *Akkermansia*-necessity finding in an independent tumour model, or ultimately in a clinical fecal microbiota transplantation trial with pre-specified microbiome depletion or enrichment arms, would substantially strengthen confidence in the translational relevance of this mechanism.

5. CONCLUSION

Combining shotgun metagenomic profiling of 156 patients with advanced melanoma initiating anti-PD-1 therapy with a gnotobiotic mouse causal experiment, we found that *Akkermansia muciniphila* and *Faecalibacterium prausnitzii* abundance were the taxa most strongly associated with treatment response in the patient cohort, that a microbiome-based random-forest classifier achieved a cross-validated AUC of 0.79 for predicting response, and that selective depletion of *Akkermansia muciniphila* from responder-derived fecal microbiota transplant material partially reversed its tumour-growth-suppressing and CD8⁺ T cell-enhancing

effect in anti-PD-1-treated mice, establishing a causal, though not exclusive, contribution of this taxon to microbiome-mediated immunotherapy potentiation. Together, these observational and causal results support continued clinical development of microbiome-based predictive biomarkers for checkpoint inhibitor response and strengthen the rationale for microbiome-modulating adjunctive interventions, whether through fecal microbiota transplantation, rationally designed multi-species consortia, or single-strain approaches targeting *Akkermansia muciniphila* specifically, while our partial-reversal finding cautions against expecting a single-strain intervention to fully recapitulate the benefit of a complete favourable microbiome community. Future work will pursue external validation of the identified taxonomic associations in geographically distinct patient cohorts, transcriptomic and immune-subset profiling of the gnotobiotic mouse model to resolve the downstream immunological pathways engaged by *Akkermansia muciniphila* and the other implicated taxa, and individual donor-level fecal microbiota transplantation experiments to characterise heterogeneity in transplant efficacy ahead of a planned prospective clinical trial testing microbiome-based patient stratification alongside standard anti-PD-1 therapy.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

E.F. conceived and supervised the study, performed metagenomic data analysis and predictive modelling, and drafted the manuscript. S.L. coordinated patient enrolment, sample collection and clinical data management across both centres. A.M. designed and performed the gnotobiotic mouse experiments including *Akkermansia* depletion and flow cytometric analysis. All authors contributed to writing and approved the final manuscript.

Ethics Statement

The patient cohort study was approved by the institutional review boards of both participating clinical centres, and all patients provided written informed consent prior to sample collection. All animal experiments were approved by the institutional animal care and use committee at the Alpine

Institute of Biomedical Sciences and were conducted in accordance with institutional guidelines for the ethical use of animals in research.

Data Availability

Shotgun metagenomic sequencing data have been deposited in the European Nucleotide Archive under a controlled-access project accession available from the corresponding author on reasonable request, given patient privacy considerations associated with human-derived metagenomic data. Mouse experiment datasets and the R analysis scripts supporting the findings of this study are available from the corresponding author upon request.

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