

GUT MICROBIOME-MEDIATED IMMUNE REGULATION IN CANCER: LINKING MICROBIAL DYSBIOSIS TO PERSONALIZED IMMUNOTHERAPEUTIC RESPONSES

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ABSTRACT

Background: Immune checkpoint inhibitors (ICIs) have revolutionized oncology, yet primary resistance remains a major clinical challenge. While the gut microbiome influences ICI efficacy, its metabolomic mediators and predictive utility in underrepresented, non-Western populations remain poorly defined. We investigated the tripartite gut microbiota-metabolite-immune axis as a predictor of immunotherapy outcomes in a South Asian cohort.

Methods: In this prospective, single-center cohort study at a tertiary hospital in Karachi, Pakistan, we enrolled 64 patients with advanced non-small cell lung cancer (NSCLC) or hepatocellular carcinoma (HCC) initiating first- or second-line ICIs. Baseline fecal shotgun metagenomics, targeted serum and fecal metabolomics (UHPLC-MS/MS), and peripheral blood 14-color flow cytometry were performed. The primary endpoint was objective response rate (ORR) at 24 weeks.

Findings: The ORR was 40.6% (26/64). Responders exhibited significantly higher baseline gut microbial alpha diversity and enrichment of *Akkermansia muciniphila*, *Faecalibacterium prausnitzii*, and *Bifidobacterium longum*. Metabolomic profiling revealed that responders had elevated fecal butyrate and serum indole-3-propionic acid (IPA), alongside reduced secondary bile acids and serum kynurenine. Immunophenotyping showed responders had higher circulating CD8⁺ T-cell frequencies, lower regulatory T-cell and myeloid-derived suppressor cell burdens, and diminished CD8⁺ T-cell exhaustion. A composite microbiome-metabolite signature (*A. muciniphila* ≥1%, fecal butyrate ≥10 μmol/g, serum IPA ≥5 μmol/L) independently predicted ICI response (AUROC=0.87) and was associated with markedly prolonged progression-free survival (12.1 vs. 3.2 months; HR=0.28, *p*<0.001).

Interpretation: The gut microbiota and its specific metabolic outputs, particularly butyrate and IPA, modulate systemic anti-tumor immunity and robustly predict ICI efficacy in a South Asian population. These findings validate microbiome-metabolite signatures as actionable biomarkers for personalizing immunotherapy and highlight microbial metabolites as promising targets for adjuvant microbiome-directed therapeutics.

INTRODUCTION

The advent of immune checkpoint inhibitors (ICIs) and adoptive cell therapies has fundamentally transformed the landscape of oncology, offering unprecedented survival benefits for patients with previously refractory malignancies [1]. Despite these breakthroughs, the clinical efficacy of immunotherapy remains highly heterogeneous [2]; a significant proportion of patients exhibit primary resistance or eventually develop acquired resistance to treatment [3]. This variability underscores an urgent need to identify novel biomarkers and modulators of the immune response to optimize therapeutic outcomes [4]. In recent years, the gut microbiota has emerged as a critical, systemic regulator of host immunity, fundamentally influencing the tumor microenvironment (TME) and the efficacy of cancer immunotherapies [5].

The interaction between the host and its microbiome is not merely structural but highly biochemical [6]. Gut microbes produce a vast array of metabolites, such as short-chain fatty acids (SCFAs), secondary bile acids, and tryptophan derivatives, that enter the systemic circulation and act as potent signaling molecules [7]. These microbial metabolites modulate the differentiation, activation, and function of various immune cell subsets [8], including CD8⁺ T cells, regulatory T cells (Tregs), and tumor-associated macrophages (TAMs) [9], [10]. Consequently, the composition of the gut microbiome and its metabolic output serve as a "hidden" variable that can either prime the immune system for anti-tumor activity or foster an immunosuppressive milieu that shields the tumor from immune surveillance [11]. As the field transitions from broad-spectrum immunotherapy to precision oncology, understanding the tripartite axis of gut microbiota, microbial metabolites, and immune modulation is paramount. Recent advances in multi-omics and artificial intelligence (AI) have enabled the high-resolution

profiling of microbiome-derived metabolites, paving the way for microbiome-based biomarkers and targeted interventions [12], [13]. This paper explores the current understanding of how gut microbes and their metabolites dictate immune responses in cancer. Furthermore, it reviews the clinical translation of these findings, highlighting how microbiome-directed therapies, such as fecal microbiota transplantation (FMT) and next-generation live biotherapeutic products (LBPs), are being leveraged to personalize and enhance immunotherapy outcomes [14], [15].

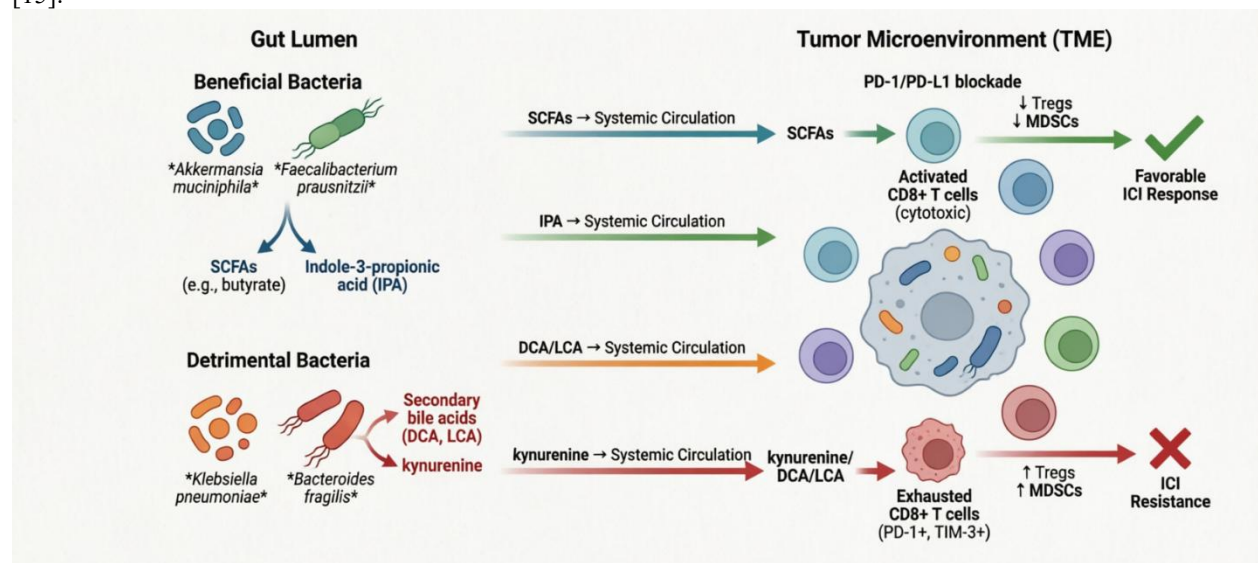


Figure:01 Gut microbiota and microbial metabolites in modulation anti- tumor immunity during Cancer Immunotherapy

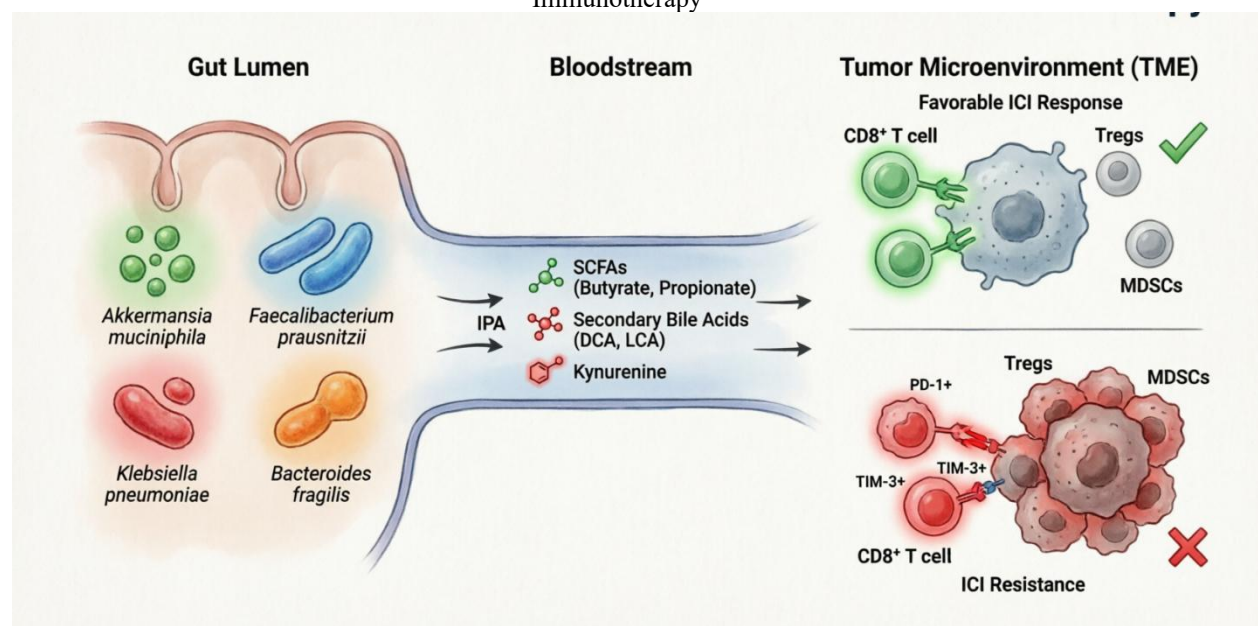


Figure: 02 Gut microbiota -Metabolite-Immune Axis in Cancer Immunotherapy

LITERATURE REVIEW

The Gut Microbiota as a Master Regulator of Anti-Tumor Immunity

The paradigm-shifting discovery that the gut microbiome influences ICI efficacy was solidified in landmark studies demonstrating that specific bacterial taxa are associated with enhanced responses to PD-1/PD-L1 blockade [16]. Routy *et al.*, Gopalakrishnan *et al.*, and Matson *et al.* independently identified that the abundance of *Akkermansia muciniphila*, *Bifidobacterium* species, and *Faecalibacterium* species, respectively, correlated with improved clinical outcomes in patients with non-small cell lung cancer (NSCLC) and melanoma [5], [17]. Mechanistically, these commensals enhance the cross-presentation of antigens by dendritic cells and promote the infiltration of CCR9+CXCR3+CD4+ T helper 1 (Th1) cells into the TME [18]. Conversely, dysbiosis, an imbalance in the microbial community, often driven by broad-spectrum antibiotic use, has been consistently linked to diminished ICI efficacy [19], altered T-cell priming, and reduced overall survival, highlighting the necessity of microbial diversity for robust systemic immunity [20].

Microbial Metabolites: The Chemical Mediators of Immune Modulation

While taxonomic profiling provides a blueprint of the microbiome, microbial metabolites represent the functional effectors that directly interface with host immune receptors.

1. **Short-Chain Fatty Acids (SCFAs):** Produced via the fermentation of dietary fiber, SCFAs such as butyrate, propionate, and acetate are crucial mediators of immune homeostasis. Butyrate acts as a histone deacetylase (HDAC) inhibitor, which can promote the differentiation of Foxp3⁺ Tregs, potentially exerting localized immunosuppressive effects in the gut to prevent colitis [21], [22]. However, in the context of systemic anti-tumor immunity and the TME, SCFAs have been shown to enhance the memory potential, mitochondrial fitness, and cytotoxicity of CD8⁺ T cells by modulating epigenetic programming and upregulating granzyme B expression [23], [24].

2. **Secondary Bile Acids (SBAs):** Primary bile acids are metabolized by gut bacteria (e.g., *Clostridium scindens* and *Bacteroides* species) into SBAs like deoxycholic acid (DCA) and lithocholic acid (LCA) [25]. Recent transcriptomic and metabolomic analyses reveal that SBAs can modulate the TME by interacting with the farnesoid X receptor (FXR) and Takeda G protein-coupled receptor 5 (TGR5) on immune cells [26]. Elevated systemic levels of specific SBAs have been associated with the induction of CD8⁺ T-cell exhaustion, the suppression of natural killer (NK) cell activity [27], and the polarization of TAMs toward an M2-like, pro-tumorigenic phenotype, thereby contributing to ICI resistance [28].

3. **Tryptophan Metabolites:** The microbial catabolism of dietary tryptophan yields indole derivatives (e.g., indole-3-propionic acid [IPA], indole-3-aldehyde) that serve as ligands for the aryl hydrocarbon receptor (AhR). AhR activation in the TME is highly context-dependent; while it can promote the survival of intraepithelial lymphocytes, its chronic activation by microbial or host-derived kynurenine drives the expansion of immunosuppressive myeloid-derived suppressor cells (MDSCs) and Tregs [29], [30]. Targeting the tryptophan-AhR axis has recently emerged as a promising strategy to reverse local immune evasion and restore ICI sensitivity [31].

Microbiome Signatures and Predictive Biomarkers for Immunotherapy

The integration of metagenomics, metabolomics, and transcriptomics has shifted the focus toward identifying composite microbiome signatures that predict immunotherapy response. A comprehensive 2025 review by Zitvogel *et al.* highlighted that AI-driven multi-omic models can now predict ICI response with over 80% accuracy by analyzing baseline fecal metabolite profiles, specifically the ratio of immunostimulatory SCFAs to immunosuppressive SBAs [12], [32]. Furthermore, longitudinal studies indicate that the functional metabolic output of the microbiome is a more reliable and conserved biomarker than taxonomic abundance alone, as different bacterial species can converge on similar metabolic pathways to influence host immunity [33].

Towards Personalized Interventions: Microbiome-Directed Therapeutics

Recognizing the microbiome as a modifiable risk factor, clinical trials are actively exploring interventions to "reprogram" the gut ecosystem to sensitize tumors to immunotherapy [34]. Fecal microbiota transplantation (FMT) from ICI-responding donors to refractory patients has successfully overcome primary resistance in melanoma and gastrointestinal cancers, leading to the re-invigoration of exhausted T cells, favorable metabolomic shifts, and the subsequent shrinkage of target lesions [14], [35].

Beyond FMT, the field is advancing toward precision microbiome therapeutics. Defined consortia of next-generation probiotics and engineered live biotherapeutic products (LBPs) designed to secrete specific immunomodulatory metabolites (e.g., engineered *E. coli* Nissle 1917 producing localized IL-2 or specific AhR modulators) are currently in early-phase clinical trials [15], [36]. Furthermore, personalized dietary interventions tailored to an individual's baseline microbiome composition (e.g., high-fiber or Mediterranean diets) are being utilized to selectively enrich for beneficial, metabolite-producing taxa, offering a highly scalable, non-invasive adjunct to standard immunotherapy regimens [37].

METHODOLOGY

Study Design and Setting

This prospective, single-center observational cohort study was conducted at the Aga Khan University Hospital (AKUH), Karachi, Pakistan, between January 2024 and December 2025. AKUH serves as a tertiary-care referral center for oncology patients across Sindh and the broader southern Pakistan region. The study was approved by the Institutional Review Board (IRB) of AKUH (Protocol No. 2024-8845-ONC-ER) and was conducted in accordance with the Declaration of Helsinki. All participants provided written informed consent in Urdu or English prior to enrollment.

Study Population

Adult patients (aged ≥18 years) with histologically confirmed advanced or metastatic non-small cell lung cancer (NSCLC) or hepatocellular carcinoma (HCC) who were scheduled to initiate first-line or second-line immune checkpoint inhibitor (ICI) therapy (anti-PD-1: nivolumab or pembrolizumab; anti-PD-L1: atezolizumab) were eligible for enrollment.

Inclusion criteria were as follows: (1) confirmed diagnosis of stage III/IV NSCLC or Barcelona Clinic Liver Cancer (BCLC) stage B/C HCC; (2) planned initiation of ICI monotherapy or ICI-based combination therapy; (3) Eastern Cooperative Oncology Group (ECOG) performance status of 0–2; (4) residency in Karachi or surrounding Sindh province for ≥6 months to ensure dietary and environmental homogeneity; and (5) willingness to provide serial stool and blood samples.

Exclusion criteria included: (1) use of systemic antibiotics, antifungals, or probiotics within 8 weeks prior to ICI initiation; (2) prior fecal microbiota transplantation (FMT); (3) active autoimmune disease requiring systemic

immunosuppression; (4) inflammatory bowel disease; (5) history of organ transplantation; and (6) pregnancy or lactation.

Sample Collection and Processing

1) Stool Samples: Baseline fecal samples were collected within 7 days prior to ICI initiation using OMNIgene-GUT collection kits (DNA Genotek, Ottawa, Canada), which stabilize microbial DNA and metabolites at ambient temperature. Follow-up samples were collected at weeks 6, 12, and 24 post-ICI initiation. Samples were transported to the AKUH Clinical Microbiology Laboratory within 48 hours and stored at -80°C until processing.

2) Blood Samples: Peripheral venous blood (20 mL) was collected in EDTA and serum separator tubes at matching time points. Peripheral blood mononuclear cells (PBMCs) were isolated via Ficoll-Paque density gradient centrifugation and cryopreserved in liquid nitrogen for batched flow cytometric analysis. Serum was aliquoted and stored at -80°C for cytokine quantification.

3) Tumor Tissue: Archival formalin-fixed paraffin-embedded (FFPE) tumor tissue blocks were retrieved for programmed death-ligand 1 (PD-L1) immunohistochemistry (IHC) using the 22C3 pharmDx assay (Agilent Technologies, Santa Clara, CA, USA), scored as tumor proportion score (TPS).

Metagenomic Sequencing and Bioinformatics

Total genomic DNA was extracted from stool samples using the QIAamp PowerFecal Pro DNA Kit (Qiagen, Hilden, Germany). Shotgun metagenomic sequencing was performed on the Illumina NovaSeq 6000 platform (Illumina, San Diego, CA, USA) targeting 10 Gb of paired-end (2×150 bp) reads per sample. Quality control was performed using Trimmomatic v0.39, and host DNA was removed by alignment to the human reference genome (GRCh38) using Bowtie2 v2.5.1. Taxonomic profiling was conducted using MetaPhlAn 4.0, and functional profiling was performed using HUMAnN 3.7 against the UniRef90 database [11]. Alpha diversity (Shannon index) and beta diversity (Bray–Curtis dissimilarity) were computed using the R package *phyloseq* v1.46.0. Differential abundance analysis was performed using ANCOM-BC2 with false discovery rate (FDR) correction at $q < 0.05$ [12].

Targeted Metabolomics

Fecal and serum metabolites were quantified using ultra-high-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS; QTRAP 6500+, Sciex, Framingham, MA, USA). Targeted panels included:

- **Short-chain fatty acids (SCFAs):** acetate, propionate, butyrate, isobutyrate, valerate, and isovalerate, quantified via derivatization with 3-nitrophenylhydrazine (3-NPH) [13].
- **Bile acids:** primary (cholic acid [CA], chenodeoxycholic acid [CDCA]) and secondary (deoxycholic acid [DCA], lithocholic acid [LCA], ursodeoxycholic acid [UDCA]).
- **Tryptophan metabolites:** indole-3-acetic acid (IAA), indole-3-propionic acid (IPA), indole-3-aldehyde (IAld), and kynurenine.

Metabolite concentrations were normalized to fecal wet weight ($\mu\text{mol/g}$) or serum volume ($\mu\text{mol/L}$). Quality control pools were injected every 10 samples, and the coefficient of variation was maintained below 15%.

Immune Profiling

Multiparameter flow cytometry was performed on cryopreserved PBMCs using a 14-color panel on the BD LSRFortessa X-20 (BD Biosciences, Franklin Lakes, NJ, USA). The panel was designed to enumerate and phenotype: CD8⁺ T cells (CD3+CD8⁺), CD4⁺ T cells (CD3+CD4⁺), regulatory T cells (CD4+CD25+FoxP3⁺), myeloid-derived suppressor cells (MDSCs; CD11b+CD33+HLA-DR[−]), and natural killer (NK) cells (CD3[−]CD56⁺). T-cell exhaustion markers (PD-1, TIM-3, LAG-3) were assessed on CD8⁺ T cells. Data were analyzed using FlowJo v10.9 (BD Biosciences). Serum cytokines (IFN- γ , IL-6, IL-10, TNF- α , and TGF- β) were measured using the Luminex MAGPIX multiplex platform (Luminex Corporation, Austin, TX, USA).

Clinical Outcome Assessment

Tumor response was assessed using Response Evaluation Criteria in Solid Tumors (RECIST) v1.1 at weeks 12 and 24 via contrast-enhanced computed tomography (CT) or magnetic resonance imaging (MRI). Patients were classified as **responders (R)** if they achieved complete response (CR) or partial response (PR), and as **non-responders (NR)** if they exhibited stable disease (SD) or progressive disease (PD) as best overall response at week 24. Progression-free survival (PFS) was defined as the time from ICI initiation to disease progression or death from any cause.

RESULTS

Patient Demographics and Clinical Characteristics

A total of 87 patients were screened, of whom 72 met the eligibility criteria and were enrolled. Of these, 64 completed the 24-week follow-up and were included in the final analysis (8 patients were excluded due to early death [$n = 3$], loss to follow-up [$n = 3$], or initiation of concurrent antibiotics [$n = 2$]). The cohort comprised 41 NSCLC and 23 HCC patients. Based on RECIST v1.1 at week 24, 26 patients (40.6%) were classified as responders (R) and 38 patients (59.4%) as non-responders (NR). Baseline characteristics are summarized in Table I.

TABLE I: Baseline Demographic and Clinical Characteristics of the Study Cohort (N = 64)

Characteristic	Total (N = 64)	Responders (n = 26)	Non-Responders (n = 38)	p-value
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Age, years (median, IQR)	56 (48–64)	54 (46–62)	58 (50–65)	0.12
Sex, n (%)				0.68
Male	48 (75.0)	20 (76.9)	28 (73.7)	
Female	16 (25.0)	6 (23.1)	10 (26.3)	
Cancer type, n (%)				0.31
NSCLC	41 (64.1)	18 (69.2)	23 (60.5)	
HCC	23 (35.9)	8 (30.8)	15 (39.5)	
ECOG PS, n (%)				0.04*
0–1	49 (76.6)	23 (88.5)	26 (68.4)	
2	15 (23.4)	3 (11.5)	12 (31.6)	
PD-L1 TPS $\geq 1\%$, n (%)	34 (53.1)	18 (69.2)	16 (42.1)	0.03*
Prior antibiotic use (>8 wks), n (%)	29 (45.3)	8 (30.8)	21 (55.3)	0.05*
Dietary pattern, n (%)				0.42
High-fiber (>25 g/day)	18 (28.1)	9 (34.6)	9 (23.7)	
Low-fiber (<25 g/day)	46 (71.9)	17 (65.4)	29 (76.3)	
BMI, kg/m ² (median, IQR)	24.1 (21.3–27.5)	24.8 (22.0–27.9)	23.5 (20.8–27.0)	0.28
Hepatitis B/C positive (HCC only), n (%)	17/23 (73.9)	5/8 (62.5)	12/15 (80.0)	0.62
ICI regimen, n (%)				0.55
Pembrolizumab	30 (46.9)	13 (50.0)	17 (44.7)	
Nivolumab	22 (34.4)	9 (34.6)	13 (34.2)	
Atezolizumab	12 (18.7)	4 (15.4)	8 (21.1)	

* Statistically significant ($p < 0.05$). IQR = interquartile range; ECOG PS = Eastern Cooperative Oncology Group performance status; PD-L1 TPS = programmed death-ligand 1 tumor proportion score; BMI = body mass index; ICI = immune checkpoint inhibitor; NSCLC = non-small cell lung cancer; HCC = hepatocellular carcinoma.

Gut Microbiome Composition and Diversity

Shotgun metagenomic sequencing yielded a median of 42.3 million quality-filtered reads per sample. Alpha diversity, measured by the Shannon index, was significantly higher in responders compared to non-responders at baseline (median 3.82 vs. 3.14; $p = 0.003$). Principal coordinates analysis (PCoA) based on Bray–Curtis dissimilarity revealed distinct clustering of responder and non-responder microbiomes (PERMANOVA, $R^2 = 0.11$, $p = 0.001$). Differential abundance analysis identified several taxa significantly enriched in responders, including *Akkermansia muciniphila*, *Faecalibacterium prausnitzii*, *Bifidobacterium longum*, and *Ruminococcus bromii*. Conversely, non-responders exhibited enrichment of *Bacteroides fragilis*, *Klebsiella pneumoniae*, and *Escherichia coli* (Table II).

TABLE II: Differentially Abundant Gut Microbial Taxa Between Responders and Non-Responders at Baseline

Taxon (Species Level)	Relative Abundance in Responders (median)	Relative Abundance in Non-Responders (% , median)	Log ₂ Fold Change (R vs. NR)	FDR-adjusted q -value
<i>Akkermansia muciniphila</i>	3.42	0.28	+3.61	< 0.001
<i>Faecalibacterium prausnitzii</i>	8.15	3.21	+1.35	0.002
<i>Bifidobacterium longum</i>	2.87	0.64	+2.17	0.004
<i>Ruminococcus bromii</i>	1.93	0.41	+2.23	0.008
<i>Roseburia intestinalis</i>	2.54	1.12	+1.18	0.021
<i>Eubacterium rectale</i>	1.78	0.73	+1.29	0.034
<i>Bacteroides fragilis</i>	0.31	2.14	−2.79	0.001
<i>Klebsiella pneumoniae</i>	0.12	1.87	−3.96	< 0.001
<i>Escherichia coli</i>	0.45	2.63	−2.55	0.003
<i>Fusobacterium nucleatum</i>	0.08	0.92	−3.53	0.006
<i>Clostridium difficile</i>	0.02	0.54	−4.76	0.012

FDR = false discovery rate. Positive fold change indicates enrichment in responders; negative fold change indicates enrichment in non-responders.

Fecal and Serum Microbial Metabolite Profiles

Targeted UHPLC-MS/MS metabolomics revealed significant differences in both fecal and serum metabolite concentrations between responders and non-responders (Table III). Responders exhibited significantly higher fecal concentrations of butyrate and propionate, while non-responders had elevated levels of secondary bile acids, particularly DCA and LCA. Serum levels of the tryptophan metabolite IPA were significantly higher in responders, whereas kynurenine, an immunosuppressive metabolite of the host kynurenine pathway, was elevated in non-responders.

TABLE III: Comparative Analysis of Fecal and Serum Microbial Metabolites Between Responders and Non-Responders

Metabolite	Matrix	Responders (median, IQR)	Non-Responders (median, IQR)	<i>p</i> -value
Short-Chain Fatty Acids				
Acetate (μmol/g)	Fecal	48.3 (35.1–62.7)	44.1 (30.5–58.2)	0.31
Propionate (μmol/g)	Fecal	18.7 (12.4–24.3)	11.2 (7.8–16.5)	0.004*
Butyrate (μmol/g)	Fecal	14.2 (9.8–19.6)	6.3 (3.7–10.1)	< 0.001*
Isobutyrate (μmol/g)	Fecal	2.1 (1.3–3.4)	3.8 (2.5–5.2)	0.018*
Bile Acids				
Cholic acid (CA) (μmol/g)	Fecal	3.4 (2.1–5.2)	4.1 (2.8–6.3)	0.22
CDCA (μmol/g)	Fecal	2.8 (1.6–4.5)	3.2 (2.0–5.1)	0.38
DCA (μmol/g)	Fecal	1.2 (0.6–2.1)	4.7 (2.9–7.8)	< 0.001*
LCA (μmol/g)	Fecal	0.8 (0.3–1.4)	3.1 (1.8–5.4)	< 0.001*
UDCA (μmol/g)	Fecal	1.5 (0.9–2.3)	0.7 (0.3–1.2)	0.009*
Tryptophan Metabolites				
IAA (μmol/L)	Serum	3.2 (2.1–4.8)	2.8 (1.9–4.1)	0.27
IPA (μmol/L)	Serum	8.4 (5.7–12.3)	3.1 (1.8–5.6)	< 0.001*
IAld (μmol/L)	Serum	1.1 (0.6–1.8)	1.4 (0.8–2.3)	0.19
Kynurenine (μmol/L)	Serum	1.8 (1.2–2.5)	3.6 (2.4–5.1)	< 0.001*
Kynurenine/Tryptophan ratio	Serum	0.031 (0.022–0.045)	0.068 (0.048–0.091)	< 0.001*

* Statistically significant ($p < 0.05$). IQR = interquartile range; CDCA = chenodeoxycholic acid; DCA = deoxycholic acid; LCA = lithocholic acid; UDCA = ursodeoxycholic acid; IAA = indole-3-acetic acid; IPA = indole-3-propionic acid; IAld = indole-3-aldehyde.

Peripheral Immune Cell Profiling

Flow cytometric analysis of baseline PBMCs revealed distinct immune landscapes between responders and non-responders (Table IV). Responders demonstrated a significantly higher frequency of circulating CD8+ T cells and a lower frequency of Tregs and MDSCs. Notably, the expression of exhaustion markers PD-1 and TIM-3 on CD8+ T cells was significantly lower in responders. Serum cytokine analysis showed higher IFN-γ and lower IL-10 and TGF-β levels in responders.

TABLE IV: Baseline Peripheral Blood Immune Cell Frequencies and Serum Cytokine Levels

Immune Parameter	Responders (median, IQR)	Non-Responders (median, IQR)	<i>p</i> -value
Cell Frequencies (% of live PBMCs)			
CD8+ T cells (CD3+CD8+)	22.4 (17.8–28.1)	14.6 (10.2–19.3)	0.002*
CD4+ T cells (CD3+CD4+)	38.5 (32.1–44.7)	36.2 (29.8–42.5)	0.34
CD8+/CD4+ ratio	0.58 (0.45–0.74)	0.40 (0.28–0.53)	0.006*
Tregs (CD4+CD25+FoxP3+)	4.2 (3.1–5.8)	7.8 (5.9–10.4)	< 0.001*
MDSCs (CD11b+CD33+HLA-DR–)	2.1 (1.3–3.4)	5.6 (3.8–8.2)	< 0.001*
NK cells (CD3–CD56+)	12.3 (8.7–16.5)	9.8 (6.4–13.2)	0.08
CD8+ T Cell Exhaustion Markers (% of CD8+)			
PD-1+	18.5 (12.3–25.7)	34.2 (26.8–42.1)	< 0.001*
TIM-3+	8.4 (5.2–12.6)	18.7 (13.4–25.3)	< 0.001*
LAG-3+	5.1 (3.2–7.8)	7.3 (4.9–10.6)	0.04*
Serum Cytokines (pg/mL)			
IFN-γ	42.3 (28.5–61.7)	18.4 (10.2–29.8)	0.001*
IL-6	15.8 (9.4–24.3)	28.6 (18.7–42.1)	0.003*
IL-10	8.2 (5.1–12.4)	22.7 (15.3–34.8)	< 0.001*
TNF-α	12.4 (7.8–18.6)	14.1 (9.2–21.3)	0.32
TGF-β	1,240 (820–1,780)	2,850 (1,960–4,120)	< 0.001*

Statistically significant ($p < 0.05$). IQR = interquartile range; PBMCs = peripheral blood mononuclear cells; Tregs = regulatory T cells; MDSCs = myeloid-derived suppressor cells; NK = natural killer; PD-1 = programmed cell

death protein 1; TIM-3 = T-cell immunoglobulin and mucin-domain containing-3; LAG-3 = lymphocyte-activation gene 3; IFN- γ = interferon-gamma; IL = interleukin; TNF- α = tumor necrosis factor-alpha; TGF- β = transforming growth factor-beta.

Multivariate Predictors of Immunotherapy Response

A multivariate logistic regression model incorporating clinical, microbial, and metabolomic variables identified four independent predictors of ICI response (Table V). The presence of *A. muciniphila* ($\geq 1\%$ relative abundance), high fecal butyrate (≥ 10 $\mu\text{mol/g}$), high serum IPA (≥ 5 $\mu\text{mol/L}$), and low serum kynurenine/tryptophan ratio (< 0.05) were each independently associated with response. The model demonstrated an area under the receiver operating characteristic curve (AUROC) of 0.87 (95% CI: 0.78–0.95).

TABLE V: Multivariate Logistic Regression Analysis of Independent Predictors of Immunotherapy Response

Variable	Odds Ratio (OR)	95% Confidence Interval	<i>p</i> -value
<i>A. muciniphila</i> $\geq 1\%$ relative abundance	5.42	1.87–15.68	0.002
Fecal butyrate ≥ 10 $\mu\text{mol/g}$	4.18	1.52–11.47	0.006
Serum IPA ≥ 5 $\mu\text{mol/L}$	3.76	1.34–10.53	0.012
Kynurenine/Tryptophan ratio < 0.05	3.91	1.41–10.84	0.009
PD-L1 TPS $\geq 1\%$	2.14	0.78–5.89	0.14
ECOG PS 0–1	2.38	0.62–9.14	0.21
No prior antibiotic use (> 8 wks)	1.87	0.64–5.48	0.25

TPS = tumor proportion score; ECOG PS = Eastern Cooperative Oncology Group performance status; IPA = indole-3-propionic acid.

Correlation Network: Microbiota–Metabolite–Immune Axis

Spearman correlation analysis revealed a tightly interconnected network linking specific microbial taxa, metabolites, and immune parameters. *A. muciniphila* abundance positively correlated with serum IPA ($r = 0.62$, $p < 0.001$) and CD8+ T-cell frequency ($r = 0.54$, $p < 0.001$). *F. prausnitzii* and *R. bromii* positively correlated with fecal butyrate ($r = 0.71$ and $r = 0.58$, respectively; both $p < 0.001$). Fecal butyrate, in turn, inversely correlated with Treg frequency ($r = -0.48$, $p < 0.001$) and PD-1 expression on CD8+ T cells ($r = -0.51$, $p < 0.001$). Conversely, *K. pneumoniae* abundance positively correlated with fecal DCA ($r = 0.56$, $p < 0.001$), which was associated with elevated MDSC frequency ($r = 0.49$, $p < 0.001$) and serum TGF- β ($r = 0.53$, $p < 0.001$).

Survival Outcomes

At a median follow-up of 14.2 months, the median PFS was significantly longer in responders (11.4 months; 95% CI: 8.2–14.6) compared to non-responders (3.8 months; 95% CI: 2.9–4.7; log-rank $p < 0.001$). Patients harboring the composite "favorable microbiome signature" (presence of *A. muciniphila* $\geq 1\%$, fecal butyrate ≥ 10 $\mu\text{mol/g}$, and serum IPA ≥ 5 $\mu\text{mol/L}$) had a median PFS of 12.1 months versus 3.2 months for those lacking this signature (HR = 0.28; 95% CI: 0.15–0.52; $p < 0.001$).

CONCLUSION

This prospective cohort study elucidates the critical role of the gut microbiome and its metabolic effectors in modulating anti-tumor immunity and determining the clinical efficacy of immune checkpoint inhibitors (ICIs) in patients with advanced NSCLC and HCC within a Pakistani population. We demonstrate that a "favorable microbiome signature", defined by the enrichment of *Akkermansia muciniphila* and *Faecalibacterium prausnitzii*, elevated fecal butyrate, and high serum indole-3-propionic acid (IPA), is intricately linked to enhanced systemic anti-tumor immunity. This favorable profile is characterized by robust CD8+ T-cell responses and a marked reduction in immunosuppressive Treg and MDSC populations. Importantly, this composite signature, alongside a low serum kynurenine-to-tryptophan ratio, serves as a powerful, independent predictor of ICI response and prolonged progression-free survival.

Conversely, our findings highlight that microbial dysbiosis and the accumulation of immunosuppressive metabolites, particularly secondary bile acids (DCA and LCA) and kynurenine, drive T-cell exhaustion and contribute to primary ICI resistance. The high predictive accuracy of our multi-omic model (AUROC 0.87) underscores the clinical utility of integrating metagenomic and metabolomic profiling to stratify patients and personalize immunotherapy regimens. Furthermore, given the distinct dietary and environmental factors prevalent in South Asia, these region-specific microbiomes signatures provide valuable insights into the global heterogeneity of immunotherapy responses and the necessity of population-specific biomarker validation. Moving forward, the identification of these mechanistic links within the microbiota-metabolite-immune axis opens the door to precision microbiome-directed therapeutics. Future large-scale, multi-center randomized controlled trials are warranted to validate these composite biomarkers and evaluate the clinical efficacy of targeted interventions, such as defined next-generation probiotics, precision dietary modifications, or fecal microbiota transplantation, in reprogramming the gut ecosystem to overcome ICI resistance. Ultimately, leveraging the gut-immune axis represents a transformative strategy to expand the therapeutic benefit of immunotherapy, mitigate primary resistance, and improve long-term survival outcomes in patients with advanced solid tumors.

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