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Local tumor microbial signatures and response to checkpoint blockade in non-small cell lung cancer

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ABSTRACT

In cancer patients, the clinical response to checkpoint-based immunotherapy is associated with the composition and functional quality of the host microbiome. While the relevance of the gut microbiome for checkpoint immunotherapy outcome has been addressed intensively, data on the role of the local tumor microbiome are missing. Here, we set out to molecularly characterize the local non-small cell lung cancer microbiome using 16S rRNA gene amplicon sequencing of bronchoscopic tumor biopsies from patients treated with PD-1/PD-L1-targeted checkpoint inhibitors. Our analyses showed significant diversity of the tumor microbiome with high proportions of Firmicutes, Bacteroidetes and Proteobacteria. Correlations with clinical data revealed that high microbial diversity was associated with improved patient survival irrespective of radiology-based treatment response. Moreover, we found that the presence of Gammaproteobacteria correlated with low PD-L1 expression and poor response to checkpoint-based immunotherapy, translating into poor survival. Our study suggests novel microbiome-specific/derived biomarkers for checkpoint immunotherapy response prediction and prognosis in lung cancer. In a broader sense, our data draw attention to the local tumor microbial habitat as an important addition to the spatially separated microbiome of the gut compartment.

Background

Immune checkpoint inhibitor (ICI) therapy, either alone or in combination with classical chemotherapeutic drugs, constitutes the mainstay of treatment for advanced non-small cell lung cancer (NSCLC), in the absence of targetable molecular driver alterations.[1,2] ICIs are powerful therapeutic agents that harness the natural specificity and adaptability of the immune system to reinvigorate anticancer immunity and tackle tumor heterogeneity[3,4] and cancer-associated immune suppression.[5] Across tumor entities, biomarkers for ICI response prediction include target antigen expression (e.g., PD-1/PD-L1),[6] tumor mutational burden (TMB),[7,8] DNA mismatch repair deficiency/ microsatellite instability,[9] and tumor T cell infiltration at baseline;[10] however, ICI responsiveness also relies on the composition and functional quality of the host microbiome.[11–13] Microbes are particularly abundant in the gastrointestinal (GI) tract,[14] and certain species of this compartment (mostly bacterial commensals) have been shown to be associated with ICI treatment efficacy through various immunological and metabolic mechanisms.[15,16] In the case of lung cancer, the GI microbiome is anatomically distant despite a postulated functional interaction via the gut-lung axis,[17,18] which theoretically compromises the relevance of gut microbes in relation to more proximal habitats.[19,20] Therefore, profiling of the local NSCLC tumor microbiome, as a reflection of the overarching pulmonary microbial landscape,[18] is important to (i) get a better understanding of local tumor–microbiome interactions and (ii) potentially establish novel biomarkers with superior predictive value in ICI-treated NSCLC.

In this proof-of-concept study, we aimed at analyzing bacterial species directly in NSCLC tissue, using mostly bronchoscopic tumor biopsies. Our study reports novel microbiome-related NSCLC biomarkers in the tumor environment and emphasizes the importance of the local tumor microbial habitat for ICI responsiveness and patient outcome.

Methods

NSCLC biopsies and patient characteristics

Bronchoscopic (n = 35) and surgical (n = 3) biopsies from 38 patients with advanced-stage NSCLC treated with ICIs were obtained from the St. Gallen Lung Biopsy Biobank, a dedicated biobank for pulmonary samples maintained and operated by the Lung Center of the Cantonal Hospital St. Gallen. Healthy adjacent lung tissue macroscopically free of tumor was obtained from NSCLC patients undergoing curative-intent surgical treatment and was used for control purpose (n = 10). Tumor biopsies were from stage III–IV NSCLC patients who

received a PD-1/PD-L1-targeting ICI (pembrolizumab, nivolumab, atezolizumab, durvalumab, or spartalizumab) in various therapy lines (first, second, or third) with or without a chemotherapy doublet (carboplatin-pemetrexed, cisplatin-pemetrexed, or carboplatin-paclitaxel) or an investigational treatment (MK-1308/anti-CTLA-4, NIS793/anti-TGF-β, NIR178/adenosine A2a receptor antagonist). Importantly, biopsies were sampled before the start of treatment. The patient population comprised 80% males and the main NSCLC histological subtypes were well-balanced (43% adenocarcinoma (ADC) and 53% squamous cell carcinoma (SCC)). PD-L1 expression status was available for 77% of the cases (12 patients with 0–20% of the cells positive, 11 patients with >20% of the cells positive) and was determined using the VENTANA PD-L1 (SP263) Assay from Roche Diagnostics (Rotkreuz, Switzerland). 30% of the patients had a known mutation in one of the following proto-oncogenes: KRAS, EGFR, ERBB2, PIK3CA, MET. Of note, the MET alteration in one patient concerned a missense mutation in exon 14 (T992I) that may have influenced immunotherapy outcome.[21] In addition, the EGFR alteration found in another patient (a single nucleotide variant in exon 18) may also have impacted immunotherapy outcome.[22] Median progression-free survival (PFS) and median overall survival (OS) were 3.1 and 15.3 months, respectively, and the overall response rate to ICI therapy was 27%. Patient characteristics are specified in Table 1.

16S rRNA gene amplicon sequencing

Bronchoscopic (n = 35) and surgical (n = 3) NSCLC biopsies as well as healthy lung control tissues (n = 10) were subjected to 16S rRNA sequencing using Illumina MiSeq technology, a platform for targeted resequencing, expression profiling and metagenomics. To this end, PCR amplification protocols were optimized, and two-step NextEra PCR libraries were generated using 341 F (5′- CCT ACG GGN GGC WGC AG −3′) and 802 R (5′- GAC TAC HVG GGT ATC TAA TCC −3′) primers specific for the V3 and V4 regions of the bacterial 16S rRNA gene. The amount of total DNA (including host and other non-bacterial DNA) was determined using PicoGreen (Thermo Fisher Scientific, Waltham, MA) and 140.2 ± 33.7 ng of DNA (mean ± SEM) were used as input for PCR amplification emanating from similarly sized biopsies. Libraries were sequenced on the Illumina MiSeq platform employing a 500 cycles MiSeq Reagent Kit v2. Paired-end reads passing Illumina quality filtering were demultiplexed and trimmed of adaptor residuals using MiSeq reporter built-in data analysis software v2.6. The read quality was further analyzed using FastQC v0.11.8 and sequencing reads were trimmed of primers using cutadapt v2.8. Forward and reverse reads were merged to reproduce the sequenced molecule using USEARCH v11.0.667 and a minimum overlap of 15 bases. Further quality filtering allowed one expected error per merged read and discarded reads with ambiguous bases or an outlier amplicon size. Samples with a minimum of 5000 merged reads were denoised using the UNOISE module of the USEARCH package, and operational taxonomic units (OTUs) were defined. OTU

abundances were filtered for possible bleed-in contaminations making use of the UNCROSS algorithm. Sequences from the RDP 16S rRNA database served as reference and taxonomies were predicted using the SINTAX module included in the USEARCH package, setting the minimum confidence threshold to .5. Ultimately, 5 NSCLC and 5 healthy lung samples did not pass the quality controls and were excluded from

Table 1. Patient characteristics (n = 30).

Parameter		Median (range)
Age (years)		67 (23–79)
Progression-free survival (months)		3.1 (.7–28.7)
Overall survival (months)		15.3 (2.6–50.9)
Sex	**Case number (n)**	**Fraction (%)**
female	6	20.0
male	24	80.0
NSCLC histological subtype		
ADC	13	43.3
SCC	16	53.3
unknown	1	3.3
Tumor stage		
III	7	23.3
IVA	7	23.3
IVB	14	46.7
unknown	2	6.7
ICI therapy line		
first	9	30.0
second	16	53.3
third	5	16.7
ICI used		
Pembrolizumab (anti-PD-1)	12	40.0
Nivolumab (anti-PD-1)	12	40.0
Atezolizumab (anti-PD-L1)	1	3.3
Durvalumab (anti-PD-L1)	3	10.0
Spartalizumab (anti-PD-1)	2	6.7
Combination anticancer therapy		
yes (chemotherapy)	5	16.7
yes (investigational medicine)	3	10.0
no (ICI monotherapy)	22	73.3
ICI treatment response		
CR	0	.0
PR	8	26.7
SD	6	20.0
PD	14	46.7
unknown	2	6.7
PD-L1 expression status		
0–20% of cells positive	12	40.0
>20% of cells positive	11	36.7
unknown	7	23.3
Known mutation in a proto-oncogene		
no	21	70.0
yes	9	30.0
KRAS G12C	1	3.3
KRAS G13C	1	3.3
KRAS G12F	1	3.3
KRAS G12D	1	3.3
KRAS G12A	1	3.3
EGFR SNV exon 18	1	3.3
ERBB2 G776delinsVC	1	3.3
PIK3CA SNV exon 9	1	3.3
MET T992I	1	3.3
Number of packyears		
0–40	13	43.3
>40	13	43.3
unknown	4	13.3

ADC, adenocarcinoma; CR, complete response; ICI, immune checkpoint inhibitor; NSCLC, non-small cell lung cancer; PD, progressive disease; PR, partial response; SCC, squamous cell carcinoma; SD, stable disease; SNV, single nucleotide variant.

subsequent analysis; thus, a total of 30 NSCLC biopsies (28 bronchoscopic, 2 surgical) and 5 healthy lung samples were finally analyzed. Three non-template controls (NTCs) were run. Signals detected in the NTCs were subtracted from the whole analysis to correct for potential reagent contamination by trace amounts of bacterial DNA as reported.[23–25] 16S rRNA gene amplicon sequencing as described in this section was performed by Microsynth AG (Balgach, Switzerland). All samples were processed in parallel, starting from DNA isolation, PCR amplification, library preparation and sequencing, thus eliminating potential batch effects.

Data analysis and statistical considerations

Phylum, class and OTU abundances were analyzed in a descriptive manner. Bacterial α-diversity was estimated using the Shannon diversity index (SDI)[26] as well as the number of unique OTUs observed. Correlation of the SDI with the number of unique OTUs was analyzed using Spearman's rank correlation coefficient. Two-group data were tested for statistical significance using the Mann–Whitney U test, and three-group data were statistically analyzed using the Kruskal–Wallis test. A Cox proportional hazard model was used to analyze phylum and class abundance with respect to PFS and OS, and Wald test p-values are reported. Time to event data (patient survival) were analyzed using Kaplan–Meier estimates and log-rank tests (X^2 statistics and associated p-values are reported). Where applicable, optimized cutoffs have been used for data analysis (e.g., to harmonize group sizes). Unless otherwise stated, quantitative data are shown as boxplots with whiskers and outliers. The nominal significance level was .05. Statistical analyses were performed using IBM SPSS Statistics 20 and the R statistical software (www.r-project.org).

Ethics statement

Patients donating their samples to the St. Gallen Lung Biopsy Biobank provided written informed consent for use of their biological material for research purpose (EKSG 11/044). The study was approved by the local Ethical Review Board (Ethikkommission Ostschweiz) under BASEC number 2019–02059.

Results

### 16S rRNA gene amplicon sequencing deciphers tumor microbial diversity

To characterize the tumor microbial habitat of NSCLC, broncho-scopic (n = 28) and surgical (n = 2) tumor biopsies as well as healthy lung control tissues (n = 5) were subjected to 16S rRNA gene amplicon sequencing.[27,28] Bacterial species from various phyla were detectable in the samples, which could be allocated to 224 individual OTUs. OTU abundances ranged from <5000 reads to >30000 reads and showed high proportions of Firmicutes, Bacteroidetes and Proteobacteria in both NSCLC biopsies and healthy lung samples (Figure 1a). In NSCLC biopsies, the number of unique OTUs observed (ranging from 3 to 109) was highly correlated with the SDI, an established measure for α-diversity taking into account both the number of species and their abundance (r = .926, p < .001, Figure 1b). In summary, our results show that 16S rRNA gene amplicon sequencing represents a suitable method to analyze bacteria in bronchoscopic NSCLC biopsies and suggest significant diversity of the tumor microbial habitat along with marked interpatient variation.

Survival benefit of patients with a higher tumor microbial diversity

Analyzing the SDI in stratified groups of patients did not reveal associations with sex (female vs. male, p = .494), the number of packyears (0–40 vs. >40, p = .650), tumor stage (III vs. IVA vs. IVB, p = .214), or the NSCLC histological subtype (ADC vs. SCC, p = 1.000) (Figure 2a-d). Along similar lines, the SDI was comparable in PD-L1^{low} (0–20%) vs. PD-L1^{high} (>20%) expressers (p = .786, Figure 2e). The SDI could not discriminate patients based on their observed radiological response to checkpoint-based immunotherapy (partial response (PR) vs. stable disease (SD) vs. progressive disease (PD)) (p = .161,
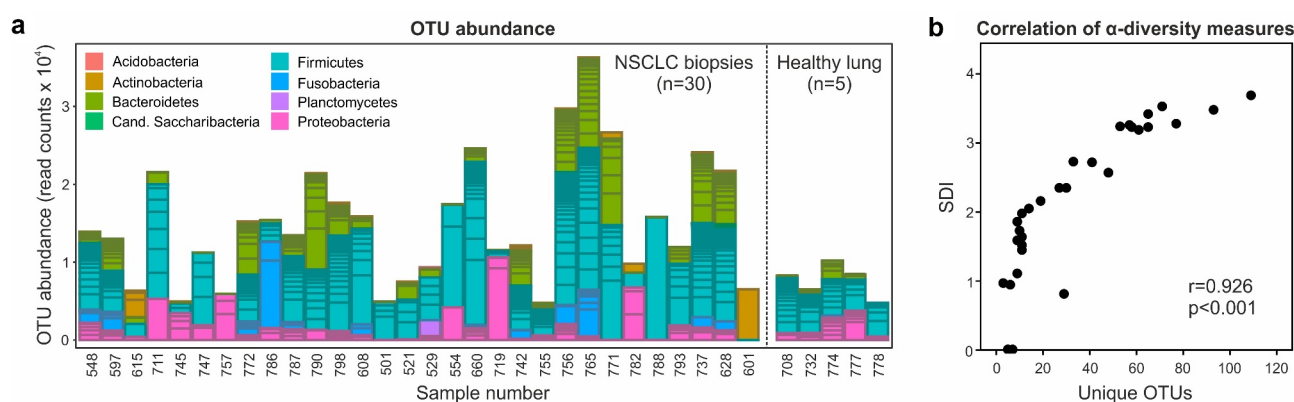
Figure 1. Microbial diversity of NSCLC tumors revealed through 16S rRNA gene amplicon sequencing. (a) OTU abundance in NSCLC and healthy lung samples and allocation to specific bacterial phyla. (b) Correlation of the SDI with the number of unique OTUs observed in NSCLC samples. NSCLC, non-small cell lung cancer; OTU, operational taxonomic unit; SDI, Shannon diversity index.
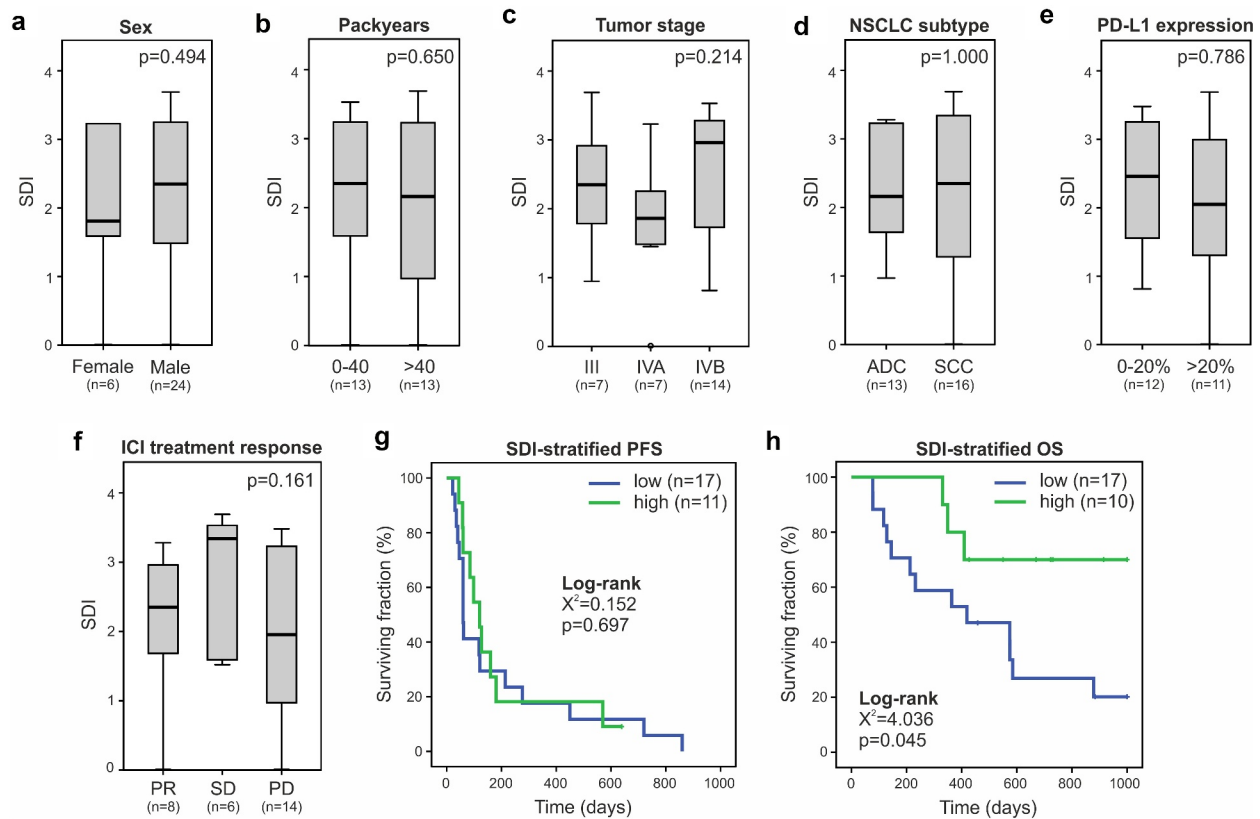
Figure 2. Tumor microbial diversity associates with NSCLC patient survival. (a-d) Analysis of the SDI in stratified subgroups of NSCLC patients. (e-h) Analysis of the SDI in terms of PD-L1 expression, ICI treatment responsiveness, and patient survival. (a-f) Boxes indicate the median (highlighted in bold) and interquartile ranges. Whiskers indicate the minimum and maximum values except in the case of outliers (outliers are indicated by circles). (G + H) cutoffs used for SDI stratification: 2.73 for PFS and 2.65 for OS. ADC, adenocarcinoma; ICI, immune checkpoint inhibitor; NSCLC, non-small cell lung cancer; OS, overall survival; PD, progressive disease; PFS, progression-free survival; PR, partial response; SCC, squamous cell carcinoma; SD, stable disease; SDI, Shannon diversity index.

figure 2f) and, accordingly, PFS was indifferent among SDI^{low} vs SDI^{high} patients ($\chi^2 = .152$, $p = .697$, Figure 2g). However, patients with a high SDI showed significantly longer OS ($\chi^2 = 4.036$, $p = .045$, Figure 2h), which indicates benefit from a higher microbial diversity irrespective of checkpoint immunotherapy responsiveness. Collectively, the diversity of the tumor bacterial metagenome correlates with specific survival metrics in ICI-treated NSCLC.

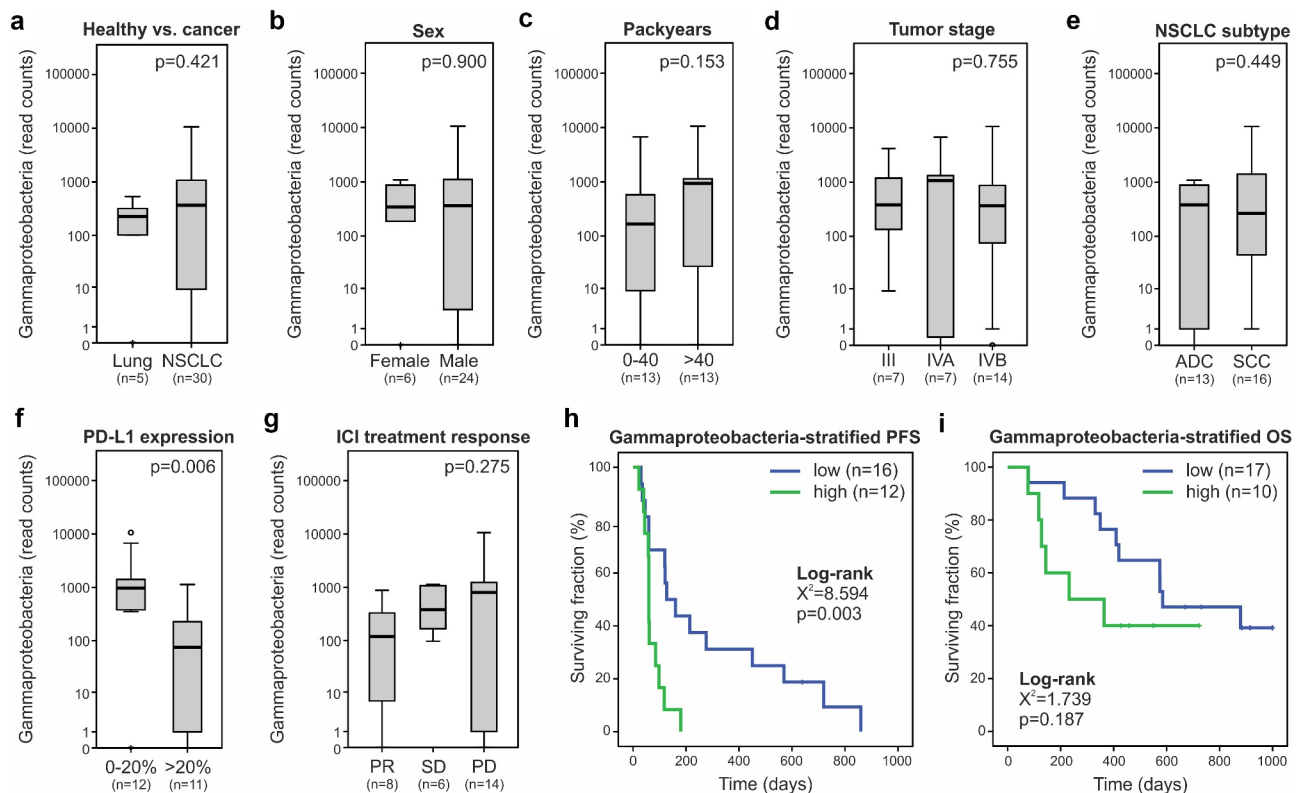### Gammaproteobacteria constitute a significant part of the NSCLC tumor microbiome

We next sought to investigate which types of bacteria would play a role in shaping or predicting the response to PD-1/PD-L1-targeted ICIs. As shown in Figure 3a, the phyla of Firmicutes, Bacteroidetes and Proteobacteria were largely dominating the scene, jointly accounting for almost 90% of the detected tumor microbiome. Other phyla still showing appreciable abundance included Fusobacteria and Actinobacteria (Figure 3a). A Cox proportional hazard model revealed that only the phylum of Proteobacteria was significantly associated with PFS ($p < .001$) and OS ($p < .001$) in our cohort and that the class of Gammaproteobacteria (accounting for almost 60% of Proteobacteria, Figure 3a), was most significantly correlated with survival ($p < .001$ for PFS and $p = .011$ for OS) (Figure 3b). In summary, Gammaproteobacteria account for roughly 7% of the total detected microbiome and are associated with survival in NSCLC patients receiving immunotherapy.

Gammaproteobacteria correlate with low PD-L1 expression and predict poor checkpoint immunotherapy responsiveness

Based on the predictions from the Cox proportional hazard model (Figure 3b), we analyzed the abundance of Gammaproteobacteria in stratified patient subgroups and also comparatively investigated their levels in healthy lung vs. NSCLC tissues. Although not reaching statistical significance, Gammaproteobacteria levels appeared to be higher in lung cancer tissue as compared to healthy lung control tissue ($p = .421$, Figure 4a). Statistical significance was also not reached when stratifying according to sex (female vs. male, $p = .900$), the number of packyears (0–40 vs. >40, $p = .153$), tumor stage (III vs. IVA vs. IVB, $p = .755$), or the NSCLC histological subtype (ADC vs. SCC, $p = .449$), even though some trends were observed (Figure 4b-e). In contrast, high abundance of Gammaproteobacteria was significantly associated with low PD-L1 expression ($p = .006$, figure 4f), a finding that was also partly reflected in the response to ICI therapy (PR vs. SD vs. PD) ($p = .275$, Figure 4g). In line with these data, patients with a high abundance of Gammaproteobacteria in their tumors showed a significantly worse PFS ($\chi^2 = 8.594$, $p = .003$, Figure 4h), with OS revealing a similar trend without reaching statistical significance ($\chi^2 = 1.739$, $p = .187$, Figure 4i). Taken together, Gammaproteobacteria appear to be enriched in the cancerous lung and their abundance in the tumor surroundings correlates with low PD-L1 expression and poor PFS and a trend toward worse OS under ICI therapy.
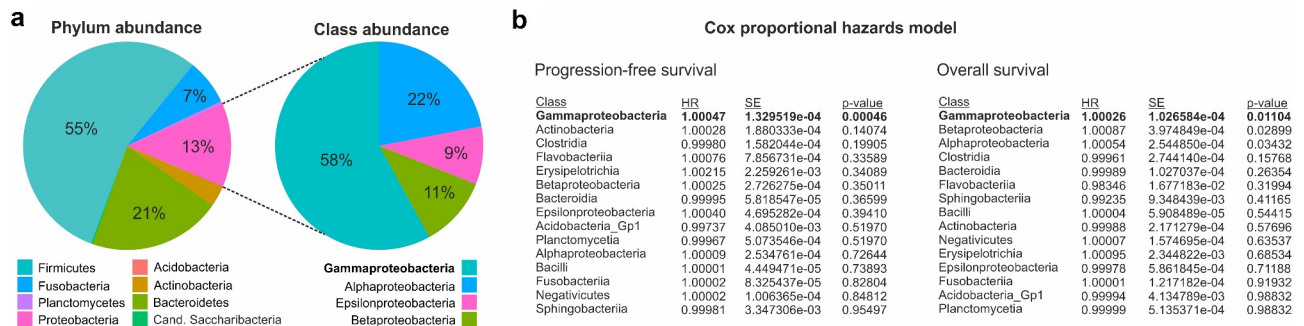
Figure 3. Gammaproteobacteria are abundant in NSCLC tumors and associate with patient survival. (a) Pie charts illustrating the relative abundance of particular bacterial phyla and classes within the total detected tumor microbiome. (b) Results of a Cox proportional hazards model for the analysis of possible associations of bacterial classes with patient survival ranked according to statistical significance. HR, hazard ratio; NSCLC, non-small cell lung cancer; SE, standard error.

b Cox proportional hazards model

Progression-free survival

Class	HR	SE	p-value
Gammaproteobacteria	**1.00047**	**1.329519e-04**	**0.00046**
Actinobacteria	1.00028	1.880333e-04	0.14074
Clostridia	0.99980	1.582044e-04	0.19905
Flavobacteriia	1.00076	7.856731e-04	0.33589
Erysipelotrichia	1.00215	2.259261e-03	0.34089
Betaproteobacteria	1.00025	2.726275e-04	0.35011
Bacteroidia	0.99995	5.818547e-05	0.36599
Epsilonproteobacteria	1.00040	4.695282e-04	0.39410
Acidobacteria_Gp1	0.99737	4.085010e-03	0.51970
Planctomycetia	0.99967	5.073546e-04	0.51970
Alphaproteobacteria	1.00009	2.534761e-04	0.72644
Bacilli	1.00001	4.449471e-05	0.73893
Fusobacteriia	1.00002	8.325437e-05	0.82804
Negativicutes	1.00002	1.006365e-04	0.84812
Sphingobacteriia	0.99981	3.347306e-03	0.95497

Overall survival

Class	HR	SE	p-value
Gammaproteobacteria	**1.00026**	**1.026584e-04**	**0.01104**
Betaproteobacteria	1.00087	3.974849e-04	0.02899
Alphaproteobacteria	1.00054	2.544850e-04	0.03432
Clostridia	0.99961	2.744140e-04	0.15768
Bacteroidia	0.99989	1.027037e-04	0.26354
Flavobacteriia	0.98346	1.677183e-02	0.31994
Sphingobacteriia	0.99235	9.348439e-03	0.41165
Bacilli	1.00004	5.908489e-05	0.54415
Actinobacteria	0.99988	2.171279e-04	0.57696
Negativicutes	1.00007	1.574695e-04	0.63537
Erysipelotrichia	1.00095	2.344822e-03	0.68534
Epsilonproteobacteria	0.99978	5.861845e-04	0.71188
Fusobacteriia	1.00001	1.217182e-04	0.91932
Acidobacteria_Gp1	0.99994	4.134789e-03	0.98832
Planctomycetia	0.99999	5.135371e-04	0.98832

Figure 4. Gammaproteobacteria correlate with low PD-L1 expression and poor patient survival under ICI therapy. (a) Comparative analysis of Gammaproteobacteria abundance in healthy lung and NSCLC samples. (b-e) Analysis of Gammaproteobacteria abundance in stratified subgroups of NSCLC patients. (f-i) Analysis of Gammaproteobacteria abundance in terms of PD-L1 expression, ICI treatment responsiveness, and patient survival. (a-g) Boxes indicate the median (highlighted in bold) and interquartile ranges. Whiskers indicate the minimum and maximum values except in the case of outliers (outliers are indicated by circles and extreme outliers are indicated by stars). (H + I) cutoffs used for Gammaproteobacteria stratification: 480 for PFS and 811 for OS. ADC, adenocarcinoma; ICI, immune checkpoint inhibitor; NSCLC, non-small cell lung cancer; OS, overall survival; PD, progressive disease; PFS, progression-free survival; PR, partial response; SCC, squamous cell carcinoma; SD, stable disease.

Discussion

The host microbiome serves important functions in health and disease and is particularly abundant in the GI tract,[14] the respiratory tract,[18] and on the skin.[29] Commensal microbes are essential gatekeepers for epithelial barrier integrity[30] and transient (e.g., antibiotics and infection) or chronic (e.g., long-term medication and underlying conditions) dysbiosis can prime to a variety of immune-related and metabolic disorders including inflammatory bowel disease, obesity, and type 2 diabetes.[31–35] In contrast, 'gain-of-function' dysbiosis can lead to the overgrowth of particular microbial strains in specific anatomical compartments, thus causing local opportunistic infections.[36,37] A high diversity of the host microbiome with a well-balanced and time-stable composition of bacteria, viruses and fungi is therefore essential to prevent disease and maintain health until old age.[38–40] Interactions of the microbiome with host cells are mediated mostly by immunological and metabolic means, and can be local, distant, or systemic.[18]

While, with the notable exception of colorectal cancer,[41] the significance of the microbiome for cancer development remains elusive, accumulating evidence suggests a pivotal role of host microbes in shaping the response to cancer therapy. Specifically, the use of antibiotics curtails ICI treatment efficacy[42–44] and also has a negative impact on the performance of classical cytotoxic drugs such as platinum and cyclophosphamide.[45] Importantly,

unresponsiveness of germ-free or antibiotic-treated mice to checkpoint inhibition can be rescued through oral supplementation with certain bacteria.[11,12] In ICI-treated melanoma, the fecal microbiome is significantly different between responding and non-responding patients,[13] and fecal microbiome transplantation (FMT) from responding patients is clinically evaluated as an ICI re-sensitizing intervention in the subgroup of treatment-refractory patients.[46] While these data clearly bear witness to a key role of host commensal microbes in cancer immunotherapy outcome, most studies have prioritized the GI microbiome irrespective of the tumor site of origin. However, the host microbiome in its totality is much wider, and more proximal tumor ecosystems may be equally important, if not dominant, in governing disease progression and treatment responsiveness.[19,20,47–51] Here, we have pictured the local tumor microbiome of NSCLC using 16S rRNA gene amplicon sequencing of tumor biopsies from patients treated with PD-1/PD-L1-targeted ICIs.

Results from this study converge on three key messages as follows: (i) 16S rRNA gene amplicon sequencing is technically feasible in bronchoscopic tumor biopsies that typically provide limited sample input and are further enriched for host cells rather than metagenomic signals (very low microbial biomass). (ii) A higher diversity of the tumor microbiome represents a prognostic factor, as it correlates with improved OS of ICI-treated NSCLC patients irrespective of the clinical response to ICI treatment or PFS. (iii) A high abundance of Gammaproteobacteria, a diverse class of gram-negative bacteria,[52] predicts low PD-L1 expression and poor response to PD-1/PD-L1-targeted checkpoint blockade, resulting in unfavorable patient survival. Importantly, the associations of SDI and Gammaproteobacteria with survival were cross-validated using random permutation of both strata based on the log-rank test X^2 statistics (**data not shown**).

While shotgun sequencing represents the gold standard for metagenomics in stool samples and additionally covers viruses, fungi and protozoa,[28] 16S rRNA sequencing is a valuable approach especially when microbial density is low such that sample composition is biased toward host cells as is the case for lung tissue[53] and fetal organs.[54] Here, we have characterized the local NSCLC microbiome as a potential mirror of the overarching pulmonary metagenomic landscape[18] using the latter approach. Our data suggest high tumoral abundances especially of Firmicutes, Bacteroidetes, and Proteobacteria. Our observation that a higher α-diversity predicts improved OS independently from the clinical response to ICI treatment is interesting and may indicate that a more diverse microbiome is selected for patients with a better health status or other factors associated with favorable outcome (e.g., less disease burden or more indolent disease). In addition, a higher diversity of the tumor microbiome may increase the chances for protective immunological cross-reactivity between microbial-derived peptide products and current or future arising tumor neoantigens,[15] indicating a more causal role of the microbiome as a mediator of a better tumor response to ICI therapy. It is challenging to uncouple prognostic vs. predictive features of α-diversity in the current study, and it cannot be inferred that a higher microbial diversity would also correlate with longer OS in untreated patients or patients not treated with ICIs but with other systemic agents. In comparison, a higher gut microbial diversity clearly represents a predictive biomarker in anti-PD-1-treated melanoma.[13] Our finding that Gammaproteobacteria correlate with low PD-L1 expression and poor ICI-related survival suggests a new potential biomarker for ICI response prediction in NSCLC while leaving unanswered questions about the underlying mechanisms. Certainly, Gammaproteobacteria represent a highly diverse class of bacteria with numerous metabolic pathways involved, and member species can act as both facultative (e.g., *E. coli*) and *bona fide* pathogens (*P. aeruginosa, S. typhimurium, H. influenzae, L. pneumophila*).[52] Whether the totality of Gammaproteobacteria downregulates PD-L1 expression and/or curtails anticancer immunity through metabolic or immunological rewiring[55] remains to be shown in future mechanistic studies.

The following limitations are applicable to the study, which lower the generalizability of the results: (i) The study is purely correlative and case numbers are limited. (ii) The study population was retrospectively selected and heterogeneous in terms of clinical parameters. (iii) Bronchoscopic biopsies provide a locoregional snapshot of the tumor and may not be representative of the tumor as a whole. (iv) The sampling and handling of bronchoscopic and surgical biopsies in non-sterile environments may have led to some degree of cross-contamination, a potential pitfall that might be more relevant to the smaller biopsy specimens. (v) No systematic data on the use of antibiotics immediately before or during checkpoint immunotherapy were available for the study population, thus prohibiting corresponding investigations. (vi) The study did not investigate non-bacterial microbes such as viruses and fungi. (vii) The bacterial signal in bronchoscopic tumor biopsies was low such that PCR amplification may have introduced some bias. In addition, results need to be interpreted in awareness of the fact that microbial signals detected in NTCs were subtracted from the test samples to account for reagent contamination as reported.[23–25] (viii) Many bacterial species were detectable in a few samples only such that species-level analyses could not be performed for statistical reasons.

We here show the technical feasibility of 16S rRNA gene amplicon sequencing-based bacterial identification in bronchoscopic tumor biopsies and report the identification of novel microbiome-specific biomarkers for prognosis and checkpoint immunotherapy response prediction in NSCLC. Our proof-of-concept study sets the stage for larger validation trials and gives impetus for endeavors to mechanistically dissect the underlying mechanisms and define therapeutic leads. In the long run, it is hoped that basic knowledge about the role of the local tumor microbiome can inspire rational combination therapies to re-sensitize to – or boost – checkpoint blockade,[56] e.g., through oral or inhaled supplementation with commensal microbes (re-installation of diversity) or the informed use of narrow-spectrum antibiotic/antiviral/antifungal agents (elimination of detrimental microbes).

Specific abbreviations used

ADC: Adenocarcinoma
CR: Complete response
FMT: Fecal microbiome transplantation
GI: Gastrointestinal
HR: Hazard ratio
ICI: Immune checkpoint inhibitor
NSCLCNon-small cell lung cancer

NTC: Non-template control
OS: Overall survival
OTU: Operational taxonomic unit
PD: Progressive disease
PFS: Progression-free survival
PRPartial response
SCC: Squamous cell carcinoma
SD: Stable disease
SDI: Shannon diversity index
SE: Standard error
SEM: Standard error of the mean
SNV: Single nucleotide variant
TMB: Tumor mutational burden

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Disclosure statement

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Data availability

Sequencing data are available from the corresponding author upon reasonable request.

Authors' contributions

Conception and design: MB, MHB
    Data collection: MB, FB, RR
    Data analysis and interpretation: MB, FB, WCA, LF, RR, SIR, MJ, MF, MHB
    Statistics: FB
    Wrote the first draft of the paper: MB, MHB
    Wrote the final version of the paper: MB, FB, WCA, LF, RR, SIR, MJ, MF, MHB
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