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ORIGINAL RESEARCH

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Combined tumor-associated microbiome and immune gene expression profiling predict response to neoadjuvant chemo-radiotherapy in locally advanced rectal cancer

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ABSTRACT

Locally advanced rectal cancer (LARC) is treated with neoadjuvant chemo-radiotherapy (nCRT) followed by surgery. A minority of patients show complete response (CR) to nCRT and may avoid surgery and its functional consequences. Instead, most patients show non-complete response (non-CR) and may benefit from additional treatments to increase CR rates. Reliable predictive markers are lacking. Aim of this study was to identify novel signatures predicting nCRT responsiveness. We performed a combined analysis of tumor-associated microbiome and immune gene expression profiling of diagnostic biopsies from 70 patients undergoing nCRT followed by rectal resection, including 16 with CR and 54 with non-CR. Findings were validated by an independent cohort of 49 patients, including 7 with CR and 42 with non-CR. Intratumoral microbiota significantly differed between CR and non-CR groups at genus and species level. Colonization by bacterial species of *Ruminococcus* genera was consistently associated with CR, whereas abundance of *Fusobacterium*, *Porhpyromonas*, and *Oscillibacter* species predicted non-CR. Immune gene profiling revealed a panel of 59 differentially expressed genes and significant upregulation of IFN-gamma and -alpha response in patients with CR. Integrated microbiome and immune gene profiling analysis unraveled clustering of microbial taxa with each other and with immune cell-related genes and allowed the identification of a combined signature correctly identifying non-CRS in both cohorts. Thus, combined intratumoral microbiome-immune profiling improves the prediction of response to nCRT. Correct identification of unresponsive patients and of bacteria promoting responsiveness might lead to innovative therapeutic approaches based on gut microbiota pre-conditioning to increase nCRT effectiveness in LARC.

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Introduction

Rectal cancer is common worldwide[1] and is mostly diagnosed at a locally advanced stage (T3–4 and/or N+).[2] Patients with locally advanced rectal cancer (LARC) are usually treated by a multimodal strategy including neoadjuvant chemo-radiation therapy (nCRT) followed by rectal low anterior resection with total mesorectal excision (TME).[3]

This combined therapeutic approach has significantly decreased rates of local recurrence and improved overall survival.[4] However, rectal surgery, in particular when combined with radiation therapy, leads to significant defecatory, urinary and sexual dysfunction.[5]

In 15–20% of the cases, nCRT ultimately results in a pathological complete response (CR), with no viable tumor cells left, as confirmed by histological analysis of the resected tissue.[6]

These patients might avoid surgery and be monitored with a "watch and wait" strategy.[7–9] New nCRT protocolos have been developed aiming at increasing response rates[10,11]but accuracy of clinical and radiological assessment of CR after nCRT remains suboptimal and local regrowth occurs in[7–9,12–14] in 20–40% of

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cases.[15–17] The identification of factors predicting response is needed to effectively tailor nCRT and provide new treatment targets.

A number of histopathologic and genetic biomarkers, including specific gene mutations, non-coding RNA expression and the presence of circulating tumor DNA, have been proposed,[18–24] but none of them has shown a sufficiently reliable predictive ability.

Recent evidence suggests that nCRT effectiveness might be associated with activation of the immune system. Indeed, tumor infiltration by CD3+, CD8+ or FOXP3+ lymphocytes has been found to be associated with favorable outcome and improved response to nCRT.[25–30] Radiation and oxaliplatin, currently used in nCRT protocols, are indeed known to induce an immunogenic form of cell death, leading to the release of damage-associated molecular patterns (DAMPs) by dying cells, ultimately inducing activation of myeloid cells, including antigen presenting cells (APCs), such as macrophages and dendritic cells.[31,32] By presenting tumor-associated antigens to T lymphocytes, APCs may promote the generation of tumor-specific immune responses.

The higher rate of response to nCRT when surgery is delayed,[33] is also consistent with the timing of induction of anti-tumor immune responses. However, factors influencing the composition of LARC immune contexture, and the activation of potentially relevant immune cell pathways require better understanding.

Intra-tumoral gut microbiota is known to modulate immune cell infiltration in human colon cancer.[34,35] Due to neoplastic alteration of the mucosal barrier, defined components of the gut microbiota do translocate into tumor tissues. Some bacterial species, such as *Fusobacterium nucleatum*, appear to support tumor progression by promoting tumor cell proliferation,[36] enhancing resistance of tumor cells to chemotherapy[37] or favoring the generation of an immunosuppressive environment.[38,39] On the other hand, abundance of other bacterial commensals has been found to be associated with high infiltration by immune cells and favorable outcome[35]or enhanced responsiveness to oxaliplatin.[40] Moreover, administration of specific bacteria has proved to effectively boost anti-tumor immune responses in CRC models.[41,42]

Little is known about LARC-associated microbiota. In a recent study, abundance and persistence of *Fusobacterium nucleatum* in LARC tissues, has been shown to correlate with unresponsiveness to nCRT.[43] In few recent studies exploring fecal microbiota, specific microbial signatures were correlated with response to nCRT[44–46]However, the predictive significance of LARC intra-tumoral microbiota has not been thoroughly investigated, and its relationship with tumor immune contexture has not been explored yet.

To fill this knowledge gap, in this work, we have characterized LARC-associated intratumoral microbiota and corresponding immune transcriptomic profiles in a cohort of patients treated with nCRT, and have analyzed the capacity of identified microbial and immune related signatures to predict response to nCRT.

Materials and methods

Patients' population

All patients consecutively admitted at the Regional Hospital of Lugano, of the Ente Ospedaliero Cantonale, between 2012 and 2019 with a diagnosis of non-metastatic LARC (T3 or T4 and/or N+, M0) and treated, as recommended by a multidisciplinary board, with nCRT (long course: 50,4 Gy over 5 weeks combined with Capecitabine, 1250 mg/m^2 daily) without interruption, followed by surgical resection, were retrospectively considered for inclusion in the Discovery Cohort.

Patients with a diagnosis of non-metastatic LARC (T3 or T4 and/or N+, M0), admitted at the Clinica Moncucco between 2013 and 2020, and treated with long course nCRT followed by surgical resection, were included in the Validation Cohort.

Patients whose diagnostic biopsy was no longer available or not adequate for further molecular characterization, or who did not provide written informed consent were excluded.

Eventually, 70/94 patients were included in the Discovery Cohort and 49/51 in the Validation Cohort (Figure 1 and Supplementary Figure S1).

The study was approved by the local Ethics Committee (Project-ID 2020–02387/CE 3759).

Clinical and pathological characteristics were retrieved from a prospectively maintained rectal cancer database and from electronic patient records. Local and systemic recurrence rates were determined from data obtained from electronic patient records and from the regional cancer registry.

Pathological response to nCRT was assessed according to the Mandard tumor regression grading system,[47,48] based on histological evaluation of tissues resected after TME. Tumor regression grade (TRG) was confirmed by an experienced pathologist (P.S.) based on histological reevaluation.

Patients were divided into two groups: complete responders (CRs), i.e. only cases with TRG-1 response, and non-complete responders (non-CRs), with TRG2–5 (see below).

Clinicopathologic characteristics were compared by Fisher's exact test.

Sample acquisition and processing

FFPE (Formalin Fixed Paraffin Embedded) tissues from diagnostic biopsies, obtained before any treatment from patients included in the study, were retrieved. Three 8 µm-thick and two 10 µm-thick serial sections were obtained for DNA and RNA extraction, respectively. The presence of malignant tissue within the selected area was confirmed by an experienced pathologist (P.S.) upon hematoxylin/eosin staining.

Following sample deparaffinization, genomic DNA (gDNA) and mRNA were extracted from consecutive slides of each specimen, using the respective QIAmp Mini kits (Qiagen, Chatsworth, CA, USA), according to the manufacturers' protocols, and were quantified by NanoDrop Onec (ThermoFisher Scientific, USA). DNA and RNA were then used for microbiome analysis and immune cell gene expression profiling, respectively (see below).

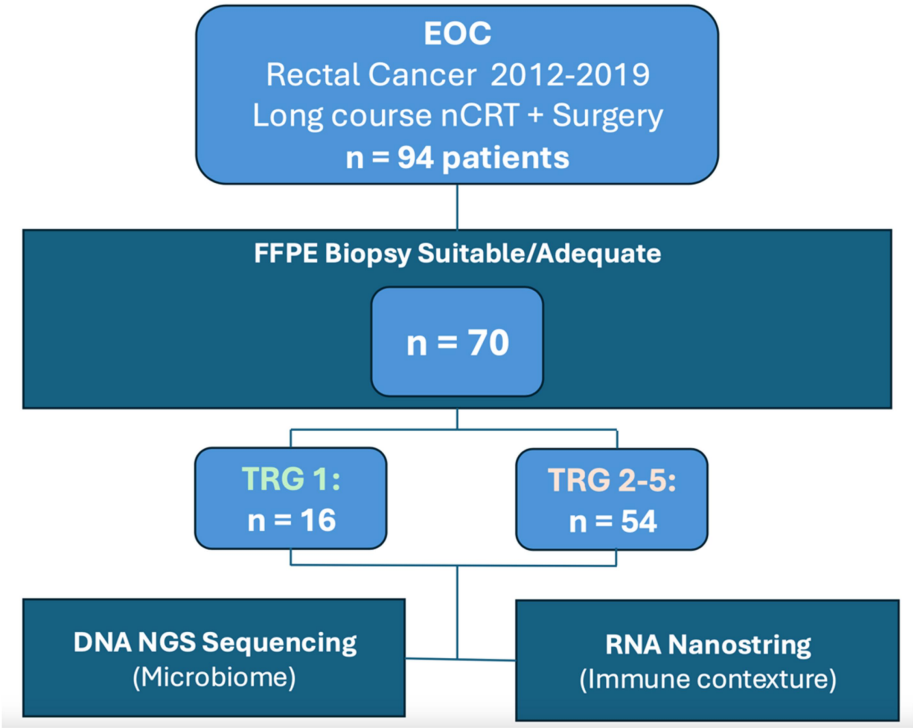

Figure 1. Consort flow diagram of discovery cohort. EOC: ente ospedaliero cantonale, nCRT: neoadjuvant chemo-Radio Therapy, FFPE: Formalin-fixed paraffin-embedded, TRG: tumor regression grade, NGS: next generation sequencing.

16S rRNA gene sequencing and intratumoral microbiome analysis

Tumor-associated microbiome analysis was conducted based on amplification and sequencing of bacterial 16SrRNA gene. Briefly, two-step PCR libraries were created using the primer pair 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806 R (5′-GGACTACHVGGGTWTCTA AT-3′) to sequence V4 hypervariable region of 16S RNA gene.

Libraries were then sequenced on the Illumina MiSeq platform using a v2 500 cycles kit resulting in 2 × 250 bp reads. Demultiplexing and trimming of Illumina adaptor residuals for the produced paired-end reads were performed using the Illumina MiSeq Reporter software (v2.6.2). Quality of reads was checked with FastQC software (v0.11.5). Reads were pre-processed using the MICCA pipeline (v.1.7.0) (http://www.micca.org).[49] Forward and reverse primers trimming and quality filtering were performed using Micca trim and Micca filter, respectively. Filtered sequences were denoised using the UNOISE algorithm[50] implemented in MICCA operational taxonomic units to determine true biological sequences at the single nucleotide resolution level by generating amplicon sequence variants (ASVs). Bacterial ASVs were taxonomically classified using classify and the Ribosomal Database Project (RDP) Classifier v2.13.[46,51] Multiple sequence alignment (MSA) of 16S sequences was performed using the Nearest Alignment Space Termination (NAST) algorithm,[52] implemented in MICCA msa with the template alignment clustered at 97% similarity of the Greengenes database[53] (release 13_08). Phylogenetic trees were inferred using the Micca tree.[54] Sampling heterogeneity was reduced by rarefying samples at the depth of the less abundant sample using Micca tablerare. Alpha (within-sample richness) and beta-diversity (between-sample dissimilarity) estimates were computed using the phyloseq R package.[55] Permutational multivariate analysis of variance (PERMANOVA) test was performed by using the adonis function in the R package vegan with 999 permutations. Linear discriminant effect size analysis (LEfSe) was performed to find features (microbial genera) most likely explaining differences between classes.[56] ASVs differential abundance testing was carried out by using the R package DESeq2,[57] on non-rarefied data.[58] Spearman's correlations were tested by using the psych R package.[55] Random Forest analyses[59] of 16S rRNA gene sequencing data were performed by using the randomForest R package, and permutation tests with 1000 permutations were performed to assess model significance.[60]

Tissue immune cell profiling

Tissue immune gene profiling was performed by using the nCounter Human PanCancer Immune Profiling Panel (NanoString Technologies), according to the manufacturer protocol. Briefly, the tissue-derived mRNA was hybridized at 65°C overnight. Following binding to streptavidin-coated surfaces and washings, cartridges were scanned by using nCounter digital analyzer (NanoString Technologies). Quality control (QC) metrics were obtained through the NanoStringQC function from nanostringr package.[61]

Samples with percent fields of view (FOV) ≤0.75, with Linearity of Positive Controls by Plate (R2) ≤0.95, with average

housekeeping gene expression ≤0.60 and with a signal-to-noise ratio >150 were removed. A total of 61 samples, including 14 CRs and 47 NCRs, passed all QC filters and were evaluable for further analysis.

Gene expression data were processed with the NanoStringNorm R package[62]using background subtraction and normalization with housekeeping genes. Batch effects associated with sequencing plate were adjusted with CombatSeq.[63]

Quality control of the validation cohort ($n = 48$) was carried out with NACHO R package [PMID: 31504159] using the default cutoffs and results were combined with a Z-score-based meta-analysis [PMID: 20616382]. A total of 24 samples, including 5 CRs and 19 NCRs, passed all QC filters and were evaluable for further analysis.

Differential gene expression analysis was performed by fitting a quasi-likelihood negative binomial generalized model to count data with EdgeR (v3.36).[64]

To test whether a condition was enriched for relevant up/downregulated pathways, the Camera approach was used together with the collection of gene-sets and Gene Ontology (GO) terms from Molecular Signatures Database (MSigDB).[65] Gene-wise moderated t-statistics and Camera tests were used to assess whether a gene-set is highly ranked relative to condition signature in terms of differential expression (logFC), accounting for inter-gene correlation.[66] To show the enrichment of gene sets among logFC ranked genes, barcode plots were produced using the function implemented in the limma package.[67] After multiple testing correction, a false discovery rate (FDR) of 1% was used as cutoff for statistical significance.

IFNγ release assay

Alloprevotella rava (strain DSM 22,548) and *Fusobacterium nucleatum* (DSM 15,643), expanded under anaerobic conditions, were incubated with peripheral blood mononuclear cells (PBMCs) from healthy donors, purified upon gradient separation, in RPMI 1640 medium supplemented with 5% human AB serum at 10:1 ratio. *IFNG* gene expression was assessed after 6 hours of stimulation by quantitative RT-PCR. Protein release in culture supernatants collected after 24 hours was measured by ELISA.

Results

Clinicopathological characteristic of the Discovery Cohort

Out of 94 consecutive patients considered for the Discovery Cohort, 70 met the inclusion criteria (Figure 1). Of those, 16 (23%) were CRs (TRG 1), and 54 (77%) were non-CRs (TRG 2:12 (17%); TRG 3:24 (34%); TRG 4:18 (26%), TRG 5:0 (0%). Clinicopathological characteristics of patients included in the study are listed in Table 1.

No significant differences were observed between CRs and non-CRs regarding median age, sex, distance of the tumor from the anal verge, clinical T or N stage, tumor grade, tumor size, CRM involvement, extramural vascular invasion, or time from nCRT to surgery (Table 1). A trend for more frequent CR in patients with mucinous adenocarcinoma was detectable (3/16 = 18.7% vs 1/54 = 1,9%, $p = 0.07$).

CRs and non-CRs are characterized by a different tumor-associated microbiota

Following the amplification and sequencing of 16SRNA gene from gDNA, a total of 13,798,954 sequences were obtained from all samples. Sequences were mapped to 4207 ASVs. A total of 153 genera with relative abundance ≥0.05% were identified.

No significant differences in the overall diversity and microbial community structure, as measured by alpha- (within-sample richness) and beta-diversity (between-sample diversity) were observed between CRs and nonCRs (Figure 2a-c). However, the two genera, i.e. *Catenibacterium* and *Coprococcus*, were significantly enriched in CRs and four genera, i.e. *Pedomicrobium*, *Neisseria*, and two unclassified genera of *Bacillales* and *Microbacteriaceae*, were more abundant in non-CRs (Figure 2d-e).

In agreement with our previous evaluation, an in-depth analysis of the tumor-associated microbiota showed the enrichment in CRs of different amplicon sequence variants (ASVs) belonging to *Catenibacterium* (ASV176) and *Coprococcus* (ASV123) genera. Moreover, *Ruminococcus* (ASV498), *Alloprevotella* (ASV280), and *Enterococcoccus* (ASV677) were also enriched (Figure 2f and Supplementary Table S1). On the other hand, 28 different ASVs belonging to, among others, *Porphyromonas* (ASV146), and *Fusobacterium* (ASV11, ASV19, ASV154), and *Oscillibacter (*ASV115) were enriched in non-CRs (Figure 2f, and Supplementary Table S1).

Response to nCRT is associated with activation of ifn-γ and ifn-α pathways

To gain insights into immune contexture features possibly associated with CR, on the same tumor samples, we performed an immune cell gene expression profiling on tissue-derived mRNA by using the PanCancer panel including 770 immune-related genes.

Upon differential analysis of individual gene expression, we could identify 41 differentially expressed genes (DEGs), with 20 genes, including, among others, *TREM2*, *PRAME*, *CXCL11*, *CTSW, FCGR1*, and C*4BPA*, upregulated in CRs, and 21 genes including *CLEC6A, SYCP1*, *CTAGE1, CTCFL, SLAMF6, IL22RA2*, and *EBI3*, overexpressed in non-CRs (Figure 3a and Supplementary Table S2). Gene set enrichment analysis identified numerous upregulated pathways in CRs versus non-CRs (Figure 3b and Supplementary Table S3), many of which were related to IFN-γ and IFN-α responses (Figure 3c).

Combined microbial-immunological signature predicts resistance to nCRT

Previous studies have demonstrated an association between the presence of defined bacterial species within tumor tissues and infiltration by specific immune cell subsets.[33,34] Therefore, we sought to investigate whether tumor-associated bacteria in LARC patients could promote specific immune-related transcriptional responses.

Indeed, immune cell exposure to bacteria associated with CR, such as *Alloprevotella*, resulted in significantly higher IFN-

Table 1. Baseline characteristics of the Discovery Cohort.

	Patients with LARC ($n = 70$)				
	CRs [TRG1] ($N = 16$)		non-CRs [TRG2–5] ($N = 54$)		p
Age (years):					
Median (IQR)	65	(57–77)	67	(62–74)	0,25
Gender, n. (%):					
Male	12	(75)	35	(64.8)	0,55
Female	4	(25)	19	(35.2)	
Dist. a.v. (cm), n. (%):					
>5	6	(37.5)	32	(59.3)	0,16
≤5	10	(62.5)	22	(40.7)	
MRI Clinical T, n. (%):					
cT2	1	(6.3)	4	(7.4)	0,45
cT3	14	(87.4)	40	(74.1)	
cT4	1	(6.3)	10	(18.5)	
MRI Clinical N, n. (%):					
cN0	4	(25)	10	(18.5)	0,56
cN1	8	(50)	35	(64.8)	
cN2	4	(25)	9	(16.6)	
AJCC Stage Baseline n. (%):					
II	4	(12.6)	10	(18.5)	0,83
III	12	(87.4)	44	(81.5)	
Grading [G] n. (%):					
G2	16	(100)	51	(94.4)	0,79
G3	0	(0)	3	(5.6)	
Histology n. (%):					
Conventional	13	(81.3)	53	(98.1)	0,07
Mucinous	3	(18.7)	1	(1.9)	
CRM Involvement n. (%):					
Positive	6	(37.5)	19	(35.2)	1
Negative	10	(62.5)	35	(64.8)	
EMVI n. (%):					
Positive	1	(6.3)	2	(3.7)	0,55
Negative	15	(93.7)	52	(96.3)	
Tumor Length (cm):					
Median (95% CI)	7	(5,8–8)	5	(4–6)	0,10
Time from nCRT to Surgery (days):					
Median (95% CI)	70	(65–72)	63	(58–70)	0,12
Surgery, n (%):					
Sphincter-Preserving	16	(100)	46	(66.6)	0,37
Non Sphincter-Preserving	0	(0)	8	(33.4)	

LARC: locally advanced rectal cancer; CRs: complete responders; non-CRs: non-complete responders; TRG: tumor regression grade; MRI:Magnetic Resonance imaging; AJCC: American Joint Committee on Cancer; CMR: Circumferential resection margin; EMVI: Extramural vascular invasion.

γ expression at both gene and protein level, as compared to *Fusobacterium*, which was abundant in non-CRs (Figure 4a).

Furthermore, combined microbiome and immune cell gene expression profiling analysis unraveled specific clustering of microbial taxa with each other and with immune cell-related genes. In particular, we observed that a cluster of microbial taxa enriched in CRs, including *Ruminococcus, Catenibacterium*, *Coprococcus*, and *Alloprevotella*, negatively correlated with the expression of *CHIT1, CLEC6A, CTCFL, CYFIP2*, and *PAX5* genes. In addition, *Ruminococcus* (ASV498) abundance positively correlated with expression of *LTK, FCGR1A, CD244* and *C4BPA* (Figure 4B). On the other hand, the cluster of microbial taxa enriched in non-CRs, comprising *Pedomicrobium*, *Hyphomicrobium and* Legionella, positively correlated with the expression of *IL22RA2, KIR3DL3, CHIT1, IL4, CTAGE1, SYCP1, CLEC6A* and *CTCFL* (Figure 4B and Supplementary Table S4). *Pedomicrobium* abundance also correlated with *CXCL14* and *SLAMF6* gene expression. Notably, the enrichment of *Neisseria* and *Streptococcus* pathobionts correlated with the expression of *IL22RA2, CLEC6A, CTCFL, CXCL11, C1R* and with the expression of *STAT1*, *CD209* and *GAGE1* genes, respectively (Figure 4B, and Supplementary Table S4).

Since we were able to associate specific microbial features with differential expression of immune-related genes, we asked whether we could predict responsiveness to nCRT by using their tumor-associated microbiome or immune transcriptional signatures. By using a Random Forest classifier, we observed that the tumor-associated microbiome is a poorer predictor of response to nCRT (Out-of-bag error, OOB = 28.57%, Accuracy = 0.715, Kappa = −0.0870, $p = 0.9$), as compared to the immune transcriptional profile (OOB = 19.67%, Accuracy = 0.822, Kappa = 0.346, $p = 0.002$). However, the combination of microbial and immune transcriptional signatures (signature 1, Supplementary Table S5A) improved the ability of the classifier to predict the response to nCRT (OOB = 14.75%, Accuracy = 0.901, Kappa = 0.672, $p < 0.0001$). Most importantly, only the combination of microbial and transcriptional data predicted non-CR to nCRT without errors (Supplementary Figure S2).

Validation of the identified immune-microbial signature in an independent cohort

To validate the predictive capacity of the identified combination signature, we performed microbiome and immune cell

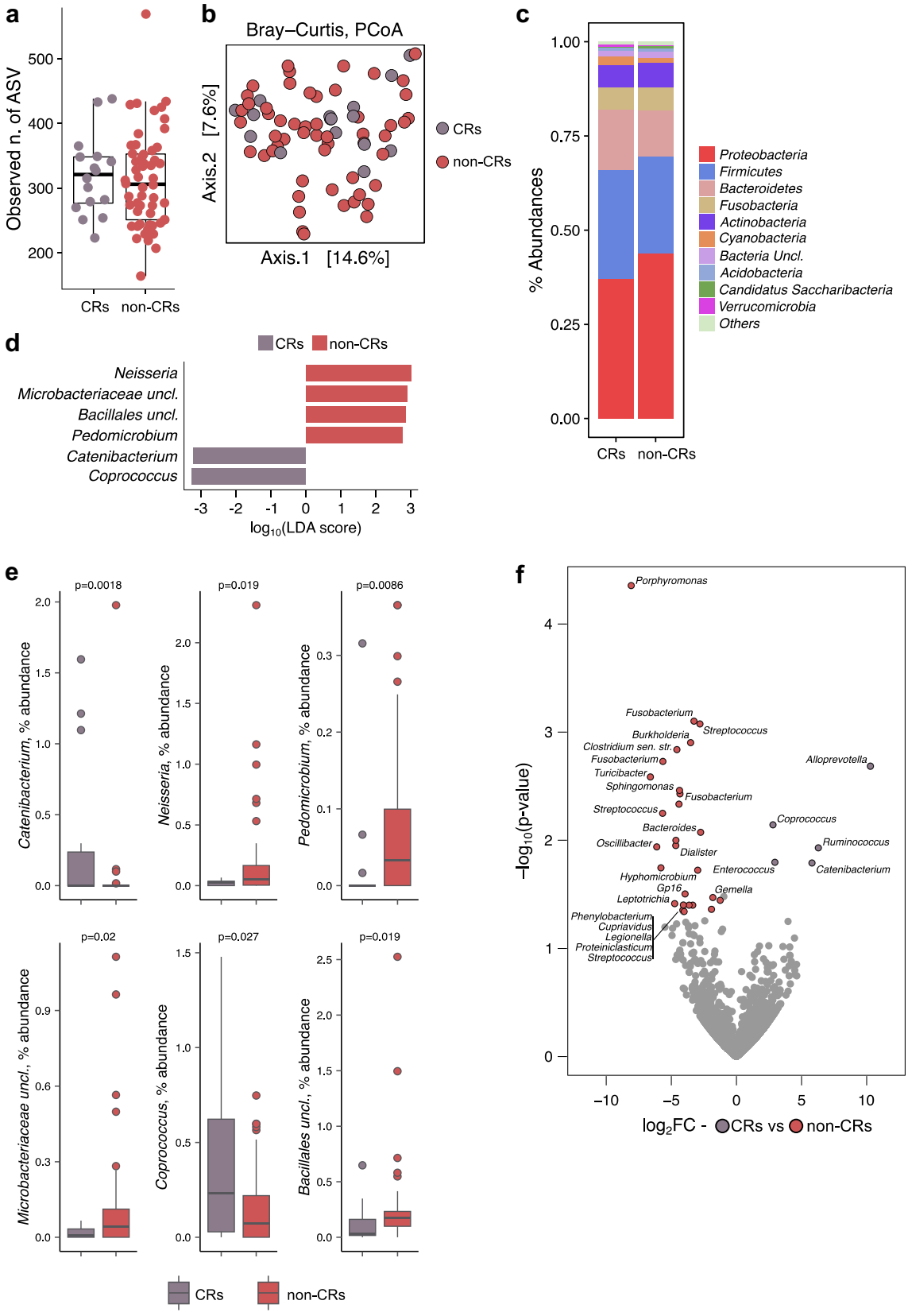

Figure 2. Tumor-associated microbiome analysis of CRs versus NCRs. a. Observed number of amplicon sequence variants (ASVs) in complete responders (CRs; $n = 16$) and non-complete responders (non-CRs; $n = 54$). b. Principal component analysis (PCoA) of microbial beta-diversity as measured by Bray–Curtis dissimilarity index. c. Mean relative abundance of the top 10 most abundant Phyla in CRs and non-CRs. All the other less abundant Phyla are reported together and labeled as "others.". d. Most discriminant bacterial genera identified by Linear discriminant analysis Effect Size (LEfSe) analysis. Positive and negative.Linear discriminant analysis (LDA) scores indicate taxa enriched in the tumor-associated microbiome of non-CRs and CRs, respectively. Only taxa having a $p < 0.05$ (Wilcoxon rank-sum test) and LDA>|2.0| are shown. e. Relative abundance of the differentially enriched genera identified by LEfSe analysis. The exact p-value of the pairwise comparisons between CRs and non-CRs are also shown (Wilcoxon rank-sum test). f. Volcano plot showing the significantly enriched ASVs ($p < 0.05$) by the DEseq2 analysis. Names of the significantly enriched bacterial ASVs classified up to the genus level are reported in Supplementary Table 2.

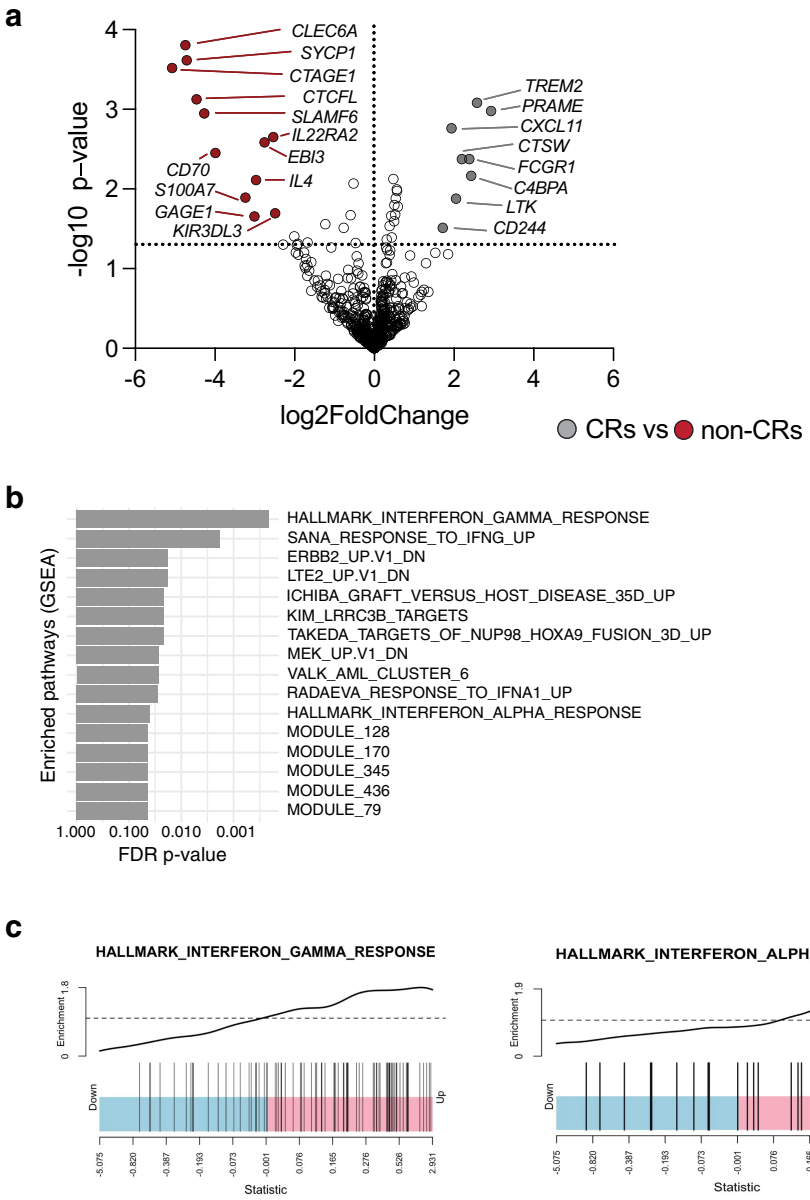

Figure 3. Responsiveness to nCRT is associated with upregulation of immune cell-related genes and activation of ifn-γ and ifn-α responses. a. Volcano plots depicting differentially expressed genes in complete responders (CRs, $n = 14$) and in non-complete responders (non-CRs, $n = 47$). b. Significantly enriched transcriptional pathways (false discovery rate, FDR, -corrected $p < 0.05$) as evaluated by gene set enrichment analysis (GSEA) of differentially expressed genes (DEGs). c. Enrichment score profiles of ifn-γ and ifn-alpha gene sets.

profiling analyses in an independent cohort of 49 patients, including 7 (14%) CRs and 42 (86%) non-CRs, referred to as Validation Cohort (Supplementary Figure S1 and Supplementary Table S6).

No significant differences in microbiome alpha- and beta-diversity were observed between CRs and non-CRs (Suplementary Figure S3). At genus level, increased abundances of *Roseburia* and *Micrococcus* were associated with CR and non-CR, respectively.

At species level, consistent with results from the Discovery Cohort, species belonging to *Ruminococcus* genus (ASV1866) were enriched in CRs, whereas ASVs belonging to *Fusobacterium* (ASV27, ASV125, ASV251), *Porphyromonas* (ASV158), and *Oscillibacter* (ASV576) genera were associated with NCRs. In addition, ASVs of *Roseburia* (ASV34), *Allisonella* (1296) and *Streptococcus* (ASV502) were also more abundant in CRs, whereas ASVs of *Turicella* (ASV178), *Selenomonas* (ASV253), and *Reyranella* (ASV299) genera were enriched in NCRs (Supplementary Figure S3C and Supplementary Table S7)

Immune cell profiling of the Validation Cohort and meta-analyses of the two cohorts confirmed significant dysregulation of 30 of 41 previously identified DEGs (Supplementary Table S8A), and unraveled the association of responsiveness with 29 additional genes (Supplementary Table S8B). Furthermore, GSEA demonstrated significant upregulation of most pathways identified in the Discovery Cohort, including IFN-γ and IFN-α pathways (Supplementary Table S9).

We therefore refined the combined immune-microbial signature based on results from Validation Cohort, i.e. upon inclusion of additional species and genes associated with response and

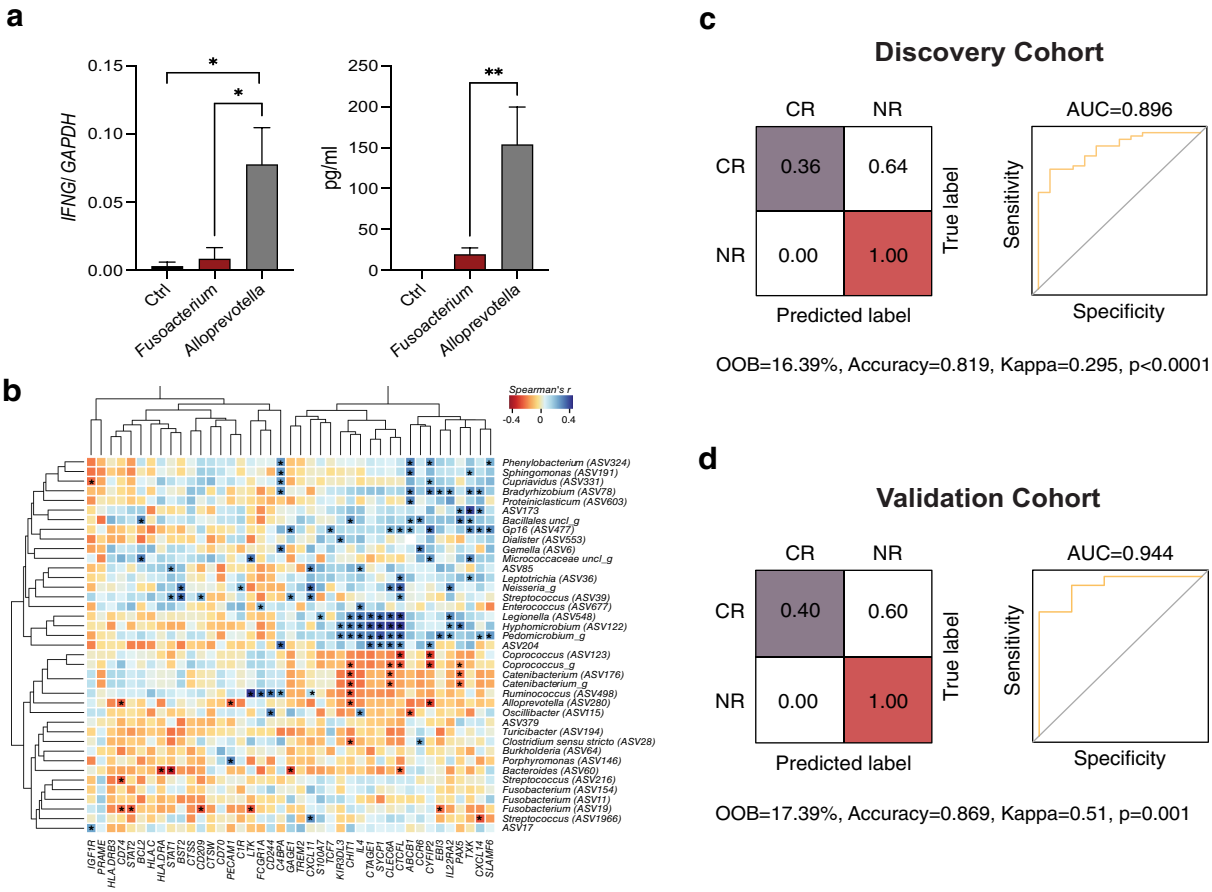

Figure 4. Predictive power of tumor-associated microbial and immunological signatures to nCRT. a. ifn-γ expression in PBMC unstimulated (ctrl) or stimulated with *Alloprevotella* or *Fusobacterium*, assessed at gene level after 6 h by quantitative PCR (left panel) and at protein level after 24 h ELISA (right panel). Results refers to average from three independent experiments performed with three different healthy donors as responders. Statistical significance was evaluated by Mann-Whitney test; $*p < 0.05$; $**p < 0.01$. b. Heatmap of Spearman's rho correlations between the relative abundance of the significantly enriched bacterial taxa identified by LEfSE and DEseq2 analysis with the DEGs identified by nanostring transcriptional profiling. The significant correlations with p-value <0.05 are indicated with an asterisk. c,d. The predictive power of the microbial-immunological signature identified based on analysis of both discovery and validation cohorts (see Supplementary Table 5, signature 2) was tested by random forest analysis. Statistics, confusion matrix and ROC curve representing the diagnostic accuracy of the random forest classification model in the discovery ($n = 61$, C) and validation cohort ($n = 24$, D) are shown. OOB: out-of-bag error; AUC: area under the curve.

exclusion of non-confirmed genes. The new signature (signature 2, Supplementary Table S5B) showed comparable predictive capacity in both cohorts, leading to a fully correct classification of non-CRs (Figure 4c,d).

Discussion

In this study, we have performed a combined analysis of intratumoral microbiome and immune gene expression profiling, and we have identified a novel combined signature predicting the response of LARC to nCRT.

We found that tumor tissue colonization by bacterial species of *Ruminococcus* genus is associated with CR to nCRT in two independent cohorts, whereas abundance of *Fusobacterium*, *Pophyromonosa*, and *Oscillibacter*, together with *Pedomicrobium*, *Neissaeria*, and *Streptococcus* species predicts non-CR. Overexpression of genes related to IFN-γ and IFN-α response was also identified as hallmark of responsiveness. Our analysis unraveled the specific clustering of microbial taxa among each other and with the expression of immune cell-related genes, leading to the identification of highly relevant predictive features. Indeed, while microbiome or immune transcriptional signatures, were per se relatively poor predictors of response, their combination resulted in improved prediction potentially leading to the correct identification of all non-CRs.

A few recent studies have evaluated the impact of components of gut microbiota on nCRT outcome in LARC. Serna et al.[43] analyzed rectal cancer tissue samples from 143 patients, specifically for the presence and abundance of *Fusobacterium nucleatum* at baseline and after nCRT, using RNA in situ hybridization and quantitative PCR. Although baseline *Fusobacterium nucleatum* abundance was not associated with response, its persistence after nCRT predicted poor outcome. Yi et al. ,[44] performed a whole-microbiome analysis on fecal samples of 84 patients. They found that butyrate producing microbes, including *Roseburia, Dorea, and Anaerostipes*, were overexpressed at baseline in responders whereas, consistent with our findings, *Fusobacterium* was associated with poor response. Teng et al. also analyzed fecal samples from 116 Chinese LARC patients and found that *Bacteroides vulgatus*-mediated nucleotide biosynthesis was associated with resistance to nCRT.[46] Finally, Sun et al.,[45] analyzed fecal and blood samples from 39 patients with

LARC before, during and after nCRT. At baseline, *Clostridium sensu stricto 1* was found to be significantly enriched in good-responders.

To this date, only the study by Takenaka et al.,[68] has analyzed the whole tumor-associated microbiome in LARC, in a group of 44 patients. *Hungatella, Flavonifractor*, and *Methanosphaera* bacterial genera were found to be enriched in CRs, whereas *Enhydrobacter, Paraprevotella* and *Finegoldia*, but not *Fusobacterium*, were overrepresented in non-CRs. However, this cohort included patients from different geographical areas (i.e 18 from Argentina and 26 from Brasil) and the composition of the intratumoral microbiota already significantly varied depending on the country of origin.[68]

Our study represents the first analysis of tumor-associated microbiome in a homogeneous LARC cohort. Our data identifies species of *Ruminococcus, together with Catenibacterium*, *Alloprevotella*, *Roseburia* and *Coprococcus*, genera as predictive of CR, whereas enrichment of species of *Fusobacterium, Pophyromonas* and *Oscillibacter* is consistently found to be associated with non-CR.

In a recent study, the abundance of *Ruminococcus* in tumor tissues has been associated with favorable outcome in colon cancer,[69] and the presence of *Alloprevotella* and *Catenibacterium* in gut microbiota of patients with different solid tumors has been reported to be associated with favorable response to treatment with immunological checkpoint inhibitors.[69,70]

On the other hand, enrichment of *Pophyromonas* species has consistently been detected in human CRC tissue[71] and has been reported to be associated with short overall survival.[72] Most recently, its abundance in feces of patients with LARC has been shown to predict poor response to neoadjuvant immunotherapy plus chemoradiotherapy.[73] *Oscillibacter* has also been previously detected in CRC tissues[74] and associated with unfavorable outcome in a CRC mouse model.[75]

Mechanisms underlying the association of these bacteria with responsiveness and their potential causative role remain to be established. Our data suggests that some of the identified bacteria may contribute to shape the tumor immune contexture.

Tumor immune gene expression profiling clearly indicated that CR to nCRT is associated with tumor infiltration by cytotoxic lymphocytes, as suggested by overexpression of genes encoding cathepsin-W, the CXCR3-binding chemokine CXCL11, and the natural killer receptor CD244, and with overexpression of gene sets related to INF-γ and IFN-alpha pathways. This is in line with previous studies reporting an association between tumor infiltration by cytotoxic CD8 and responsiveness to nCRT[25–30] and activation of proinflammatory pathways, including type I IFN, in responders.[76,77]

It is remarkable that bacteria associated with CR promoted in vitro expression of INF-γ significantly more effectively than bacteria associated with non-CR, thus supporting the capacity of these bacteria to directly influence the production of proinflammatory cytokines by immune cells.

Also, overexpression of myeloid cell markers, including TREM2, the high-affinity immunoglobulin gamma Fc receptor I, and the complement component 4 binding protein alpha, suggests that tumor-infiltrating myeloid cells, possibly including macrophages, dendritic cells, and neutrophils, also promote responsiveness to nCRT in LARC.

Previous studies in experimental models have shown that specific components of gut microbiota critically influence response to chemotherapeutic drugs, including oxaliplatin, by modulating tumor-infiltrating myeloid cell functions.[40] Bacteria abundant in CRs, might also synergize with DAMPs released by nCRT-treated cancer cells, to promote myeloid cell activation and enhance their antigen presentation capacity, ultimately favoring development of lymphocyte-mediated antitumor responses. In our cohort, overexpression of HLA-C and HLA-DR molecules and of tumor-associated antigen PRAME was also associated with CR, consistent with the possible induction of tumor-specific immune response. In line with this hypothesis, abundance of *Ruminococcus* within colon cancer-associated microbiota has been found to positively correlates with tumor infiltration by CD103+ dendritic cells, possibly favoring induction of protetctive immune responses.[69] On the other hand, the presence of *Oscillibacter* in the fecal microbiota has been reported to inversely correlated with expression of proinflammatory cytokine genes, including IFN-γ, in CRC mouse models.[75]

Thanks to our integrated analysis of intratumoral microbiome and immune profiling, we could also detect correlations between microbial taxa and immune-related genes. In particular, we found direct correlations between abundance of bacterial genera enriched in nonCRs, in particular *Pedomicrobium*, *Hypomicrobium*, *Neisseria*, and *Bacillales*, and expression levels of genes encoding for proteins involved in downmodulation of immune responses, including the IL-22 receptor antagonist (encoded by *IL22RA*), the inhibitory cytokine IL35B (encoded by *EBI3)* and the inhibitory NK cell receptor KIR3DL3 (encoded by *KIR3DL3*), thus suggesting that these bacteria may induce immunosuppressive effects possibly contributing to nCRT unresponsiveness.

In addition to providing insights into the potential bacteria-immune cell interplay occurring within LARC tissues, integration of microbiome and immune profiling allowed the identification of a combined signature displaying improved predictive power as compared to individual microbiome- or immune-related signatures.

In the study by Sun et al., integrated analysis of microbiome- and immune-related features was also found to predict the response to nCRT with higher accuracy in a cohort of 39 patients.[45] However, this work was based on the evaluation of fecal microbiota and immune cell parameters in peripheral blood, which may not necessarily mirror the dynamics occurring within the tumor microenvironment. Nevertheless, baseline high levels of *Clostridium senso strictu 1* were also found to be associated with poor response, consistent with our data.

If confirmed, our findings may have a high clinical relevance. The signature identified in our study could correctly classify all non-CRs, with no error in two independent cohorts. Such a prediction could influence treatment decisions as to the benefit or type of neoadjuvant treatment. Also, this signature could help design pre-neoadjuvant microbiome conditioning by eradicating unfavorable bacterial species, followed by administration of bacteria favoring nCRT effectiveness.

Limitations of our investigation should be acknowledged. Although it represents the largest study so far exploring the LARC intratumoral microbiome and immune contexture in combination, the number of patients included in the discovery and in the validation cohort is still relatively small. Moreover, although the patients' population under investigation is rather homogenous, and treated with a standardized nCRT protocol and surgery, the fact that all patients are from the same region limits the generalization of the identified signature. Indeed, previous studies evaluating microbiome-related signatures as biomarkers of responsiveness to anti-cancer treatments, such as immunological checkpoint inhibitors, have highlighted discrepancies in the signatures identified in patients' cohort belonging to different geographical areas.[78]

Intra-tumoral microbiome analysis was necessarily based on sequencing of 16S gene, due to low intra-tissue bacterial loads. While this method allows to identify bacteria that actually infiltrate tumor tissues, it does not provide information about their functional properties. Similarly, large scale immune gene expression profiling provides a comprehensive analysis of the functional orientation of tumor microenvironment but does not inform on individual immune cell subsets. Further multicentric prospective studies, integrating analysis of fecal and intratumoral microbiome, and large-scale immunophenotyping of tumor infiltrating immune cells, are warranted to shed more light on the tumor-microbiota-immune cell interplay within tumor microenvironment. Visualization of potential interactions between microbial species, tumor cells and immune cells by emerging spatial transcriptomics and proteomics[34] may also provide additional information about potentially direct interactions of bacteria predictive of response to nCRT and tumor infiltrating immune cells.

In addition, our analysis has been performed on pre-treatment diagnostic biopsies only, without taking into account changes potentially occurring after treatment, which could also be of predictive value.[45] Nevertheless, the identification of predictive parameters prior to therapy is critical for the implementation of innovative interventional strategies, such as gut microbiota conditioning, aimed at enhancing nCRT efficacy.

In conclusion, we have provided evidence that combined analysis of intratumoral microbiome and tumor immune gene expression profiling results in the identification of markers predicting responses to treatment with improved accuracy. The possibility to identify unresponsive patients together with the identification of bacterial species associated with responses may point toward the development of innovative "pre-neoadjuvant" treatments, based on gut microbiota modulation, to increase the effectiveness of nCRT in LARC.

Abbreviations

nCRT	neoadjuvant chemoradiotherapy
LARC	Locally Advanced Rectal Cancer
CR	Complete response
Non-CR	non complete response
CRs	Complete Responders
non-CRs	non-complete responders
TRG	tumor regression grade
TME	Total Mesorectal Excision
ASV	amplicon sequence variant
DEG	Differentially expressed gene
LEfSe	Linear discriminant analysis Effect Size
LDA	Linear discriminant analysis
OOB	out-of-bag error
AUC	area under the curve

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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R.R. designed and performed experiments, analyzed data, and wrote the manuscript. F.S. designed and performed data analysis and wrote the manuscript. F.B. contributed to design of experiments; performed microbiome and gene expression data analysis; wrote the manuscript; C.B., J.D., E.S., M.V., C.C., and F.M. contributed to implementation of experiments and data analysis; S.E., P.S., M.F., A.C., F.M. J.G. S.G.P., A.F-P., S.D. D. contributed to the collection and analysis of tissue samples and clinical data; F.F. contributed to design of experiments and data analysis, and wrote the manuscript; P.E.M-H. oversaw the study and wrote the manuscript; G.I., and D.C. conceived the study, oversaw the study, designed experiments, guided research personnel, performed data analysis, wrote the manuscript

Data availability statement

The data underlying Figures 2–4 are presented in the Supplementary Data. Raw data of microbiome and gene expression analysis are available upon request.

Ethical statement

- Approval of the emended protocol by the ethics committee (2020 -02,387-em.1-e-aggiunta-centro-pos-2021-06-21)
- Copy of the original study protocol (ORL-CHIR-011_protocol final) submitted the ethics committee,
- Initial approval of the original protocol by the ethics committee with request of additional information (2020 -02,387-ti-prot-req -2020.10.29)
- Final approval of the original protocol by the ethics committee (2020 -02,387-ti-ev-requisiti-2020-11-09)
- Copy of the study protocol emended upon inclusion of the new cohort (ORL-CHIR-011_protocol_v2)

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