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Brief Correspondence

Stool Microbiome Signature Associated with Response to Neoadjuvant Pembrolizumab in Patients with Muscle-invasive Bladder Cancer

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Abstract

Neoadjuvant pembrolizumab has been shown to be a valid treatment for patients affected by muscle-invasive bladder cancer (MIBC), as demonstrated in the PURE-01 clinical trial (NCT02736266). Among the tumor-extrinsic factors influencing immunotherapy efficacy, extensive data highlighted that the microbiome is a central player in immune-mediated anticancer activity. This report aimed to investigate the composition and role of stool microbiome in patients enrolled in the PURE-01 clinical trial. An orthotopic animal model of bladder cancer (MB49-Luc) was used to support some of the findings from human data. An analysis of stool microbiome before pembrolizumab was conducted for 42 patients, of whom 23 showed a pathologic response. The information in the preclinical model of orthotopic bladder cancer treated with anti–PD-1 antibody or control isotype was validated. Linear discriminant analysis effect size and linear models were used to identify the bacterial taxa enriched in either responders or nonresponders. The identified taxa were also tested for their association with event-free survival (EFS). Survival at 31 d after tumor instillation was used as the study endpoint in the preclinical model. Responders and nonresponders emerged to differ in terms of enrichment for 16 bacterial taxa. Of these, the genus *Sutterella* was enriched in responders, while the species *Ruminococcus bromii* was enriched in nonresponders. The negative impact of *R. bromii* on anti–PD-1 antibody activity was also observed in the preclinical model. EFS and survival of the preclinical model showed a negative role of *R. bromii*. We found different stool bacterial taxa associated with the response or lack of response to neoadjuvant pembrolizumab. Moreover, we provided experimental data about the negative role of *R. bromii* on immunotherapy response. Further studies are

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needed to externally validate our findings and provide mechanistic insights about the host-pathogen interactions in MIBC.

Patient summary: Using prepembrolizumab stool samples collected from patients enrolled in the PURE-01 clinical trials, we identified some bacterial taxa that were enriched in patients who either responded or did not respond to immunotherapy. Using an animal model of bladder cancer, we gathered further evidence of the negative impact of the *Ruminococcus bromii* on immunotherapy efficacy. Further studies are needed to confirm the current findings and test the utility of these bacteria as predictive markers of immunotherapy response.

The introduction of immune checkpoint inhibitors (ICIs) for the treatment of urothelial carcinoma of the bladder has provided an effective and safe therapeutic alternative in the genitourinary oncology toolbox, both in the metastatic and in the organ-confined disease [1]. Indeed, the efficacy of ICIs as monotherapy in the neoadjuvant setting, measured as a complete pathologic response (ie, ypT0N0), has been reported to be ~1/3 of treated patients [2,3]. Nevertheless, a proportion of patients does not benefit from immunotherapy. Therefore, it is of paramount importance to understand which tumor-intrinsic and tumor-extrinsic mechanisms drive resistance to therapy.

Among the tumor-extrinsic factors that can mediate ICI activity, extensive data provided solid evidence that the microbiome is a central player in immune-mediated anticancer activity [4,5]. For instance, Matson and colleagues [6] collected fecal specimens from a cohort of 42 melanoma patients before ICI treatment, with a 38% response rate in their cohort. Of all, they found specific bacterial microbiome phenotypes associated with a clinical response; in particular, *Bifidobacterium longum*, *Collinsella aerofaciens*, and *Enterococcus faecium* were enriched in responders. Fecal microbiome transplant (FMT) in tumor-bearing germ-free mice using feces collected from responders was able to improve antitumor immune activity, ultimately resulting in increased tumor control and immunotherapy efficacy. Using a similar design, Gopalakrishnan and colleagues [7] analyzed specimens from 112 melanoma patients treated with anti–PD-1 agents and found bacterial communities differentially enriched in responders (*Clostridiales*, *Ruminococcaceae*, and *Faecalibacterium*) or nonresponders (*Bacteroidales* and *Escherichia coli*). Moreover, the "responder" microbiome" was associated with a higher concentration of effector cytotoxic T cells, while the "nonresponder" microbiome" favored increased levels of Tregs and a more inhibitory soluble cytokine profile. In this setting, we aimed to investigate the composition and the role of the stool microbiome in patients who underwent neoadjuvant pembrolizumab for muscle-invasive bladder cancer in the PURE-01 clinical trial (NCT02736266) [2,8].

For these analyses, fecal pretherapy specimens were available from 42 patients from the PURE-01 study, with 23 patients showing a pathologic response (21 complete responses, ypT0N0, and two partial responses, ypT<2N0; Supplementary Table 1 and Supplementary material). Alpha and beta diversity metrics did not differ between responders and nonresponders (Supplementary Fig. 1A and 1B). Visual inspection of the data at different taxonomic levels (Supplementary Fig. 1C) did not identify major batch effects.

At the level of taxonomic analysis, linear discriminant analysis (LDA) effect size (LEfSe) was performed to characterize the bacterial taxa differently expressed between responders and nonresponders. LEfSe identified 16 bacterial taxa with an LDA score of $\geq$2.5 that were differentially enriched between responders and nonresponders (Fig. 1A). Among them, the genus *Sutterella* was enriched in responders, while the species *Ruminococcus bromii* was enriched in nonresponders. Linear regression analyses were performed to study the potential role of smoking in response to therapy, retaining taxa present with a relative abundance of >1% in at least 10% of patients. Of the 16 taxa identified by LEfSe, seven taxa, including the genus *Sutterella* and the species *R. bromii*, remained associated with the response to therapy independently of smoking status (Fig. 1B and Supplementary Table 2). Among the identified taxa, the phylum *Proteobacteria* (p = 0.018) and the species *R. bromii* (p = 0.12) showed the best association with event-free survival (EFS [8]), when patients' cohort was dichotomized in "high" versus "low" abundance based on the median relative abundance value for each single taxon identified by LEfSe (Fig. 2A and 2B, and Supplementary Fig. 2).

To further investigate the potential impact of the identified bacterial taxa on bladder cancer immunotherapy, we first investigated whether the syngeneic, orthotopic MB49-Luc mouse model (Supplementary Fig. 3A) could be a valid prototype to study the microbiome and the response to immunotherapy. In this framework, female C57BL/6J mice were instilled intravesically with MB49-Luc cells and randomized to receive a single injection of either anti–PD-1 antibody or the isotype control (Supplementary Fig. 3B), when the tumor became evident at the bioluminescence assay (Supplementary Fig. 3C). A survival analysis showed longer survival of mice treated with anti–PD-1 antibody than the group treated with the control isotype (p = 0.019; Supplementary Fig. 3D). Then, this murine model was used to test the negative effect of *R. bromii* on pembrolizumab antitumor activity; the murine intestinal flora was enriched by oral gavage of live *R. bromii* after a short-term vancomycin treatment and treated with anti–PD-1 (Supplementary Fig. 3E). The short-term pretreatment with vancomycin did not modify the survival probability

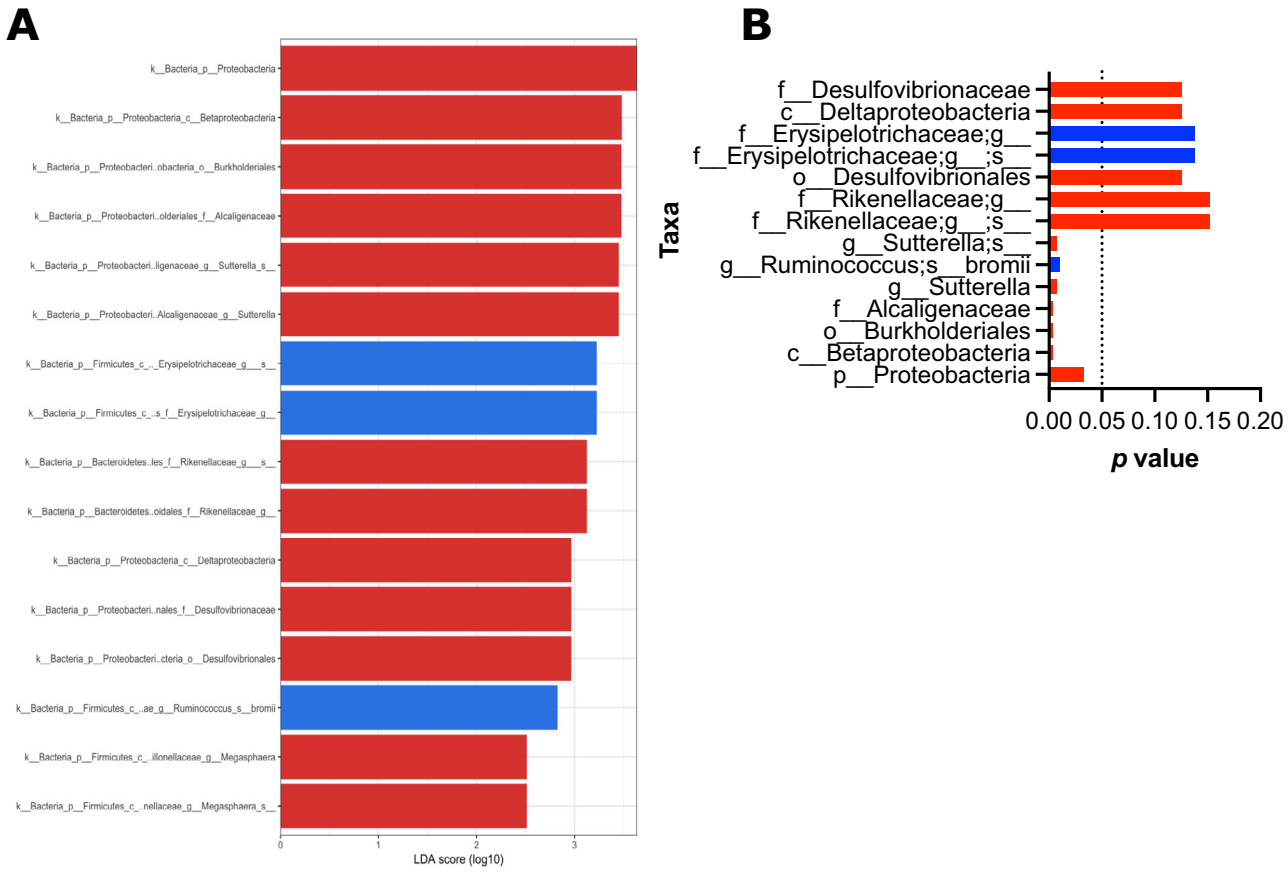

Fig. 1 – The gut microbiome associated with neoadjuvant pembrolizumab response in human bladder urothelial carcinoma. (A) Linear discriminant analysis effect size (LEfSe) analysis pinpointed 16 bacterial taxa differentially enriched in responders and nonresponders. (B) Additional analysis using a linear model considering smoking status as a covariate showed which taxa identified by LEfSe were still associated with response to therapy (the taxa g_Megasphaera and g_Megasphaera_s__were not considered in the linear model because their relative abundance was not >1% in at least the 10% of patients). LDA = linear discriminant analysis.

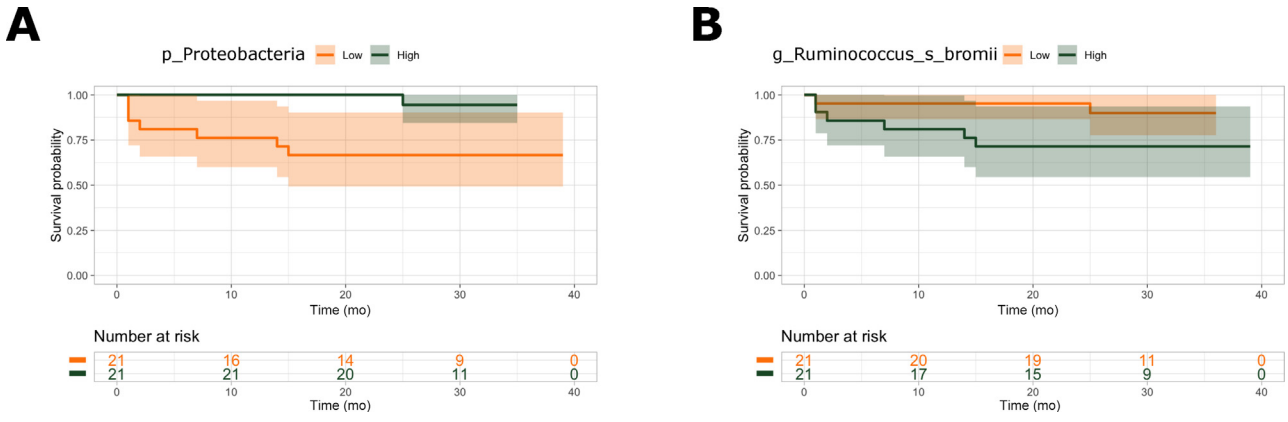

Fig. 2 – Bacterial taxa–based survival analyses. Event-free survival analyses in the PURE-01 trial dividing patients in high versus low (A) p_*Proteobacteria* ($p = 0.018$) and (B) *R. bromii* ($p = 0.12$) abundance, using the median relative abundance of each taxon as a threshold.

associated with the anti–PD-1 or isotype treatment (Supplementary Fig. 4). Therefore, we observed some evidence that the enrichment of the murine gut microbiome with the *R. bromii* (Fig. 3A) decreased anti–PD-1 efficacy in treated mice (Fig. 3B), although differences between groups did not meet conventional levels of statistical significance ($p = 0.066$).

In the intention-to-cure cohort of the PURE-01 trial, we found that the gut microbiome of nonresponder patients was characterized by a higher relative abundance of *R. bro-*

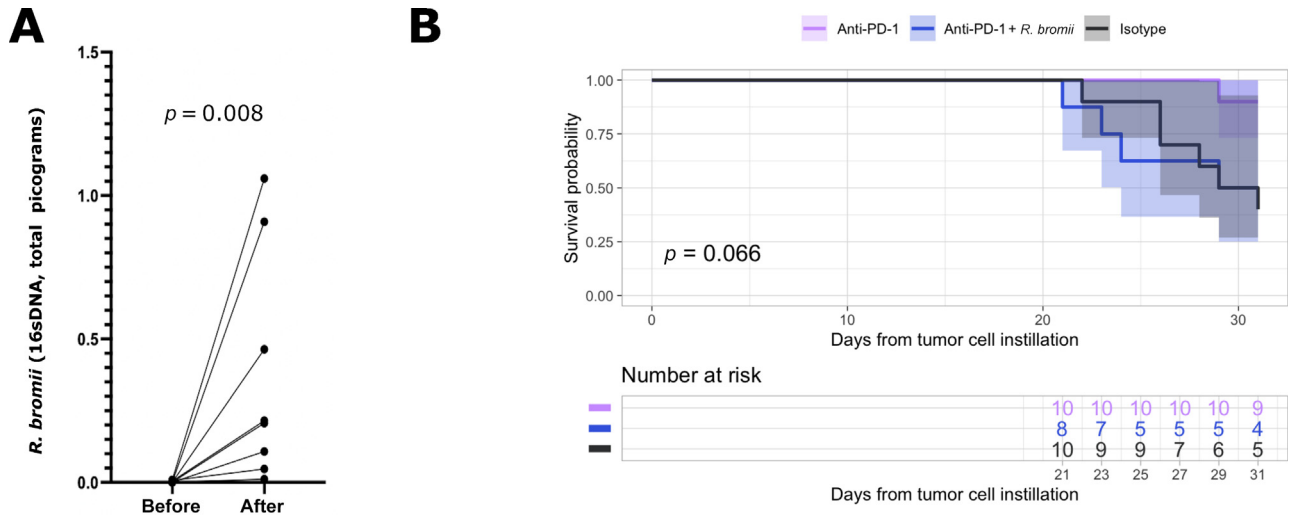

Fig. 3 – *R. bromii* administration reduced survival in a preclinical orthotopic bladder cancer model treated with anti–PD-1 therapy. (A) Line plot showing total picograms of *R. bromii* in the feces before and after oral administration (*n* = 8). (B) Survival analysis testing the potential negative effect of *R. bromii* (*p* = 0.066) on anti–PD-1 therapy *in vivo*.

mii, while responders were enriched in the phylum *Proteobacteria* and the genus *Sutterella*, among other differentially expressed bacteria. The genus *Sutterella* is a member of the order *Burkholderiales*, and it currently comprises around ten species. It is known that the enrichment of *Sutterella* in the gut promotes an increased degradation of local immunoglobulin type A (IgA), favoring a potential invasion of epithelial cells by local bacteria [9,10]. Indeed, the ability of *Sutterella* spp. to adhere to the intestinal epithelium by binding to extracellular matrix proteins, such as collagen I, fibronectin, and laminin, may suggest an immunomodulatory role. On the contrary, *Ruminococcaceae* is a family of Gram-positive, obligate anaerobic bacteria that includes important human commensal species, including *R. bromii* and *Faecalibacterium prausnitzii*, both frequently encountered in the field of cancer microbiome. Contrasting results have emerged from studies investigating the role of *Ruminococcus* spp. in immunotherapy [6,7,11,12]. Indeed, the recent results of one of the first-in-human clinical trials to test whether FMT from human responding donors can affect the response to anti–PD-1 immunotherapy further highlighted how the role of *Ruminococcus* spp. is more complex than expected [13]. While patients receiving stools from donor 1 showed objective responses in 3/5 cases, no responses were seen in patients who received FMT from donor 2. Of note, stools from donor 2 used for FMT were enriched in *Ruminococcaceae*, and a post-treatment gut microbiome analysis from patients who received FMT from donor 2 found a high abundance of *R. bromii*. This study, in line with previous reports and our human and murine data, further suggests a potential detrimental role of *R. bromii* in patients treated with pembrolizumab. As the urinary bladder is not completely sterile and has a local microbiome, future studies are needed to investigate the role of that environment in mediating a response to immunotherapy locally [5,14]. The main limitation of our study is that stool specimens were available only for a small subset of patients enrolled in the PURE-01 trial, thus limiting our analysis, and limiting the association of response to therapy with smoking status but not with other variables.

In conclusion, our study on an intention-to-cure population highlighted different bacterial taxa associated with the response to neoadjuvant pembrolizumab. These data lay the foundation for further investigations into the predictive role of gut microbiome and the potential actionability and druggability of intestinal microbiome to increase the response to ICIs.

Author contributions: Massimo Alfano had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Pederzoli, Necchi, Alfano.
Acquisition of data: Pederzoli, Venegoni, Marandino, Bandini, Alchera, Locatelli, Raggi, Giannatempo, Lazarevic.
Analysis and interpretation of data: Pederzoli, Riba, Provero, Alfano.
Drafting of the manuscript: Pederzoli, Riba, Locatelli, Necchi, Alfano.
Critical revision of the manuscript for important intellectual content: All authors.
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Peer Review Summary

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