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ORIGINAL ARTICLE

Baseline gut microbiota predicts clinical response and colitis in metastatic melanoma patients treated with ipilimumab

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Background: Ipilimumab, an immune checkpoint inhibitor targeting CTLA-4, prolongs survival in a subset of patients with metastatic melanoma (MM) but can induce immune-related adverse events, including enterocolitis. We hypothesized that baseline gut microbiota could predict ipilimumab anti-tumor response and/or intestinal toxicity.

Patients and methods: Twenty-six patients with MM treated with ipilimumab were prospectively enrolled. Fecal microbiota composition was assessed using 16S rRNA gene sequencing at baseline and before each ipilimumab infusion. Patients were further clustered based on microbiota patterns. Peripheral blood lymphocytes immunophenotypes were studied in parallel.

Results: A distinct baseline gut microbiota composition was associated with both clinical response and colitis. Compared with patients whose baseline microbiota was driven by *Bacteroides* (cluster B, $n = 10$), patients whose baseline microbiota was enriched with *Faecalibacterium* genus and other Firmicutes (cluster A, $n = 12$) had longer progression-free survival ($P = 0.0039$) and overall survival ($P = 0.051$). Most of the baseline colitis-associated phylotypes were related to Firmicutes (e.g. relatives of *Faecalibacterium prausnitzii* and *Gemmiger formicilis*), whereas no colitis-related phylotypes were assigned to Bacteroidetes. A low proportion of peripheral blood regulatory T cells was associated with cluster A, long-term clinical benefit and colitis. Ipilimumab led to a higher inducible T-cell COStimulator induction on CD4+ T cells and to a higher increase in serum CD25 in patients who belonged to *Faecalibacterium*-driven cluster A.

Conclusion: Baseline gut microbiota enriched with *Faecalibacterium* and other Firmicutes is associated with beneficial clinical response to ipilimumab and more frequent occurrence of ipilimumab-induced colitis.

Key words: ipilimumab, colitis, melanoma, microbiota

Introduction

Immunotherapy has become a major therapeutic strategy in oncology. The trail was blazed by ipilimumab, an antibody directed against Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), which demonstrated a significant survival benefit in patients with metastatic melanoma (MM) [1, 2]. It has previously been reported that 22% of MM patients treated with ipilimumab have prolonged survival [3].

Ipilimumab reactivates T cells and therefore affects a much wider repertoire of T cells than those involved only in the anti-cancer response [4, 5]. This probably explains why CTLA-4

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blockade is associated with immune-related adverse events (irAE) [6, 7]. Colitis is the most frequent irAE associated with CTLA-4 blockade [7, 8] and severe colitis may need anti-TNF therapy and sometimes colectomy [8, 9]. In this context, reliable biomarkers of efficacy and/or toxicity ensuring the risk/benefit ratio optimization of this drug would be of paramount interest. Colitis due to ipilimumab shares several features with inflammatory bowel disease (IBD) [9]. In IBD, dysregulated inflammation is associated with a dysbiotic composition of the intestinal microbiota, which is proposed to be the antigenic driver of the disease [10–12]. In addition, studies carried out in mice have suggested that the microbiota composition is critical to promote an anti-tumor immune response to anti CTLA-4 and anti PDL-1 monoclonal antibodies [13, 14].

Based on these data, we hypothesized that the ipilimumab-induced anti-cancer response, as well as immune-related enterocolitis associated with this agent, may depend on the gut microbiota. This prospective study was aimed to test this hypothesis in patients with MM treated with ipilimumab.

Patients, materials and methods

Data and materials availability

All data and materials are available from the corresponding author upon request. DNA sequence reads from this study have been submitted to the NCBI under the Bioproject ID PRJNA379764 and are available from the Sequence Read Archive (Biosamples accession numbers SAMN06620410 - SAMN06620492).

Patients

Patients with MM treated with ipilimumab were prospectively enrolled at the Gustave Roussy Cancer Campus between March 2013 and December 2014. Patients were informed of the study and consented to participate. This study was approved by the Kremlin Bicêtre Hospital Ethics Committee (GOLD study: SC12-018; ID-RCB-2012-A01496-37) and all procedures were carried out in accordance with the Declaration of Helsinki. Patients had a pre-specified clinical workup; feces and blood were collected at baseline (V_1), before each ipilimumab infusion (V_2, V_3, V_4), at the end of treatment (i.e. 3 weeks after the last infusion; V_5) and, if present, at the time of colitis occurrence (V_{Tox}). Ipilimumab was administered intravenously every 3 weeks at a dose of 3 or 10 mg/kg and could be continued after V_4, at a maintenance dose of one infusion every 12 weeks, in patients whose disease was controlled (response or stable disease). When immune-mediated enterocolitis (≥grade III) was suspected, patients were referred to the Gastroenterology Department of Bicêtre Hospital. The diagnosis of ipilimumab-induced colitis was made in patients who had endoscopic signs of inflammation and no other cause of colitis, such as ischemia and infection (stool tests for bacterial pathogens and *Clostridium difficile* toxin had to be negative).

Response to ipilimumab was assessed by several criteria. Long-term clinical benefit was defined by response decrease of tumor burden ≥50% relative to baseline, according to immune related response criteria [15, 16] or stable disease (decrease of tumor burden of less than 50% but with less than 25% increase relative to nadir) for more than 6 months. Patients with poor benefit were defined as patients with a lack of long-term benefit, i.e. with progression-free survival (PFS) of <6 months (with immune related progressive disease defined as a confirmed increase in tumor burden ≥25% relative to nadir) [15, 16]. All responses were confirmed by a subsequent assessment no less than 4 weeks from the date first documented. Use of antibiotics was also documented before each ipilimumab injection and is included in supplementary Table S3, available at *Annals of Oncology* online.

Bacterial composition assessment by high-throughput sequencing

Fecal samples were collected anaerobically at baseline (V_1), before each ipilimumab infusion (V_2, V_3, V_4), at the end of the treatment (V_5) and at the time of colitis (V_{Tox}) and were kept at −80 °C until analysis. Total DNA was extracted from fecal sample aliquots (∼150 mg), as previously described using both physical and chemical lysis [17]. Culture-independent 16S rRNA gene sequencing was carried out on 83 fecal samples ($n = 26$ patients; supplementary Table S3, available at *Annals of Oncology* online). Both 454 pyrosequencing (Life Sciences, a Roche company, Branford, CT) and MiSeq (Illumina, Inc., San Diego, CA) technologies were applied on the V_3–V_4 16S rRNA gene region. Gut bacterial composition was determined prospectively without knowledge of colitis or of clinical benefit, which was determined at the end of the study. Details of bacterial composition assessment by high-throughput sequencing and statistical analyses can be found in supplementary materials, available at *Annals of Oncology* online.

Fresh whole blood immune monitoring and soluble immune markers

Blood samples were collected at baseline (V_1), before each ipilimumab infusion (V_2, V_3, V_4), at the end of treatment (V_5) and at the time of colitis (V_{Tox}). Phenotyping was carried out on fresh whole blood or on fresh peripheral blood mononuclear cells isolated by Ficoll density gradient and frozen for later analyses (see supplementary materials, available at *Annals of Oncology* online). All serum samples were stored at −80 °C until further analysis of soluble immune markers of inflammation (IL-6, IL-8, IP-10, MCP-1, TNFα, sCD25) and bacterial translocation (sCD14). Details of immune monitoring can be found in supplementary materials, available at *Annals of Oncology* online. We monitored memory T cells and inducible T-cell COStimulator (ICOS) induction on T cells because previous studies had demonstrated that both could be related to clinical responses [18]. Regulatory T cells play a crucial role in the maintenance of immune homeostasis in the gut and are supposed to be a major target for anti-CTLA-4 treatment [19]. The heterodimer α4β7 plays a crucial role in the intestinal homing of T cells; we monitored $\alpha4^+\beta7^+$ T cells to gain some insight in the mechanism of ipilimumab-induced colitis. We monitored soluble immune markers of inflammation and bacterial translocation as a complement to microbiota composition. Indeed, microbiota composition affects gut barrier function, local as well as systemic immunity [20–25, 26].

Statistical analyses

A formal sample-size calculation was not carried out for this pioneering study. Associations between microbiota dominant profile and immunological parameters were assessed with the Spearman correlation coefficient and a two-sided Wilcoxon test. No adjustment for multiple comparisons was made because of the exploratory component of the analyses. Fisher's exact test was used to examine the association between microbiota clusters and immune parameters; microbiota clusters and colitis, as well as microbiota clusters and clinical responses.

Overall survival (OS) and PFS according to intestinal microbiota (cluster A versus cluster B) and according to the number of conventional $CD4^+$ T cells (low number of conventional CD4 versus high number of conventional CD4) were estimated using the Kaplan–Meier method and compared using the log-rank test.

When considering the occurrence of toxicity, progression and death lead to informative censoring, because when patients are censored at progression or death, their risk of toxicity could differ from that of patients who are not censored. Competing risk analysis is a method to deal with competing events, in order to avoid selection bias induced by informative censoring.

Therefore, toxicity cumulative incidence function (CIF) was estimated through a competing risk analysis in which toxicity was the event of interest, death or progression was the competing event and patients alive without progression or toxicity at their last follow-up were censored from the date of last follow-up. The toxicity CIFs in the different gut microbiota clusters and for the different conventional CD4 levels were estimated using the SAS macro %CIF and compared using the stratified Gray's test [27, 28]. These analyses were carried out using SAS software, Version 9.4 (SAS Institute, Inc., Cary, NC).

Results

Clinical characteristics of the patients

Fifty-five patients were included in the study and followed-up for at least 6 months. Among them, 13 patients did not receive ipilimumab, while 4 patients received only a single infusion of ipilimumab and died soon thereafter due to their melanoma; they were therefore excluded from further analyses. Among the 38 remaining patients, 26 provided fecal samples at baseline (i.e. before the first dose of ipilimumab) for microbiota analysis. Nine of these 26 patients (34.6%) had a long-term clinical benefit and seven (26.9%) developed an immune-mediated colitis. The clinical characteristics of patients with or without colitis and with or without long-term clinical benefit are shown in supplementary Tables S1 and S2, available at *Annals of Oncology* online, respectively.

Microbiota composition is not modified by ipilimumab treatment, except at the time of colitis

The gut microbiota was not significantly modified over the course of the ipilimumab treatment. Main bacterial phyla, Firmicutes and Bacteroidetes, remained stable over time (supplementary Figure S1A, available at *Annals of Oncology* online). Even though bacterial genera proportions differed between the different time points, they were not significantly altered by ipilimumab treatment (Wilcoxon test $P > 0.05$; supplementary Figure S1B, available at *Annals of Oncology* online). Likewise, diversity was not altered by ipilimumab injections as assessed by both Shannon and Simpson indices (supplementary Figure S1C, available at *Annals of Oncology* online). Altogether these data indicate that ipilimumab does not induce a gut microbial dysbiosis in patients with MM.

Seven patients provided fecal samples both at baseline (V_1) and during colitis (V_{tox}). Immune-related colitis was associated with a shift in the gut microbial composition as highlighted on the principal component analysis (PCA; Figure 1A). These differences were significant at the family level (Wilcoxon test, $P = 0.0049$) and at the genus level ($P = 0.0059$). The proportions of seven genera were significantly reduced by at least twofold at V_{tox}, compared with V_1 (Figure 1B). They all belonged to the Firmicutes phylum (*Ruminococcus*, *Lachnospiracea incertae sedis*, *Blautia*, *Clostridium IV*, *Eubacterium*, unclassified *Lachnospiraceae* and *Pseudoflavonifractor*) and were dominant members of the microbiota. Similarly, 18 bacterial isolates, mostly Firmicutes, were significantly reduced between V_1 and V_{tox} (paired Student's *t*-test $P < 0.05$, Figure 1C). Finally, colitis was associated with a decreased bacterial diversity, as reflected by a lower Shannon diversity index at the time of colitis ($V_{1\ Shannon} = 5.58$, $V_{tox\ Shannon} = 4.65$; paired Student's *t*-test $P = 0.042$).

Baseline microbiota composition is associated with subsequent clinical response and immune-related colitis

At V_1, inter-class PCA based on genera composition showed a significant clustering of patients depending on their clinical benefit in response to ipilimumab, either long-term clinical benefit or poor clinical benefit (Monte–Carlo test, $P = 0.0090$; Figure 2A). This was also true at finer taxonomic levels such as species or OTUs composition ($P = 0.0050$ and $P = 0.0080$ respectively). Moreover, fecal microbiota at V_1 allowed the correct classification of 84.6% of patients into either the long-term or poor clinical benefit category, and the accuracy reached more than 99% (5000 bootstraps) for patients with poor benefit, as assessed by a Random Forest analysis. The receiver-operating characteristic curve provided an area under the curve (AUC) of 0.77, which indicates a fair classification based on overall microbial composition. Main genera accounting for this stratification are *Faecalibacterium*, *Gemmiger*, *Bacteroides* and *Clostridium* XIVa, and when proportions of these genera were solely considered for testing the stratification the classification power increased and the score was nearly excellent (AUC = 0.895; supplementary Figure S2, available at *Annals of Oncology* online). While high proportions of *Bacteroides* were present at baseline in patients with poor clinical benefit (Wilcoxon test, $P = 0.034$), *Faecalibacterium* percentages were significantly higher in patients with long-term benefit ($P = 0.0092$; Figure 2B). Baseline fecal microbiota composition was also significantly associated with patients' OS and survival longer than 18 months (Wilcoxon test, $P = 0.011$; Figure 2C). All patients with an OS longer than 18 months could indeed be classified based on their gut microbial composition at V_1 and all showed higher proportions of OTUs affiliated to Firmicutes: *Ruminococcus* and *Lachnospiraceae* genus (relatives of *Faecalibacterium prausnitzii* L2-6, *Gemmiger formicilis* and *butyrate-producing bacterium* SS2-1; Figure 2D). It should be noted that although prescription of antibiotics may alter microbiota composition, antibiotics before ipilimumab treatment does not influence baseline dominant microbiota and none of the potentially predictive OTUs or bacterial taxon were associated with antibiotic use (supplementary Figure S3, available at *Annals of Oncology* online).

When the microbiota composition of patients was considered at baseline independently from their clinical characteristics and without *a priori* hypotheses (*k*-means clustering algorithm and Calinski–Harabasz index), three groups of patients emerged that were defined as clusters: clusters A, B and C. The inter-class PCA based on genera composition depending on belonging to any of these three clusters highlighted that this stratification was significant (Monte–Carlo test, $P = 0.00099$; Figure 3A). Microbiota of patients belonging to cluster A ($n = 12$ at baseline) was driven by *Faecalibacterium* and other Firmicutes (unclassified *Ruminococcaceae*, *Clostridium* XIVa and *Blautia*). Cluster B ($n = 10$ at baseline) was enriched in *Bacteroides*, and cluster C ($n = 4$ at baseline) was enriched in *Prevotella* (Figure 3B). Clinical characteristics of patients according to microbiota clustering are described in supplementary Table S4, available at *Annals*

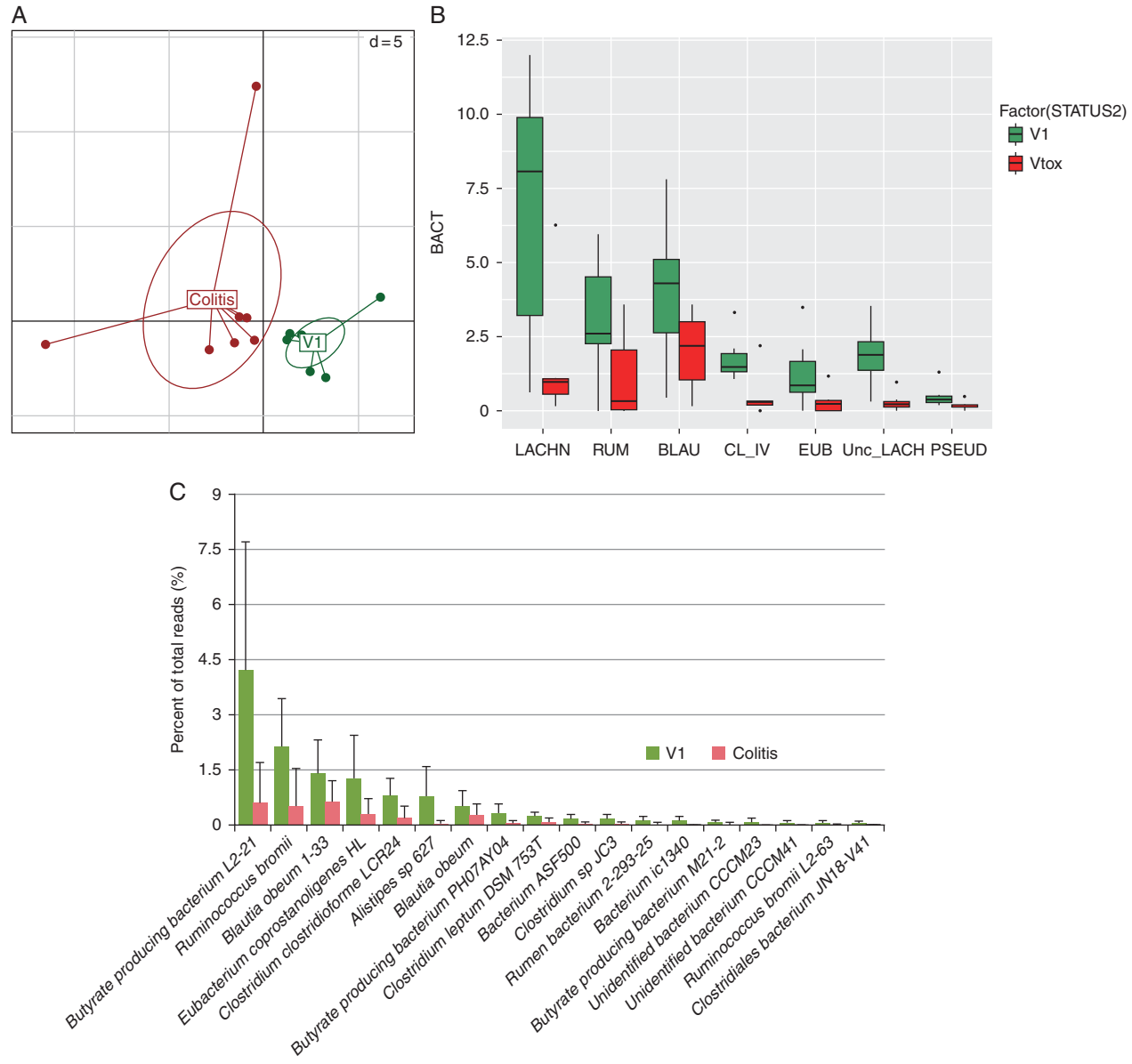


Figure 1. Gut microbiota at baseline and during colitis in patients with metastatic melanoma treated with ipilimumab. (A) Principal component analysis representation of patient distribution based on bacterial genera composition between baseline visit (V_1) and colitis. Seven patients had fecal samples collected at both baseline and time of colitis onset. Monte–Carlo simulated *P*-value=0.0059. Component 1 explains 13.59% of variance; component 2 explains 11.45% of variance. (B) Relative abundance of dominant (>1% of total reads) gut microbial genera significantly reduced during colitis (V_{tox}) when compared with baseline (V_1). LACHN, *Lachnospiracea incertae sedis*; RUM, *Ruminococcus*; BLAU, *Blautia*; CL_IV, *Clostridium* IV; EUB, *Eubacterium*; Unc_LACH, unclassified *Lachnospiraceae*; PSEUD, *Pseudoflavonifractor* (paired *t*-test $P<0.05$ for all genera). (C) Proportions as percent of total reads of significantly impacted bacterial isolates during colitis (V_{tox}) when compared with baseline (V_1). Only significant (paired *t*-test $P<0.05$) data are presented.

of Oncology online. Cluster A was associated with longer PFS than cluster B ($P=0.0039$; Figure 3D) and, to a lesser extent, longer OS ($P=0.051$; Figure 3C). Most of the 22 patients from the Bacteroides-driven cluster B (80%) received subsequent treatments, including anti-PD-1 agents in 50% of the patients while 58% of patients from the *Faecalibacterium*-driven cluster A were treated with subsequent therapies, but only 25% of them received anti-PD-1 agents. Cluster A was also associated with a higher proportion of long-term clinical benefit (8/12; 67%) compared with cluster B (0/10; $P=0.0017$, Fisher's exact test).

At the phyla level, microbiota of patients prone to develop colitis was enriched in Firmicutes at baseline (Wilcoxon test, $P=0.009$), while high proportions of Bacteroidetes was observed in patients who did not develop colitis ($P=0.011$; Figure 4A). In competing risk analysis, belonging to cluster A (driven by *Faecalibacterium* and other Firmicutes) tended to be associated with a shorter colitis-free cumulative incidence compared with cluster B (driven by *Bacteroides*) (Gray's test; $P=0.054$, Figure 4B). Using linear discriminant analysis effect size (LEfSe) analysis, 15 bacterial OTUs were detected as potential biomarkers

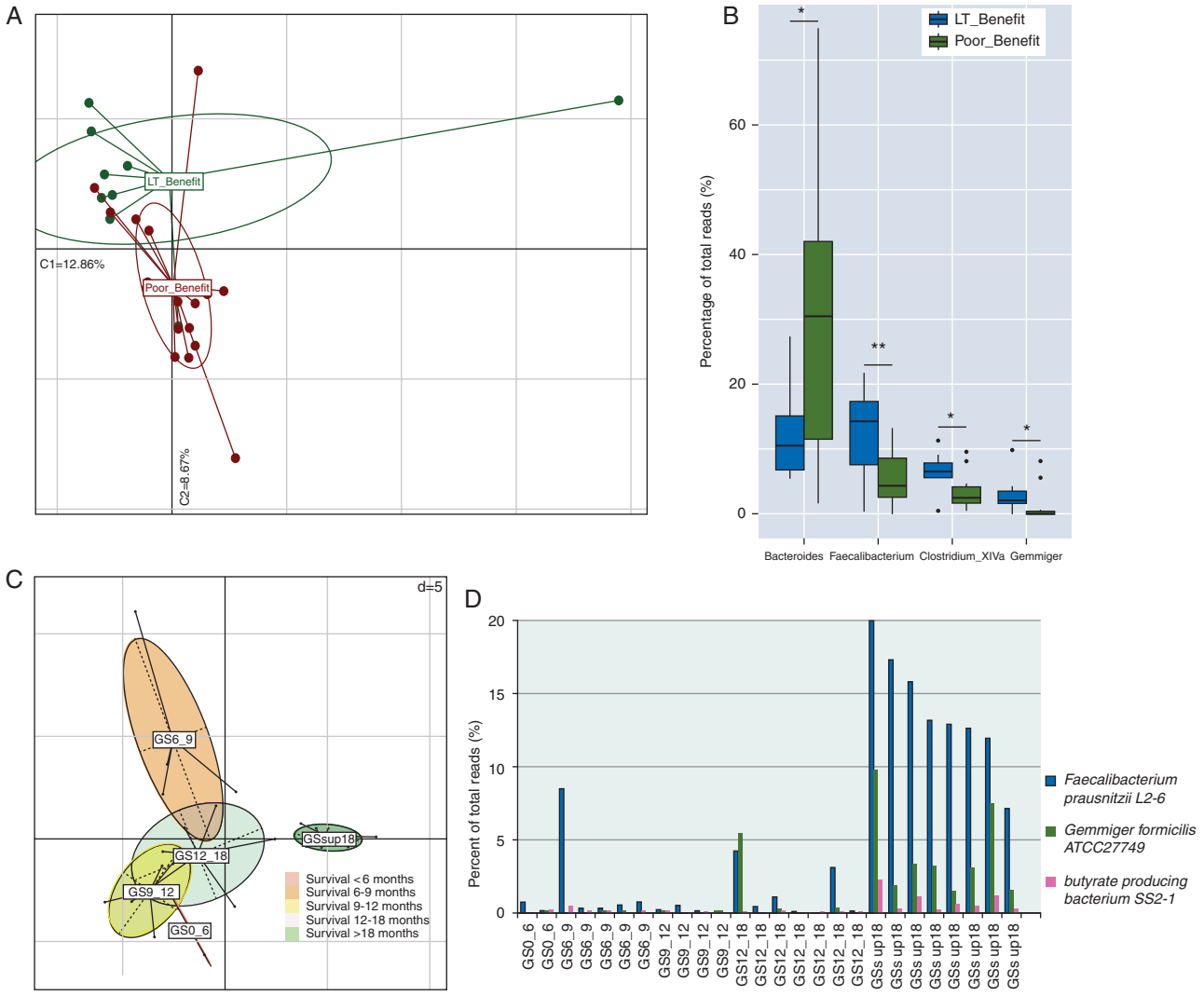


Figure 2. Baseline gut microbiota as a predictor of response to ipilimumab. (A) Principal component analysis representation of patients' distribution based on bacterial genera composition at baseline (V_1) depending on the benefit of ipilimumab treatment; LT Benefit: long-term benefit (in green) versus Poor Benefit (in dark red). Component 1 explains 12.86% of variance; component 2 explains 8.67% of variance. Monte–Carlo simulated *P*-value=0.0090. (B) Boxplot of the percentages of four dominant (>1% of total reads) genera differentially represented between both groups, i.e. *Bacteroides*, *Faecalibacterium*, *Clostridium* XIVa and *Gemmiger*; LT_Benefit, long-term benefit versus Poor Benefit; *P <0.05; **P <0.001. (C) Inter-class principal component analysis representation of patient distribution based on bacterial genera composition at baseline (V_1) depending on overall survival time following ipilimumab treatment; GS0_6: overall survival ranging from 0 to 6 months (n=2), GS6_9: overall survival ranging from 6 to 9 months (n=5), GS9_12: overall survival ranging from 9 to 12 months (n=4), GS12_18: overall survival ranging from 12 to 18 months (n=7), GSsup18: overall survival superior to 18 months (n=8). Monte–Carlo simulated *P*-value= 0.011. (D) Percentages of three specific OTUs highlighted as biomarkers at baseline (V_1) of overall survival duration >18 months. Each patient's microbiota are presented in the graph. GS0_6: overall survival ranging from 0 to 6 months (n=2), GS6_9: overall survival ranging from 6 to 9 months (n=5), GS9_12: overall survival ranging from 9 to 12 months (n=4), GS12_18: overall survival ranging from 12 to 18 months (n=7), GSsup18: overall survival >18 months (n=8).

either of colitis-free progression or colitis onset during ipilimumab treatment (Figure 4C). Five (out of six) OTUs from the Bacteroidetes phylum (such as *B. uniformis, B. vulgatus* and *Parabacteroides distasonis*) were associated with absence of colitis, whereas OTUs associated with subsequent colitis related mostly to the Firmicutes phylum (8/9 OTUs). Several of these potential colitis-predictive baseline OTUs (relatives of *F. prausnitzii* L2-6, *butyrate producing bacterium* L2-21 and *G. formicilis* ATCC 27749*)* were also associated with longer OS.

Baseline immune parameters related to microbiota clustering, clinical benefit and ipilimumab-induced colitis

Twenty-six patients had both immune monitoring and microbiota composition analysis at V_1. Patients from cluster C could not be included in this analysis due to the low number of patients belonging to this cluster ($n = 4$). Since patients who belonged to cluster A ($n = 12$) and cluster B ($n = 10$) had no differences in

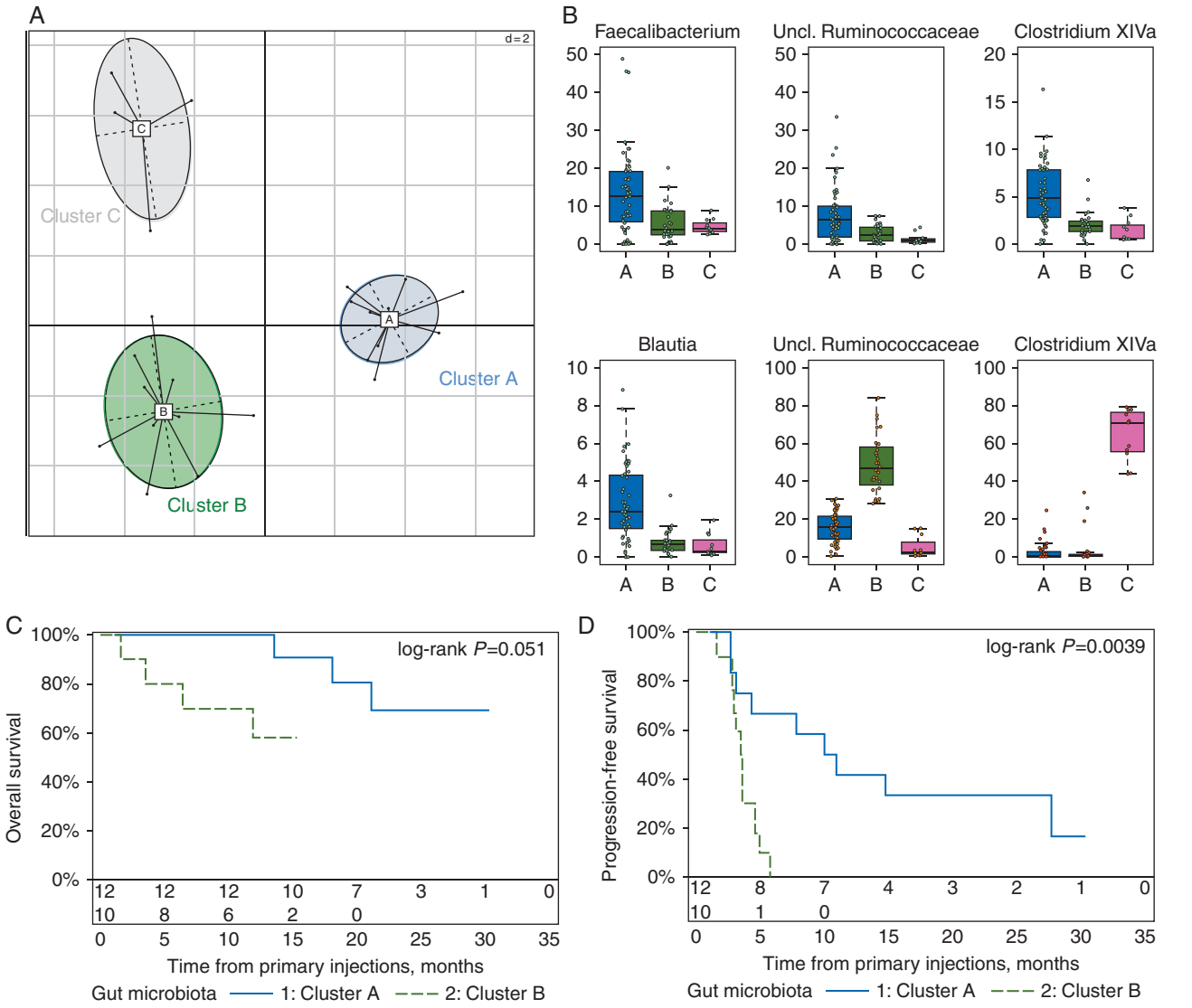
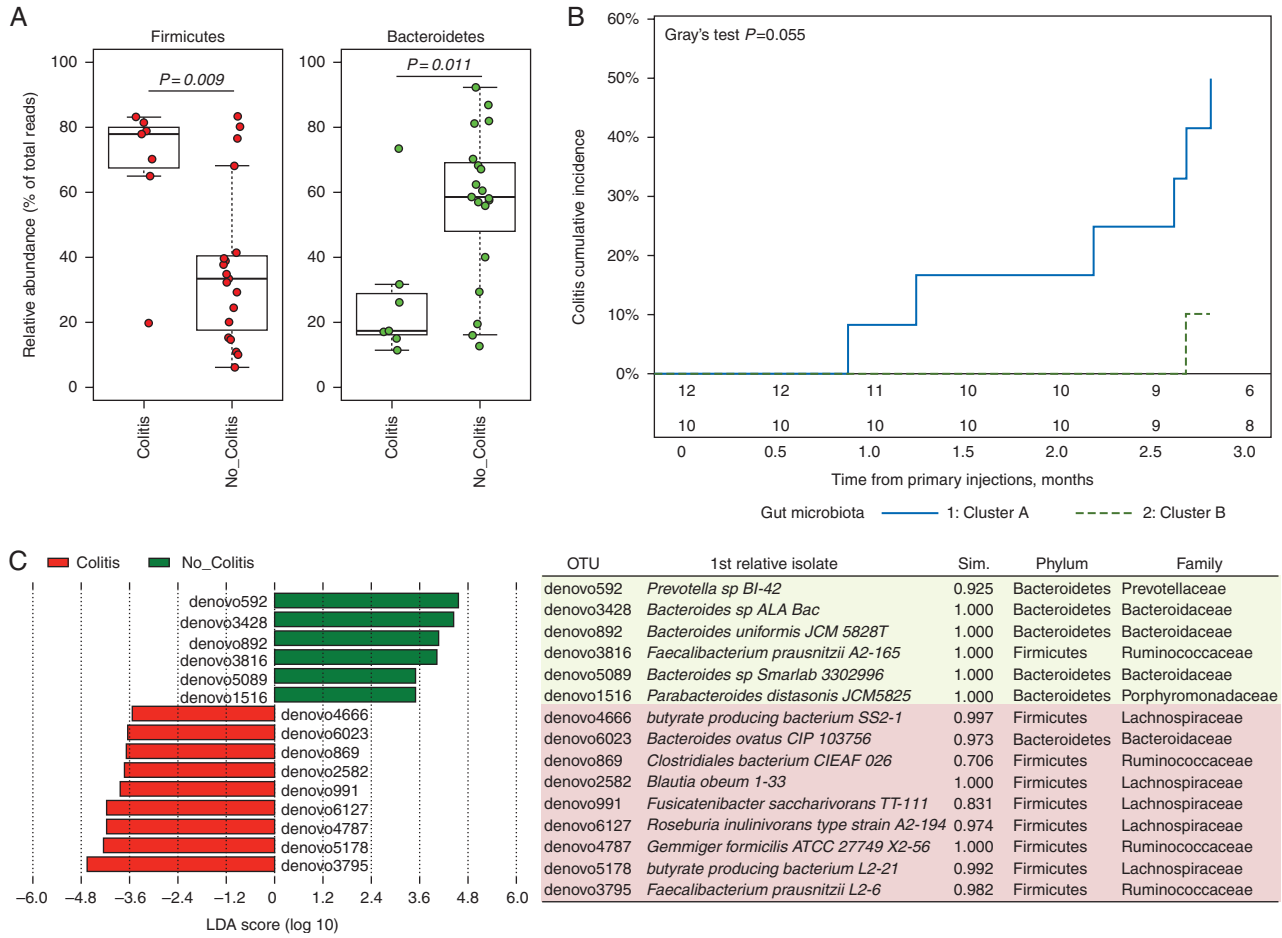


Figure 3. Baseline Gut microbiota composition predicts clinical response to ipilimumab. (A) Inter-class principal component analysis representation of patient's stratification into 3 statistically robust clusters (A, B and C) at baseline (V_1). Monte–Carlo simulated *P*-value=0.00099. (B) Bacterial genera discriminating the 3 different clusters at baseline were assessed with a Random Forest analysis, and confirmed with a Wilcoxon test. Percentage of reads for each of these six genera is represented for each cluster. Genera significantly (Wilcoxon test) overrepresented in patients from cluster A are in blue, genus significantly overrepresented in patients from cluster B is in green, and genus significantly overrepresented in patients from cluster C is in grey. (C) Baseline gut microbiota composition and overall survival (OS). Kaplan–Meier survival curves of patients classified into two groups according to clusters, cluster A versus cluster B. (D) Baseline gut microbiota composition and progression-free survival. Kaplan–Meier curves of patients classified into two groups according to clusters, cluster A versus cluster B. *P* values are indicated on each graph.

absolute counts of peripheral-blood $CD4^+$ and $CD8^+$ T cells (supplementary Table S5, available at *Annals of Oncology* online), the main T cell subpopulations were considered as a percentage. Patients who belonged to the *Faecalibacterium*-driven cluster A had a low proportion of baseline regulatory T cells (Tregs), and had a significantly lower proportion of baseline $\alpha4^+\beta7^+$ $CD4^+$ ($P=0.045$) and $\alpha4^+\beta7^+$ $CD8^+$ ($P=0.034$) T cells, compared with those who belonged to the *Bacteroides*-driven cluster B (Figure 5A).

Patients with long-term benefit tended to have a lower frequency of regulatory T cells ($P=0.056$) and a lower frequency of $\alpha4^+\beta7^+$ $CD4^+$ ($P=0.015$) and $\alpha4^+\beta7^+$ $CD8^+$ ($P=0.0081$) T cells, compared with those with poor clinical benefit (Figure 5B). Although serum concentrations of several biomarkers of inflammation (IL-6, IL-8, IP-10, MCP-1, TNFα, sCD25) and sCD14 considered as a marker of bacterial invasion (translocation) were measured, none of these were associated with any cluster or clinical benefit at baseline (supplementary Tables S5 and S6, available at *Annals of Oncology* online).

Patients who developed ipilimumab-induced colitis tended to have significantly higher absolute numbers of CD4+ T cells ($P=0.053$) and had significantly lower levels of IL-6, IL-8 and



OTU	1st relative isolate	Sim.	Phylum	Family
denovo592	*Prevotella sp BI-42*	0.925	Bacteroidetes	Prevotellaceae
denovo3428	*Bacteroides sp ALA Bac*	1.000	Bacteroidetes	Bacteroidaceae
denovo892	*Bacteroides uniformis JCM 5828T*	1.000	Bacteroidetes	Bacteroidaceae
denovo3816	*Faecalibacterium prausnitzii A2-165*	1.000	Firmicutes	Ruminococcaceae
denovo5089	*Bacteroides sp Smarlab 3302996*	1.000	Bacteroidetes	Bacteroidaceae
denovo1516	*Parabacteroides distasonis JCM5825*	1.000	Bacteroidetes	Porphyromonadaceae
denovo4666	*butyrate producing bacterium SS2-1*	0.997	Firmicutes	Lachnospiraceae
denovo6023	*Bacteroides ovatus CIP 103756*	0.973	Bacteroidetes	Bacteroidaceae
denovo869	*Clostridiales bacterium CIEAF 026*	0.706	Firmicutes	Ruminococcaceae
denovo2582	*Blautia obeum 1-33*	1.000	Firmicutes	Lachnospiraceae
denovo991	*Fusicatenibacter saccharivorans TT-111*	0.831	Firmicutes	Lachnospiraceae
denovo6127	*Roseburia inulinivorans type strain A2-194*	0.974	Firmicutes	Lachnospiraceae
denovo4787	*Gemmiger formicilis ATCC 27749 X2-56*	1.000	Firmicutes	Ruminococcaceae
denovo5178	*butyrate producing bacterium L2-21*	0.992	Firmicutes	Lachnospiraceae
denovo3795	*Faecalibacterium prausnitzii L2-6*	0.982	Firmicutes	Ruminococcaceae

Figure 4. Gut microbiota composition at baseline predicts ipilimumab-induced colitis. (A) Boxplot of relative abundance of the two dominant phyla Firmicutes and Bacteroidetes at baseline between patients prone to or resistant to ipilimumab-induced colitis. A Wilcoxon test has been applied to assess significance and *P*-values are indicated on the graph. (B) Colitis cumulative incidence (Gray's test) of patients classified into two groups according to clusters, cluster A versus cluster B. *P* values are indicated on the graph. (C) OTUs predictive of colitis development during ipilimumab treatment. LEfSe uses linear discriminant analysis (LDA) to estimate the effect size of each differentially abundant feature (i.e. bacterial OTU). On the left panel, OTUs in red are biomarkers of colitis development (Colitis). OTUs in green are biomarkers of the absence of colitis development (No_Colitis). Best biomarkers show the highest absolute LDA score with a minimal threshold of 3.5 (i.e. OTU denovo592 for the No_Colitis group and OTU denovo3795 for the Colitis group). Right panel indicates the taxonomic affiliation of each of these OTUs. Sim.: similarity between the OTU read and the first assigned isolate 16S sequence, assessed by the RDP Sab_score. The RDP S_ab score is a percentage of shared 7-mers between two sequences.

sCD25 at baseline compared with patients without colitis (Figure 5C; supplementary Table S7, available at *Annals of Oncology* online). In competing risk analysis, patients with high numbers of conventional $CD4^+$ T cells had a shorter colitis-free cumulative incidence, compared with those with low numbers of conventional $CD4^+$ T cells ($P = 0.0038$, supplementary Figure S4, available at *Annals of Oncology* online). The median percentage of Tregs were lower before introduction of ipilimumab in patients who developed colitis during the course of ipilimumab compared with patients without colitis (2.5% versus 8.4%, $P = 0.018$). However, absolute numbers of Tregs were not significantly different (supplementary Table S7, available at *Annals of Oncology* online). None of the other $CD4^+$ T cell subpopulations at baseline were related to colitis onset (supplementary Table S7, available at *Annals of Oncology* online).

The ICOS molecule is significantly up-regulated on $CD4^+$ T cells after ipilimumab treatment in patients who belong to *Faecalibacterium*-driven cluster A

Since immune parameters were monitored longitudinally, specific changes in immune activation during the course of ipilimumab treatment were assessed in patients from cluster A versus B. During ipilimumab treatment, ICOS was significantly increased on conventional $CD4^+$ T and Treg cells in patients who belonged to cluster A (Figure 6A and B), but not in those who belonged to cluster B (Figure 6A and B). Given that ICOS is induced on $CD4^+$ T cells after IL-2 stimulation, concentration of sCD25, a marker of IL-2 impregnation [29, 30], was surveyed during the course of ipilimumab treatment. Patients who belonged to cluster A showed a greater increase over time in sCD25 than patients from cluster B, as demonstrated by a higher $V_{2/3}$ to V_1 ratio compared with patients from cluster B (Figure 6C).

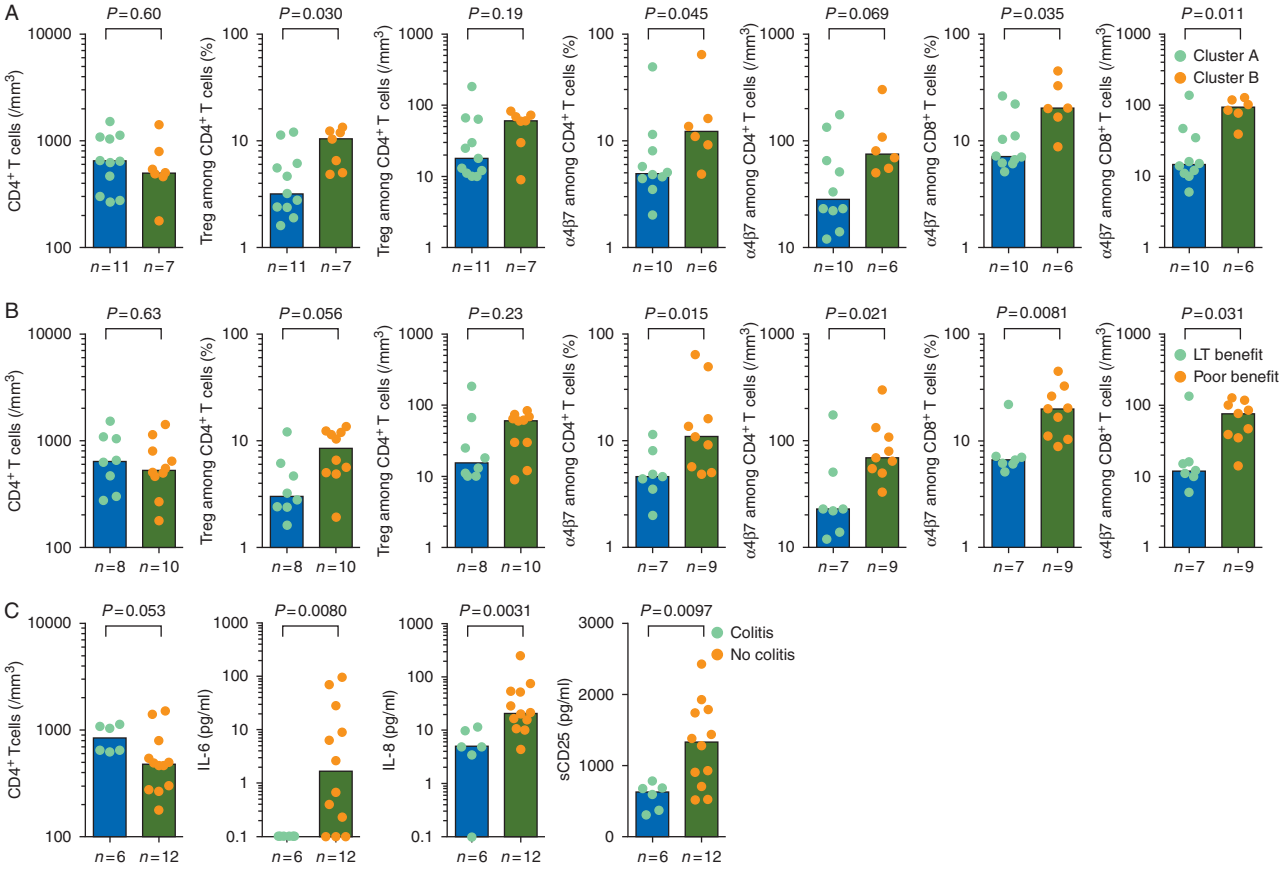


Figure 5. Baseline systemic immune status and microbiota composition. (A–C). Baseline (V_1) percentages and absolute numbers of fresh whole blood $CD4^+$ T cells, Treg cells (% $CD4^+CD3^+CD25^+Foxp3^+$ within $CD4^+CD3^+$ cells); $\alpha4^+\beta7^+$ among $CD4^+$ T and $CD8^+$ T cells, serum concentrations of IL-6, IL-8 and sCD25 were analyzed and compared between patients belonging to cluster A and patients belonging to cluster B (A); long-term clinical benefit (LT benefit) and poor clinical benefit (poor benefit) (B); patients with colitis (Colitis) and patients without colitis (No colitis) (C). Each dot represents one patient. *P* values are indicated on each graph; ns means "not significant"; Mann–Whitney tests were used.

Discussion

This study shows that a distinct baseline gut microbiota composition is associated with an anti-cancer response and immune-mediated colitis in patients with MM treated with ipilimumab. Colonization by Firmicutes and more specifically by OTUs related to *F. prausnitzii* L2-6, *butyrate producing bacterium* L2-21 and *G. formicilis* ATCC 27749 is associated with both anti-cancer response and immune-related colitis. There is a higher representation of Bacteroidetes (mostly *Bacteroides* genus) in patients who have a poor anti-cancer response and who remain colitis-free. Dubin et al. recently showed an increased representation of bacteria belonging to the Bacteroidetes phylum among patients who remain free of ipilimumab-induced colitis in an American cohort of 34 patients [31]. Our results confirm these data in an independent cohort. Moreover, in our study, *Bacteroides* genus was also associated with poor response to anti-CTLA-4 treatment. Recent studies conducted in mice have shown that the efficacy of CTLA-4 and PDL-1 blockade is influenced by gut microbiota [13, 14]. In mice, anti-CTLA-4 treatment induced a microbial dysbiosis [13] that was not observed in our patients except at the time of ipilimumab-induced colitis. Furthermore, while we observed that higher proportions of *Bacteroides* at baseline were associated with minimal benefit of ipilimumab in patients, fecal microbiota transplantation (FMT) from patients with MM into mice recipients revealed that specific species from the *Bacteroides* genus (*B. fragilis* and/or *B. thetaiotaomicron*) could elicit the immunostimulatory effects of CTLA-4 blockade [13]. However, FMT of human feces in mice have several limitations due to a bias in the reconstitution of the microbiota and is more distant from the clinical setting than our study. In our study, we directly assessed the anti-cancer efficacy of ipilimumab in patients with MM. Finally, since specific bacterial groups were targeted by quantitative PCR in FMT experiments in mice, one cannot exclude that other species were involved in ipilimumab efficacy in this setting. In our dataset, *B. fragilis* and *B. thetaiotaomicron* were not abundantly represented at baseline (supplementary Figure S5, available at *Annals of Oncology* online). Overall, these bacterial species were more abundant in patients with poor clinical benefit who remained colitis-free.

We found that the *Faecalibacterium*-driven cluster A and clinical benefit were associated with low baseline percentage of circulating $\alpha4^+\beta7^+$ T cells and $CD4^+$ Tregs. In addition, high proportions of *F. prausnitzii*, low baseline percentage of circulating $CD4^+$ Tregs and low baseline levels of systemic inflammatory proteins, such as IL-6, IL-8 and sCD25, were associated with subsequent colitis. Commensal bacteria, such as *F. prausnitzii*, and

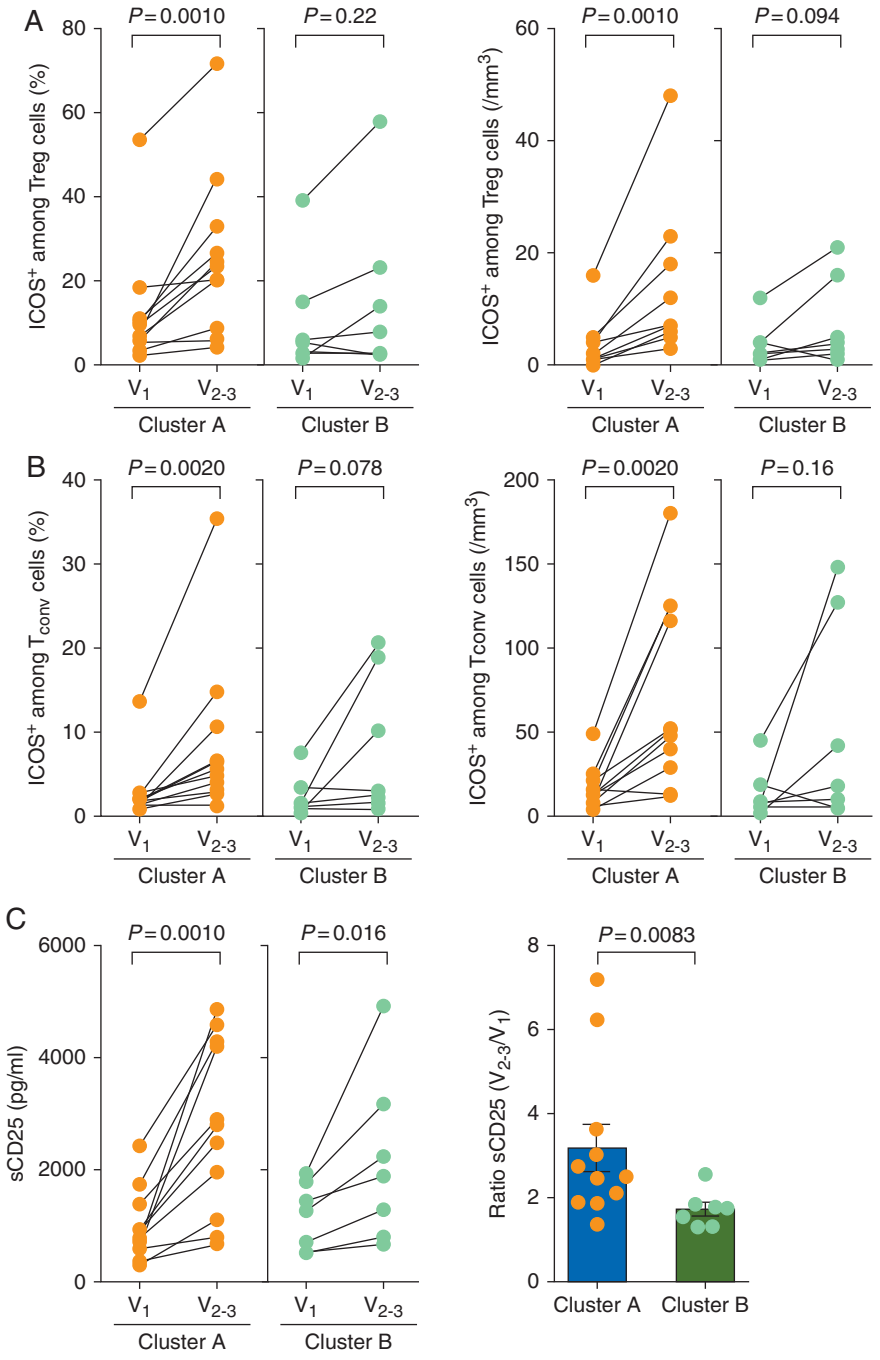


Figure 6. Ipilimumab-induced blood immune modifications and baseline microbiota composition. The expression of ICOS on $CD4^+$ T cells was monitored in fresh whole blood before ipilimumab treatment (V_1) and after one or two injections of ipilimumab (V_{2-3}). Serum concentration of sCD25 was monitored before ipilimumab treatment (V_1) and after one or two injections of ipilimumab (V_{2-3}). Percentage of conventional $CD4^+$ T cells (Tconv) was defined as $\%CD3^+CD4^+$ T excluding Treg cells. Graphs depict specific changes in Treg cells (A), Tconv cells (B) and sCD25 (C) over the course of ipilimumab treatment in patients belonging to cluster A (white circles) and patients belonging to cluster B (black circles). For sCD25, V_{2-3} to V_1 ratio was calculated [(sCD25 at V_{2-3})/(sCD25 at V_1)] for patients from clusters A and B (C, right). Each dot represents one patient. *P* values are indicated on each graph. Wilcoxon matched-pairs signed rank (A, B and C, left) or Mann–Whitney (C, right) tests were used.

several butyrate-producing bacteria promote the development of tolerogenic macrophages and dendritic cells, as well as induced and thymic Treg accumulation, and thereby contribute to the anti-inflammatory balance of the intestines [32, 33]. It has been suggested that *F. prausnitzii* have an anti-inflammatory role in IBD. Low abundance of *F. prausnitzii* and high amounts of Enterobacteriaceae predicted subsequent relapse in patients who had received an ileocaecal resection for Crohn's disease [23]. Although it is currently hypothesized that dysbiosis precedes IBD, until now it has not been possible to have access to pre-

illness microbiota in patients with IBD. The anti-inflammatory role of *F. prausnitzii* is based upon studies carried out in CD siblings and patients who already have the disease, whereas we studied patients before they received ipilimumab. Future studies will determine whether there is a specific microbiota associated with the subsequent development of IBD, and if it is close or far from that of patients who will develop colitis during ipilimumab treatment. However, our microbiota and immune monitoring data seem consistent. Baseline serum concentrations of pro-inflammatory cytokines were low in patients who developed colitis and in those who belonged to cluster A—this is in line with the anti-inflammatory role of *F. prausnitzii* and other Firmicutes. Previous studies have described correlations between specific components of the gut microbiota and systemic pro-inflammatory cytokine levels [21]. In our study, higher systemic inflammatory proteins and high proportions of Bacteroidetes at baseline were associated with resistance to colitis. *Bacteroides* enrichment has been shown to be associated with low-grade systemic inflammation, as opposed to anti-inflammatory species, such as *F. prausnitzii* [22–24, 34].Taken together, these data suggest that the concentration of systemic inflammatory proteins at baseline might reflect microbiota composition and predict colitis in patients treated with ipilimumab. High proportion and numbers of $\alpha4^+\beta7^+$ T cells before ipilimumab treatment was associated with cluster B and poor benefit in patients. A previous paper has studied microbiota composition and blood T cell phenotype in patients with Crohn's disease, their related siblings and healthy controls. It showed that patients and siblings had a lower fecal concentrations of *Faecalibacterium prausnitzii* and a higher percentage and numbers of circulating $\beta7^+$ T cells [35], as we observed in patients from cluster B in our cohort. Ipilimumab blocks CTLA-4, a major peripheral tolerance mechanism. Regulatory T cells have a critical role in regulating the development of colitis and Treg cells express high levels of CTLA-4. It has been showed that *F. prausnitzii* is associated with an augmentation/induction of regulatory T cells in the gut. Thus, we can speculate that *F. prausnitzii* could be associated with a sequestration of $\alpha4^+\beta7^+$ T cells and Tregs within the intestines and, possibly, tumor beds. Treg cells harbor high levels of CTLA-4 and are therefore vulnerable to ipilimumab [36]. Consequently, ipilimumab could induce the inactivation of intratumoral and gut-associated CTLA-4^+ Treg cells leading to effector T cell activation and therefore anti-cancer response as well as colitis development.

In addition, patients who belong to the *Faecalibacterium*-driven cluster A have a higher ICOS induction on $CD4^+$ T cells and a higher increase in sCD25 (Figure 6). These results are reminiscent of previous studies where ICOS induction on $CD4^+$ T cells during the course of ipilimumab treatment has been proposed as a biomarker of ipilimumab efficacy in patients with cancer [18, 37, 38]. sCD25 is a biomarker that has been associated with IL-2 impregnation [30] and IL-2 is a major growth factor for T cells, particularly regulatory Treg cells which respond to IL-2 treatment even at low dosage. CTLA-4 engagement prevents ICOS expression [29] while IL-2 is a great inducer of ICOS expression on T cells, particularly on Treg cells that express constitutionally high affinity IL-2R chain (CD25 or IL-2Rα) [39–42]. Taken together, these observations suggest that gut microbiota composition at baseline could predict which patients will adequately respond to ipilimumab.

It can be argued that colitis could have been a time-dependent confounder on the causal relationship between microbiota composition and clinical response. However, it could induce an opposite bias, with a decrease of the benefit in the patients with colitis in whom ipilimumab treatment is stopped prematurely. We conducted a sensitivity analysis in the subgroup of patients who remained colitis-free. At the OTU level, the PCA was statistically significant ($P = 0.048$), and there was a trend at the genera level (Monte–Carlo P-value = 0.084). The tendency for the 4 specific genera showed a similar trend, but differences were not significant anymore. While *F. prausnitzii* L2-6 was still significantly associated with patients having an OS longer than 18 months ($P = 0.031$), significance was lower for *G. formicilis* ($P = 0.064$) and for butyrate-producing bacterium SS2-1 ($P = 0.10$) in this sub-cohort. In conclusion, trends between baseline microbiota composition and response to ipilimumab were similar in these small subgroups and in the whole group.

Our results suggest that baseline gut microbiota composition may be an important determinant of response and colitis associated with anti-CTLA-4 treatment. Further studies are needed to compare the predictive value of microbiota with that of other markers, and to explore these parameters in the context of various types of tumors and anticancer treatments. The combination of anti-CTLA-4 and anti-PD1 blockade was recently approved for the treatment of patients with MM and is also in development in other tumor types based on increased response rates (50%–60%) and prolonged PFS [43, 44]. The results in terms of OS will be available in the following months. However, this combination is much more toxic than each of the drugs given as monotherapy, with close to 60% grades 3–5 adverse events, many of them being severe colitis, and interruption of treatment in 40% of patients because of toxicity [43, 44]. One of the main objectives of several research teams in this field is thus to identify baseline parameters that could help to identify patients who need the combination versus those who could be controlled with a monotherapy and not expose them to the risk of developing severe adverse events. Microbiome prospective studies similar to our present study will therefore be critical to conduct in patients treated with anti-PD1 drugs alone or in combination with ipilimumab to identify potential favorable versus hostile flora in the context of these distinct immunotherapies. It will also be very important to conduct such studies in various tumor types to evaluate the impact of the microbiome in different tumors and with different drugs, since checkpoint inhibitors are now being investigated in a wide range of tumors.

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Disclosure

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