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# 1 **Cancer and immunotherapy: A role for microbiota composition**

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15 **Keywords:** gut microbiota, immunotherapy, cancer, immune checkpoint blockers, biomarkers

16 **Abbreviation:** Antibiotics (ATB), colorectal cancer (CRC), complete response (CR), cytotoxic  
17 T-lymphocyte-associated protein-4 (CTLA-4), European Medicines Agency (EMA), U.S.  
18 Food and Drug Administration (FDA), immune checkpoint blockers (ICB), immune-related  
19 adverse events (IrAE), Inducible T-cell COStimulator (ICOS), non-small cell lung cancer  
20 (NSCLC), overall survival (OS), partial response (PR), programmed-cell death protein-1  
21 receptor (PD-1), programmed death-ligand 1 (PD-L1), progression-free survival (PFS), short  
22 chain fatty acids (SCFA), tape measure protein (TMP), tumor necrosis factor (TNF).

23 **Abstract (127 words)**

24 Human microbiota plays a key role in preserving homeostasis; therefore, alteration in its  
25 composition is associated with susceptibility to various diseases. Recent findings suggest that  
26 gut microbiota may influence response to cancer treatment, especially immune checkpoint  
27 blockers (ICB). The development of ICB has changed cancer patients' outcomes and has  
28 allowed sustained recovery. Unfortunately, some patients do not respond to ICB and microbiota  
29 may be a promising new biomarker to identify patients that will have benefit from ICB. This  
30 review presents relationship between microbiome composition or microbiota-derived  
31 metabolites and response to ICB or immune-related adverse events. Furthermore, we will  
32 present different strategies to modulate microbiota composition in patients in order to enhance  
33 ICB efficacy or dampen their toxicities which could lead to the emergence of interesting  
34 complementary treatments.

35

36

## 37 **Introduction**

38 The human microbiota is composed of around  $3 \times 10^{13}$  microorganisms (bacteria, viruses, fungi,  
39 and protozoa) in the epithelial barrier surfaces and has a commensal relationship with the host.  
40 Individual's microbes composition changes over time due to host genetics [1], diet [2],  
41 medication [3], lifestyle, geographical localization [4] or age, leading to a significant  
42 interindividual variation. Gut microbiome has several physiologic functions and participates in  
43 compounds hydrolysis [5], vitamin production [6], gut integrity and control of pathogen  
44 colonization [7] , protection against systemic infections [8] and immune system development  
45 [9]. It is now well established that intestinal dysbiosis, defined as an imbalance of the bacterial  
46 ecosystem, and digestive and even extra-digestive diseases are linked [10]. Commensal  
47 microbiota contributes to individual's immunity, affecting overall health. Disruption in the  
48 homeostasis of microbial communities induces local chronic inflammation and activation of the  
49 immune system, both mechanisms that could play a role in tumorigenesis [11–13]. Several  
50 evidences support the role of some bacteria, as viruses, in carcinogenesis and cellular dysplasia.  
51 *Salmonella typhimurium* might be correlated with the onset of gallbladder cancer [11],  
52 *Helicobacter pylori* influences the pathogenesis of gastric cancer [14] through chronic  
53 inflammation and influence over the local microenvironment to promote carcinogenesis.  
54 Growing evidence shows that gut microbial dysbiosis contributes to the development of  
55 colorectal cancer (CRC). A study identifies different compositions between tumoral tissue and  
56 neighboring tumor healthy tissue. *Firmicutes*, *Fusobacteria*, *Lactococcus* and *Fusobacterium*  
57 were found in abundance in CRC tissue compared to healthy one while *Pseudomonas* and  
58 *Escherichia-Shigella* were under-represented in tumoral tissue compared to healthy one [15].  
59 As evidences suggest that microbiota composition could confers susceptibility to certain  
60 cancers, it may also influence cancer outcomes [16]. Over the years, gut microbiota  
61 composition has emerged as a new target to shape efficacy and toxicity of cancer therapy [17]

62 and nowadays, data support, even in patients, its role as a predictive biomarker of response to  
63 chemotherapy or immunotherapy or for susceptibility to treatment-related toxicities [18–26].  
64 Consequently, there is a growing interest in targeting these microbes to improve therapeutic  
65 anticancer strategies.

## 66 **Gut microbiota modulation of ICB treatment efficacy and toxicity**

67 In the last decade, ICB have changed the therapeutic strategies of several solid cancers by  
68 enhancing antitumor immune response [27]. These FDA- and EMA-approved monoclonal  
69 antibodies target co-inhibitory molecules to block pathways of negative regulation of the  
70 immune system: Cytotoxic T-lymphocyte-associated protein-4 (CTLA-4), programmed-cell  
71 death protein-1 receptor (PD-1) or its ligand (PD-L1) down-regulate the immune response when  
72 its activation become harmful to the host. Tumor cells and immune cells in the tumor  
73 microenvironment overexpress these molecules to escape antitumor immune surveillance [28].  
74 Blocking these receptors or ligands can restore a patient's immune anti-tumoral response. ICBs  
75 have had a major impact on cancer treatment, but a non-negligible proportion of patients do not  
76 respond to these immunotherapies, some cancers show primary or secondary resistance to ICBs  
77 and response rate differs between patients and cancer localizations. As gut microorganisms play  
78 a role in the balanced ecosystem and in shaping normal and pathological immune response,  
79 contributing to carcinogenesis, microbiome might play a role in anti-tumor immunosurveillance  
80 and has then emerged as a potential biomarker for response to ICBs. Some evidences indicate  
81 that it could be part of the underlying reasons for non-responsiveness, as particular taxa have  
82 been identified in ICB responders' gut microbiota or stools in preclinical models [18,29,30] and  
83 patients [19,20,22–26].

### 84 • **Evidence in preclinical mouse models**

85 One of the first study demonstrating a relationship between gut microbiota and response to  
86 immunotherapy was conducted in mice injected subcutaneously with cancer cells and treated

87 with CpG-oligonucleotide, a ligand of Toll-like receptor 9 and an antibody directed against the  
88 immunosuppressive interleukin-10 receptor. This association of immunotherapies induces  
89 tumor necrosis factor (TNF) secretion by tumor-associated myeloid cells followed by a CD8+  
90 T cell response leading to tumor hemorrhagic necrosis. Antibiotic (ATB)-treated or germ-free  
91 mice showed an impaired production of proinflammatory cytokines by immune cells. Gavage  
92 with *Alistipes* species restore TNF-dependent tumor response to immunotherapy whereas  
93 *Lactobacillus* species dampened this response (Figure 1) [18]. A second study explored the role  
94 of microbiota composition in tumor-bearing mice treated with anti-CTLA-4. In this study, anti-  
95 CTLA-4 activity was inhibited in germ-free or ATB-treated mice compared to controls. A  
96 single injection of anti-CTLA-4 significantly affect the microbiome composition with a relative  
97 enrichment of *Bacteroides thetaiotaomicron*, *Bacteroides uniformis* and to a less extent  
98 *Bacteroides fragilis*. Recolonization of germ-free or ATB-treated mice with these species  
99 restored the anticancer response to anti-CTLA4, and increased Th1 immune responses in the  
100 tumor-draining lymph nodes and maturation of intratumoral dendritic cells (Figure 1). In this  
101 study, authors also suggested that anti-CTLA-4-induced dysbiosis was linked to the severity of  
102 gut inflammation and intestinal lesions [29]. Sivan et al. observed that microbiota influences  
103 anti-PD-L1 response in a melanoma animal model. Mice harboring different microbiota had  
104 different outcomes regarding antitumor immunity and melanoma growth. Abundance of  
105 *Bifidobacterium species* in gut microbiota was found to be correlated with T-cell response and  
106 treatment with anti-PD-L1 in combination with oral feeding with these bacteria led to nearly  
107 abolishing tumor outgrowth by increasing dendritic cells maturation and antitumor CD8+ T  
108 cells activity (Figure 1) [30]. Altogether, these pioneering studies demonstrated the link  
109 between microbiota composition and response or IrAE to ICB in preclinical models.

110 • **Evidence in patients with cancer**

111 Numerous clinical studies investigated microbiota's influence in patients with solid cancer  
112 treated with ICBs. The first study in patients investigated microbiota composition and the onset  
113 of ipilimumab-induced colitis in metastatic melanoma patients. The composition of microbiota  
114 was assessed before ipilimumab initiation; microbial richness was found to be similar between  
115 patients who developed anti-CTLA-4-induced colitis and those who remained colitis free.  
116 However, Bacteroidetes phylum was underrepresented in the patients who developed immune-  
117 mediated colitis [21]. Similar to this work, a study in patients with metastatic melanoma and  
118 treated with ipilimumab confirmed that patients with baseline microbiota enriched with  
119 Bacteroidetes phylum were protected from ipilimumab-induced colitis [20]. In mice, anti-  
120 CTLA-4 treatment induced a microbial dysbiosis after the first injection [29] that was not  
121 observed in patients except at the time of ipilimumab-induced colitis [20]. Patients with  
122 metastatic melanoma with baseline microbiota enriched with *Faecalibacterium* genus and other  
123 *Firmicutes* had longer progression-free survival (PFS) and overall survival (OS) compared to  
124 patients whose baseline microbiota was driven by *Bacteroides* genus. At the species level,  
125 *Faecalibacterium prausnitzii* was more represented in stools at baseline in patients that will  
126 benefit from ipilimumab treatment. Moreover, a lower proportion of peripheral blood  
127 regulatory T cells was observed in patients with microbiota enriched with *Faecalibacterium*.  
128 Finally, ipilimumab treatment led to a higher ICOS induction on CD4+ T cells in patients whose  
129 baseline microbiota was enriched with *Faecalibacterium* showing a link between gut  
130 microbiota composition and immunodynamics of ipilimumab (Figure 1) [20]. Another study  
131 realized in 39 patients with metastatic melanoma and treated with ipilimumab, nivolumab,  
132 ipilimumab plus nivolumab, or pembrolizumab found a number of bacteria in the Firmicutes  
133 phylum (*Faecalibacterium prausnitzii*, *Holdemania filiformis*, and *Dorea formicigenerans*)  
134 associated with anti-CTLA-4 plus anti-PD-1 clinical efficacy [19]. Gopalakrishnan et al.

135 investigated the composition of gut microbiome of patients with metastatic melanoma treated  
136 with anti-PD-1. Higher germs diversity and abundance of *Ruminococcaceae* were found in  
137 responding patients, and high diversity in the stool microbiome was correlated with  
138 significantly prolonged PFS contrasting with low stool diversity and abundance of  
139 *Bacteroidales* in non-responder patients (Figure 1). At the genus level, patients with high  
140 *Faecalibacterium* abundance had significantly prolonged PFS while patients with high  
141 abundance of *Bacteroidales* had shortened PFS [22]. All these studies were conducted in  
142 patients with metastatic melanoma however ICB are also indicated in a wide range of tumors  
143 and recent studies suggested that good or hostile microbiota composition may vary from a  
144 tumor location to another. In patients with non-small cell lung cancer (NSCLC) or urothelial  
145 carcinoma and treated with anti-PD-1, ATB treatment was associated with shortened PFS and  
146 OS. Baseline microbiota analysis showed a higher richness in patients with clinical benefit.  
147 Overrepresentation of unclassified and classified *Firmicutes*, as well as *Akkermansia* and  
148 *Alistipes* was found in responding patients. At the species level, *Akkermansia muciniphila* was  
149 the most significantly associated with favorable clinical outcome in these epithelial tumors [23].  
150 A recent work on advanced renal cell carcinoma patients treated with nivolumab, demonstrated  
151 that the alpha-diversity (measure of microbiome diversity applicable to a single sample) and  
152 beta-diversity (measure of similarity or dissimilarity of two communities i.e. between samples)  
153 of the patient's stool composition might be used as biomarkers to differentiate responder from  
154 non-responder population, and to predict which patient could have a PFS longer than a year  
155 [24]. The use of ATB treatments can affect the gut microbiome composition thus it is very  
156 difficult to differentiate the chicken from the egg. In patients with NSCLC treated with anti-  
157 PD-1/PD-L1 monotherapy, ATB treatment was associated with decreased alpha diversity at  
158 baseline and patients who did not receive ATB had feces enriched with Clostridia, specifically  
159 *Ruminococcaceae* UCG 13, Clostridiales, and *Agathobactera*. To determine whether the



160 composition of the microbiota influences response to ICB even in the absence of ATB, its  
161 composition was explored in eubiotic patients by excluding those who received ATB [25]. In  
162 ATB-free patients, decreased alpha diversity associated with shorter OS, *Ruminococcaceae*  
163 UCG 13 and *Agathobacter* were overrepresented in patients with clinical benefit. *Akkermansia*  
164 species was not associated with improved clinical outcomes but was associated with less severe  
165 irAE profile (Figure 1) [25]. Interestingly, *Agathobacter*, that was associated with a favorable  
166 outcome, was also associated with more severe irAE [25].

## 167 **Microbiota-derived metabolites and immunotherapy-induced response or** 168 **IrAE**

169 As described above, gut microbiome composition influences outcomes and IrAE of ICB, but  
170 how it could have an impact on a distant tumor lesion remains unclear. Gut microbiota  
171 influences immunity mainly through its metabolites, such as short chain fatty acids (SCFA) as  
172 acetate, propionate and butyrate, indoles derivatives, polyamines, water-soluble vitamins and  
173 others, produced in very large amounts in the colon through bacterial fermentation of dietary  
174 fibers [31,32]. Some studies have therefore investigated whether these metabolites could be  
175 related to the composition of the microbiota and to response or IrAE to ICB. Shotgun  
176 metagenomic sequencing for metabolic pathway reconstruction on patients treated with  
177 ipilimumab with and without colitis revealed that spermidine/putrescine polyamine transport  
178 system and three modules involved in the biosynthesis of B vitamins ((riboflavin (B2),  
179 pantothenate (B5) and thiamine (B1)) were more abundant in colitis-free patients [21]. This  
180 observation seems reminiscent of several data in inflammatory bowel disease where blood B  
181 vitamins (thiamine and riboflavin) concentration was significantly lower [33]. Thus,  
182 determination of these vitamins in blood could be an interesting tool for the early detection of  
183 anti-CTLA-4-induced colitis. To date, no data have been provided to support this hypothesis,

184 but since validated clinical routine tests exist, it seems interesting to include these  
185 measurements in the biological assessment of patients before the start of ICB. A recent study  
186 explored the role of systemic SCFA (acetate, propionate and butyrate) and ipilimumab  
187 outcomes [34]. As described before, gut microbiota enriched in *Faecalibacterium* is linked to  
188 clinical benefit [20] and these microorganisms are SCFA producers. Coutzac and colleagues  
189 showed that *Faecalibacterium* were inversely correlated with serum concentration of butyrate,  
190 and high serum butyrate (and propionate) concentration was associated with shorter PFS and  
191 OS in two independent cohorts of patients with metastatic melanoma and treated with  
192 ipilimumab [34]. In mice, butyrate gavage was able to limit anti-CTLA-4 anti-tumor efficacy  
193 through the inhibition of dendritic cell maturation and restrained anti-CTLA-4-induced tumor-  
194 specific T cells. In patients, high blood concentration of butyrate (and propionate) was  
195 associated with higher concentration of regulatory T cells before ipilimumab initiation, and  
196 patients with low butyrate concentrations at baseline had higher increase of memory CD4+ T  
197 cells as well as ICOS induction following ipilimumab, compared to patients with high serum  
198 butyrate [34]. Interestingly, a work from Ferrere et al. [35] described that ketogenic diet is  
199 associated with increase of ketone bodies such as  $\beta$ -hydroxybutyrate and acetoacetate.  
200 Ketogenic diet or treatment with  $\beta$ -hydroxybutyrate could synergized with anti-CTLA-4 + anti-  
201 PD-1 therapy in mice. It is described that ketogenic diet affect microbiome composition with  
202 an increase in the abundance of *Firmicutes* and *Actinobacteria* and a decrease in the abundance  
203 of *Bacteroidetes*. Moreover, under these ketogenic conditions, plasma ketone bodies levels  
204 increased markedly, whereas plasma SCFA such as propionate and butyrate levels decreased  
205 dramatically [36]. Finally Mager et al. showed that inosine could modulate response to anti-  
206 CTLA-4 associated to the Toll-like receptor 9 agonist CpG [37]. In this study, authors  
207 demonstrated that *Bifidobacterium pseudolongum* could significantly enhance efficacy of ICB  
208 through production of the metabolite inosine. Inosine could activate Th1 in an adenosine A2A

209 receptor-dependent fashion and required a costimulus (CpG agonist) along with anti-CTLA-4  
210 treatment. In this work, it has to be stressed out that in the absence of CpG or other costimulus,  
211 inosine increased tumor weight and reduced anti-CTLA-4-induced antitumor immunity  
212 demonstrating that the effect of inosine was context-dependent [37]. Indeed and as  
213 demonstrated by authors through *in vitro* experiments, the effect of inosine in terms of induction  
214 or inhibition Th1 differentiation was context dependent; in the presence of exogenous IFN- $\gamma$ ,  
215 inosine strongly boosted Th1 differentiation of naïve T cells while in the absence of IFN- $\gamma$ ,  
216 inosine inhibited Th1 differentiation [37]. Altogether, these studies provided a vision on the  
217 relationship between composition of the gut-microbiota and clinical response to ICB via  
218 microbiota-derived metabolites.

219

## 220 **Strategies to modulate the microbial composition of the gut microbiota in** 221 **patients with cancer and treated with ICB**

222 Human microbiome is investigated as a novel therapeutic target to support treatment with ICB  
223 as a personalized treatment to improve prognosis of cancer patients. Main approaches of gut  
224 microbiota's modulation to potentiate immunotherapy response in cancer patients are presented  
225 in Figure 2.

### 226 • **Fecal microbiota transplantation (FMT)**

227 FMT is the most direct method to change the composition of gut microbiota. FMT is a therapy  
228 that restores intestinal microbial diversity with the transfer of fecal material from a healthy  
229 individual into the gastrointestinal tract of a patient. FMT has demonstrated remarkable clinical  
230 efficacy against *Clostridium difficile* infection and it is now recommended for the treatment of  
231 this infection for recurrent or refractory stage [38–40]. Interest is growing in the therapeutic  
232 potential of FMT for other diseases, e.g., inflammatory bowel diseases or hematologic

233 malignancies. Several studies have highlighted microbiome's role in the pathogenesis of  
234 autoimmune and inflammatory diseases. The pathogenesis is not fully understood, but may  
235 implicate environmental factors (life-style, diet, drugs, and infections) and certain genetic  
236 backgrounds. Several preclinical studies have experimented FMT, as already discussed in the  
237 first part of this review, and regarding patients treated with ICB, some data are starting to  
238 indicate that FMT could have a potential interest. Wang et al. reported a first case of refractory  
239 ICB-associated colitis successfully treated with FMT from healthy donors, in two patients with  
240 different solid tumor [41]. Clinical trials combining FMT and ICB treatment are ongoing to  
241 assess its role on limiting ICB-induced toxicities or improving clinical outcomes  
242 (NCT04038619, NCT04130763, NCT03353402, NCT03341143). Results from two of these  
243 clinical trials have been recently published. These phase I trials have assessed safety and  
244 efficacy of FMT, where stools from patients who have previously responded to ICB, were  
245 transferred to ICB-resistant patients with metastatic melanoma. In the first study, stool from  
246 two donors (donor 1 and donor 2) with complete response (CR) for at least 1 year following  
247 anti-PD-1 treatment were used. FMT was preceded with microbiota depletion with oral ATB  
248 treatment. FMT was then performed with a colonoscopy followed by the administration of oral  
249 stool capsules before reinduction of anti-PD-1 therapy. Maintenance FMT with oral stool  
250 capsules was realized along with anti-PD-1 every 14 days with a total of six combined treatment  
251 cycles. None of the 10 patients who had FMT developed any moderate-to-severe irAEs (grade  
252 2 or greater) even if five patients did developed irAEs during their first ICB-line. Objective  
253 response to treatment were demonstrated in three recipients, one CR and two partial response  
254 (PR). All patients who experienced an objective response received FMT from the donor number  
255 one in this study. No specific response-inducer microbiota characteristics could be evidenced  
256 [42]. In a second study, stool from 7 donors, 4 with CR and 3 with PR following anti-PD-1  
257 treatment were used [43]. Sixteen melanoma patients, all refractory to anti-PD-1 therapy, were

258 included and received a single FMT performed with a colonoscopy followed by anti-PD-1  
259 therapy every 3 weeks until disease progression or intolerable toxicity. Among the fifteen  
260 patients evaluable for clinical response, one patient experienced a CR and 2 patients  
261 experienced a PR. This study showed that FMT successfully colonized the gut of responding  
262 patients and could reprogrammed the tumor microenvironment to overcome primary resistance  
263 to anti-PD-1 in some patients with advanced melanoma. These results suggest that FMT in  
264 melanoma patients seems to be safe and to have a clinical benefit in ICB-resistant patients.  
265 However, the number of patients treated remains very low and these clinical trials were not  
266 randomized; thus, it cannot be ruled out that the resumption of anti-PD-1 therapy is responsible  
267 for objective clinical responses independent of FMT. Further controlled studies should allow  
268 us to answer this question. Moreover, we have to remain cautious about security of FMT, as  
269 this therapy can increase the risks of transmitting other pathogens, although transplant origin is  
270 from healthy subjects with a supposedly well-balanced microbiome [40]. Recently, in a pilot  
271 FMT study treating patients with steroid-refractory intestinal acute graft-versus-host-disease  
272 [44] , a patient died of cerebral hemorrhage caused by thrombocytopenia. No adverse events  
273 were observed during and after FMT, but this event is to be pointed out. More recently, one  
274 immunocompetent patient died, and a second was infected, after receiving the same donors'  
275 stools preparations containing extended-spectrum  $\beta$ -lactamase-producing *Escherichia coli* [45].  
276 These events stressed out the necessity to constantly improve donor screening to limit  
277 transmission of microorganisms that could lead to adverse infectious events especially in fragile  
278 patients such as cancer patients.

279       • **Antibiotics**

280 As ATB inhibit bacteria growth or induce certain strains death, they can alter microbiota  
281 diversity and composition, which may play a role on tumorigenesis and on tumor's response to

282 treatment. Presence of *F. nucleatum* had a close relationship with the poor prognosis of CRC  
283 patients and probably protected tumor against immune cell attack [46,47]. Using ATB would  
284 be a way to decrease *F.nucleatum* in CRC patients as generally, clinically isolated *F. nucleatum*  
285 strains are sensitive to several ATB, such as metronidazole, clindamycin, and some  $\beta$ -lactamin  
286 ATB [48]. A study has shown that metronidazole treatment effectively decreased tumor  
287 volumes in CRC patient-derived xenograft mice model [49]. Several studies have investigated  
288 ATB role on ICB efficacy, as presented in the first part of our work. They presented a  
289 correlation between ATB-pretreatment, short relapse, and survival with shorter PFS and OS  
290 [23,50]. Routy et al. observed that ATB compromised efficacy of anti-PD-1 treatment in  
291 patients with advanced NSCLC, renal cell carcinoma or urothelial carcinoma. They studied  
292 fecal samples' microbiome, segregating responders from non-responders and demonstrated that  
293 an overrepresentation of *Firmicutes*, as well as *Akkermansia* and *Alistipes* were associated with  
294 favorable clinical outcome in patients with NSCLC and renal cell carcinoma [23]. A recent  
295 paper demonstrated that ATB treatment in NSCLC patients could reduce the alpha-diversity  
296 (within sample diversity) of the patient's stool composition thus, it remains difficult to  
297 determine if microbiota composition at baseline or ATB-use could be a biomarker of ICB  
298 efficacy. A meta-analysis of observational studies with 826 patients treated with ATB (28,6%)  
299 out of 2889 patients with solid cancers and treated with ICB showed negative outcomes in  
300 ATB-treated patients, with shorter PFS and OS [51]. Broad spectrum ATB can have detrimental  
301 effect on ICB efficacy; research on targeted ATB to selectively increase immune-potentiating  
302 bacteria might be a potential lead to improve current ICB therapies.

303       • **Prebiotics, Probiotics, and postbiotics**

304 Intervention strategies on microbiome are currently investigated, using probiotics, prebiotics,  
305 symbiotics and postbiotics to favorably modulate patient's gut microbiota. Various clinical and  
306 experimental studies show the role of these microbiota manipulations in cancer prophylaxis and

307 treatment, throughout mechanisms like apoptosis, antioxidant activity, immune response and  
308 epigenetics [52,53]. Prebiotics are nondigestible, oligosaccharides food ingredients that affect  
309 the host by selectively stimulating the growth and activity of bacteria in the colon that have the  
310 potential to improve host health. Probiotics refer to live microorganisms that show beneficial  
311 effects on the host health by modifying microflora of the host. Postbiotics are non-viable  
312 bacterial products or functional bioactive compounds, produced during fermentation from  
313 probiotics microorganisms, which may be used to promote health. They include many  
314 compounds as metabolites, short-chain fatty acids (SCFAs), microbial cell fractions, functional  
315 proteins, extracellular polysaccharides, cell lysates, teichoic acid, peptidoglycan-derived  
316 muropeptides and pili-type structures [54,55]. The effect of these microbe-targeted therapies  
317 has been investigated with anti-cancer therapeutic strategies such as chemotherapy,  
318 radiotherapy or surgery, but studies with ICB remain scarce, although many encouraging results  
319 *in vitro* reported effects of these therapies on the immune system [56]. Concerning prebiotics,  
320 Liu et al. recently presented a case of murine colon cancer treated with anti-PD-L1 therapy and  
321 prebiotic bilberry anthocyanin combo, this association was accompanied with improved tumor  
322 CD8+ T-cell infiltration and control of the tumor growth. Their results also showed that  
323 prebiotic might play a role in potentiating ICB treatment by restoring microbiota diversity  
324 [57]. The immunomodulating role of probiotics [58,59] has led to increased interests toward  
325 their potential role in enhancing antitumor immunity. Traditional probiotics generally show few  
326 improvements, but next generation probiotics could be interesting therapeutic tool. They have  
327 an immunomodulatory effect and certain bacterial strains can modulate dendritic cells  
328 maturation stage and increase IL-10 release [60,61]. They may also induce a change in the  
329 macrophage phenotype, from pro-inflammatory M1 to anti-inflammatory M2 [62]. Certain  
330 strains, in particular *Lactobacillus acidophilus* [63] and *Lactobacillus casei* BL23 [64], have  
331 shown antitumor properties in mouse models. Other strains such as *Enterococcus hirae* and

332 *Barnesiella intestinihominis* increase tumor infiltration of CD8+ T lymphocytes [65]. Several  
333 studies in mice have shown that the use of immunotherapies combined with probiotics such as  
334 *Bifidobacterium spp*, *Akkermansia muciniphila* or *Enterococcus hirae* increases clinical  
335 efficacy in mice as already discussed [23,30]. However, we must be vigilant and data observed  
336 in mice must be validated in patients before encouraging the use of probiotics. Indeed,  
337 preliminary results presented a 70% lower chance of response to ICB for patients who took  
338 commercially available probiotics. These commercial probiotics were associated to low  
339 diversity of gut microbiome, previously found to be linked to poorer response to ICB [66].  
340 Postbiotics have been shown to allow modulation of inflammatory and anti-inflammatory  
341 activities by affecting immune cell migration, adhesion, cytokine expression, cell proliferation  
342 and activation, and apoptosis. As already discussed, postbiotics such as butyrate, propionate  
343 [34] or inosine [37,67] could modify ICB clinical efficacy. These recent findings support the  
344 possible utilization of specific bacterial therapies or microbiota modulation in addition to ICB  
345 to enhance their efficacy but few studies presented their link with immune-mediated side  
346 effects, such as diarrhea, colitis, or dermatologic events. More studies are needed to confirm  
347 the therapeutic potential of these approaches and to clarify the mechanisms. Identification of  
348 molecular pathways implicated in the activity of such microbiota-derived metabolites could  
349 help in the identification of new targets that could enhance ICB activity in patients.

350 • **Bacteriophages**

351 Bacteriophages are viruses that infect and replicate only in bacterial cells. They play an  
352 important role in regulating bacterial populations and are naturally present in the microbiome  
353 to insure equilibrium. They could be engineered for therapeutic purposes [68] but no clinical  
354 trials on human have started yet to our knowledge and preclinical studies remain scarce. A  
355 prophage is a bacteriophage genome inserted and integrated into the circular bacterial DNA  
356 chromosome. Recently, one dominant epitope of bacteriophage tail length tape measure protein



357 (TMP1) encoded by the genome of Siphoviridae bacteriophages, was found in *Enterococcus*  
358 *hirae* microbial genome thus an *Enterococcus hirae* prophage. TMP1 coded by *Enterococcus*  
359 *hirae* prophage microbial genome has been found to be an epitope which could induce a  
360 memory CD8+ T cell response which cross-reacts with cancer antigen (Figure 2). The clinical  
361 relevance of this molecular mimicry theory has then been investigated in patients with lung or  
362 renal cancer. In patients, presence of bacteriophage was associated with better survival after  
363 anti-PD-1 immunotherapy. Cancer patients in whom the TMP phage sequence was found in the  
364 microbiota at baseline, showed a better response to PD-1 blockade [69]. This study highlights  
365 the importance of microbiota-derived peptides, which can cross-react with tumor antigens.  
366 Furthermore, the characterization of antigens and bacteriophages that transmit these cross-  
367 reactive antigenic specificities to commensal bacteria may allow the design of new and specific  
368 vaccine candidates to boost ICB efficacy.

## 369 **Conclusion**

370 Results from preclinical and clinical studies did not identify abundance of one specific bacteria  
371 that could be a common biomarker of ICB response. Different bacteria species can have  
372 opposite effects on anticancer treatment, and high inter-individual variation in its composition,  
373 diet, medications, and geographical localization have to be considered. However, a common  
374 point seems to be found in most studies, the richness and diversity of the gut microbiome  
375 appeared to be associated with better clinical outcome. Several hypotheses were raised about  
376 microbiota composition and response to ICB. First, antigens from gut microorganisms could  
377 stimulate anticancer immune response on their own through cross-reactivity (i.e. antigen  
378 mimicry between gut bacteria and tumor antigens), or provide a synergistic effect with ICB  
379 through the modulation of the immune system and/or inflammation at distant site. More results  
380 are needed to better understand microbiota mechanisms and its role in ICB efficacy. Precision

381 medicine in cancer treatment would involve modulation of the microbiota through different  
382 strategies to improve patients' outcomes. Current means available for modulating the response  
383 to immunotherapy using the microbiota seem promising but have also various limits, such as  
384 immune-mediated side effects and lack of safety data.

## 385 **Figure Legends**

386 **Figure 1. Gut microbiota modulation of ICB treatment efficacy and toxicity. A-C.** Data  
387 from mouse preclinical models. Main phylum genus or species that have been described as  
388 associated with response or resistance to CpG combined with a blocking IL-10 receptor (IL-  
389 10R) antibody (**A**) or blocking CTLA-4 antibodies (**B**) or blocking anti-PD-L1 antibodies (**C**)  
390 are presented. **D-G.** Data from patients treated with ICB. Main phylum genus or species that  
391 have been described as associated with response/resistance/IrAE, in patients with metastatic  
392 melanoma and treated with ipilimumab ( $\alpha$ CTLA-4, **D**), blocking anti-PD-1 antibodies ( $\alpha$ PD-  
393 1, **E**) or combo treatment ipilimumab and nivolumab ( $\alpha$ CTLA-4+ $\alpha$ PD-1, **G**) are presented.  
394 Main phylum genus or species that have been described as associated with response or  
395 resistance, in patients with epithelial tumors as NSCLC and renal cell carcinoma and treated  
396 with  $\alpha$ PD-1 or  $\alpha$ PD-L1 (**F**) are also presented.

397 **Figure 2: Strategies to modulate microbiota composition in cancer patients treated with**  
398 **ICB. A.** FMT using stools from patients with complete or partial response (CR or PR) to another  
399 non-responder or who has ICB-induced colitis. **B.** All possible strategies. Antibiotics could be  
400 used if certain cocktails are validated to specifically deplete bacteria associated with resistance  
401 or toxicities. The value of FMT needs to be validated in controlled clinical trials. Prebiotics  
402 such as bilberry anthocyanins could enhanced ICB anti-tumor effect; probiotics such as  
403 *Faecalibacterium*, *Bifidobacterium*, *Enterococcus hirae*, *Akkermansia muciniphila*,  
404 *Lactobacillus* or *Burkholderia* could modulate the diversity of the gut microbiota and enhance  
405 anti-tumor immunity; postbiotics with some having a beneficial impact like inosine and others  
406 having a detrimental impact, such as butyrate or propionate, targeting specific pathways  
407 associated with these detrimental effects could enhance ICB efficacy. **C.** Prophage can activate  
408 immune response through molecular mimicry between phage-encoded and cancer antigens and

409 improve survival of ICB-treated patients. Discovery of these phage-encoded antigens may  
410 allow the discovery of new targetable tumor antigens.

411

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413

414 **DECLARATION**

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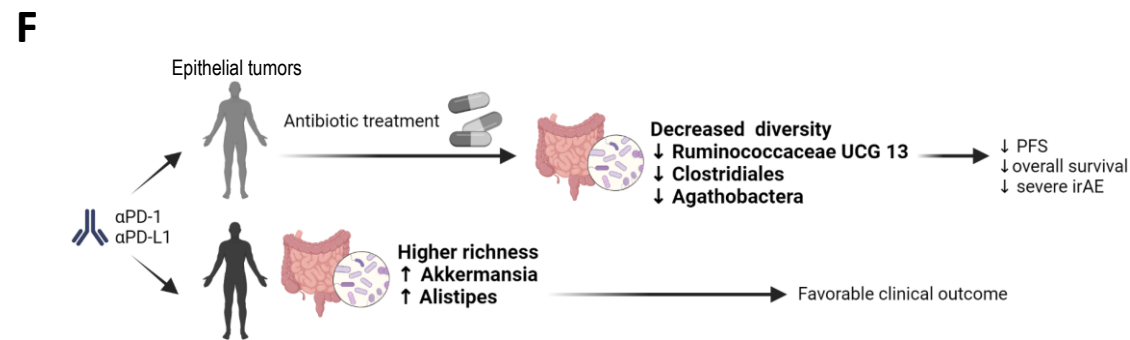
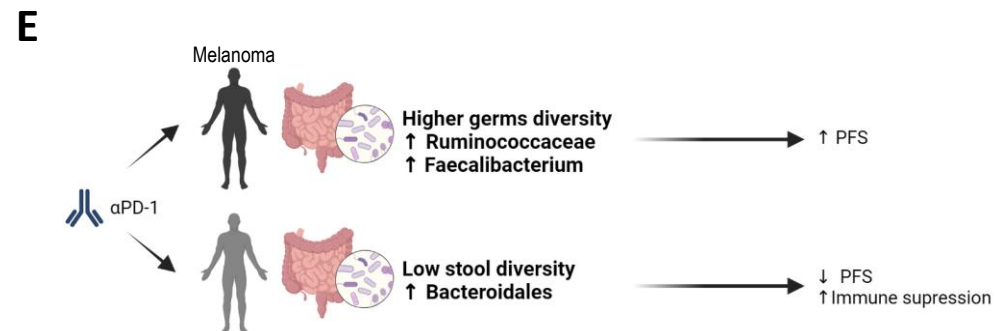
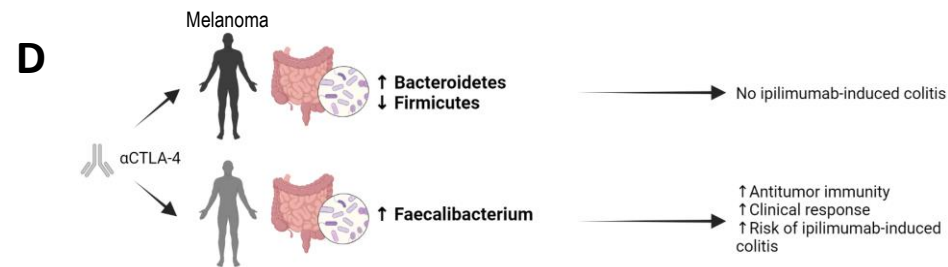
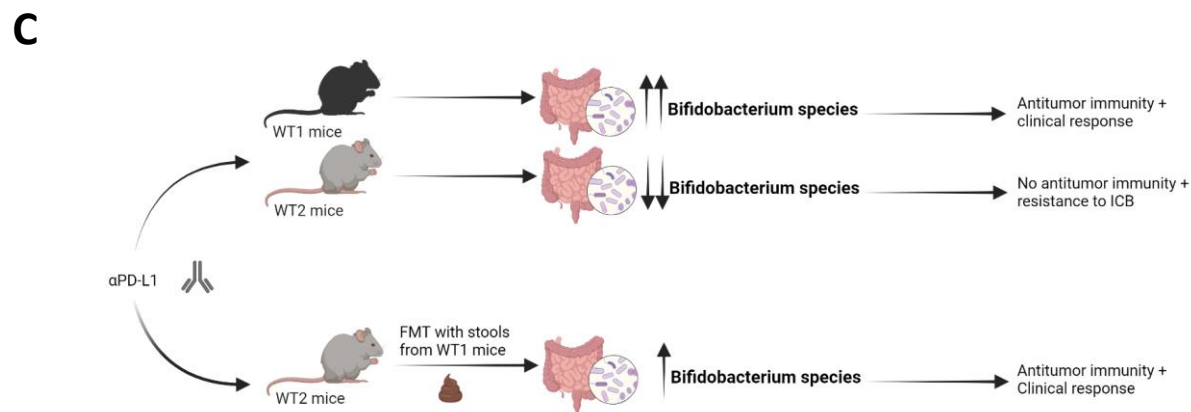
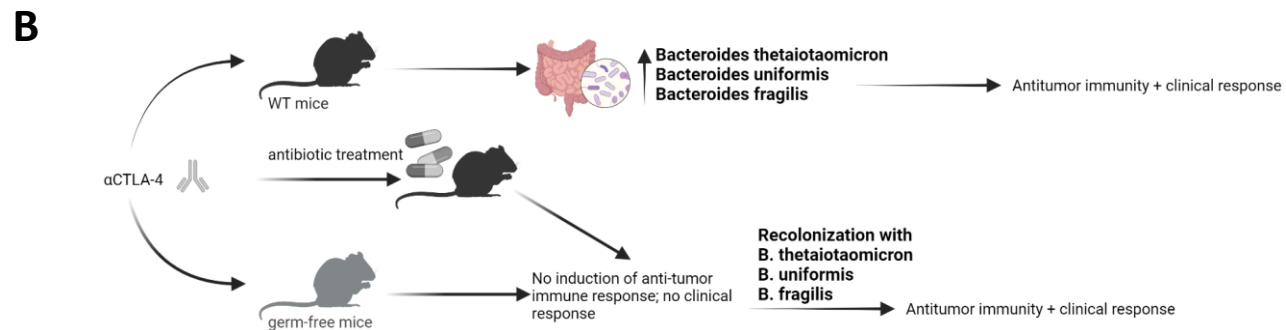
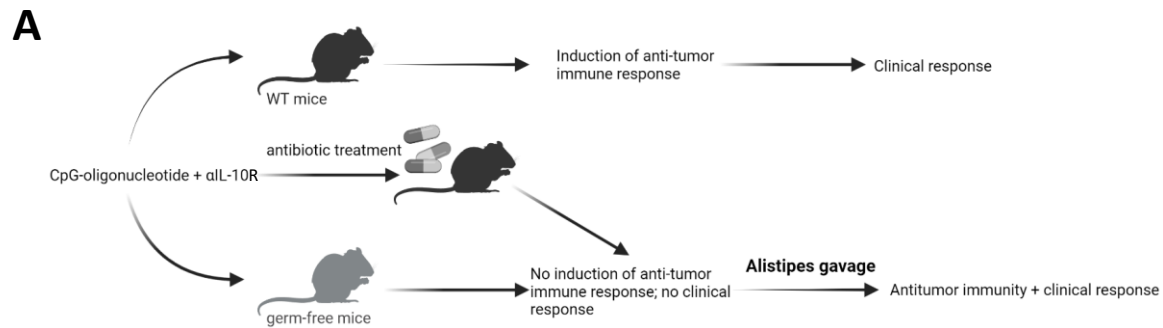
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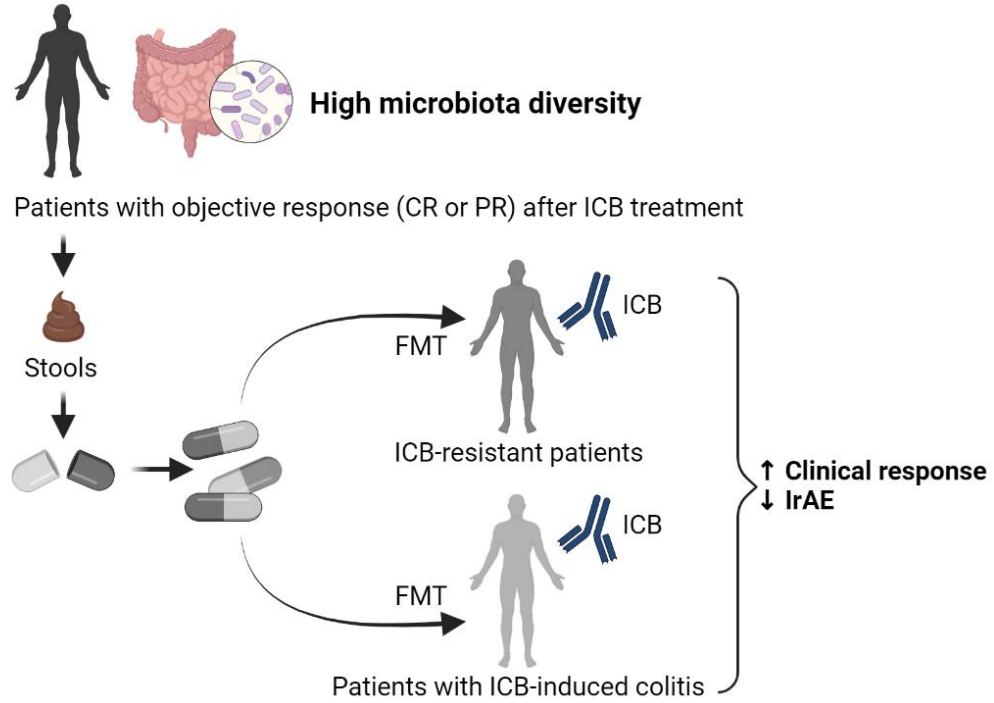
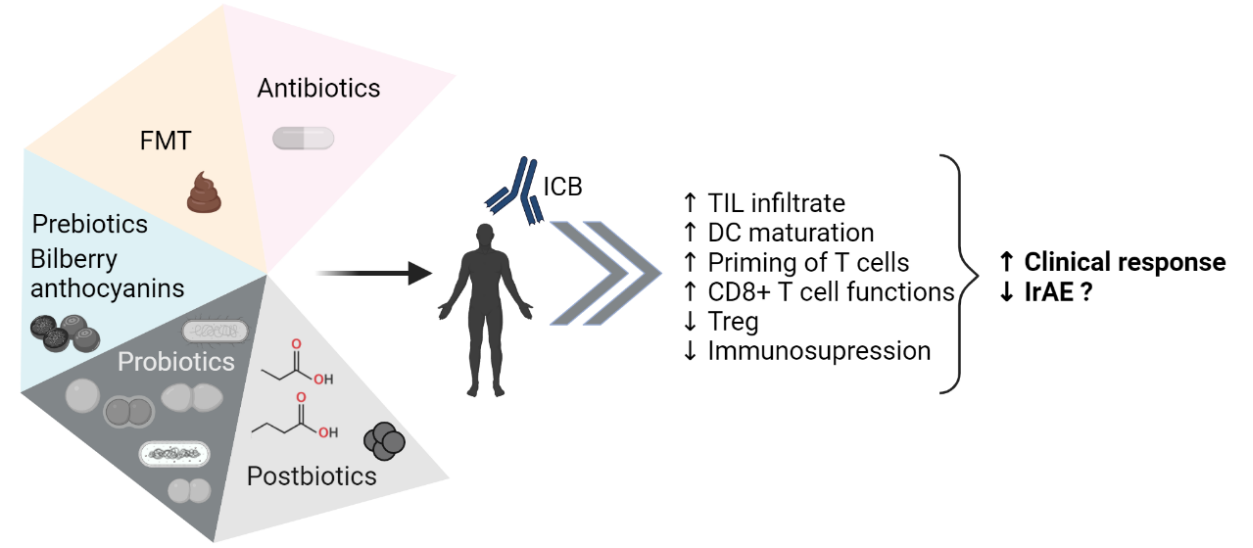
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