Microbiota composition, as a marker of responsiveness to anti-PD1/PD-L1/PD-L2 antibodies and use of microbial modulators for improving the efficacy of an anti-PD1/PD-L1/PD-L2 Ab-based treatment

FIELD OF THE INVENTION

The present invention relates to the field of anticancer treatment. In particular, the present invention concerns the role of the gut microbiota in the efficacy of cancer treatments and provides methods for determining if a patient is likely to benefit from a cancer treatment, more precisely, treatment comprising administration of an antibody directed against immune checkpoint blockers PD1, PD-L1 or PD-L2 alone or together with CTLA4. The present invention also provides probiotics to improve the efficacy of such a treatment in patients in need thereof.

BACKGROUND AND PRIOR ART

The gastrointestinal tract represents the largest compartment of the immune system. It is exposed to food, commensal antigens, and is the portal of entry for many pathogens. One hundred trillion organisms (mainly bacteria, phages but also archae, fungi and parasites) collectively referred to as the gut microbiota colonize the human intestine (Hugon *et al.*, 2016). The intestinal microbiota comprises microbial populations that colonize the human gastrointestinal tract at increasing densities (from the top to bottom) and has been shown to play a crucial role in human health (Blaser, 2016; Gensollen *et al.*, 2016). Indeed, the gut microbiota exerts a myriad of fundamental functions, such as the degradation of nutrients to provide energy source, the elimination of xenobiotics, the education of the immune system, the growth and terminal differentiation of epithelial cells, the intestinal peristalsis, and the production of antimicrobial peptides to eradicate pathogens and ensure colonization resistance (Goodrich *et al.*, 2016; Rooks and Garrett, 2016).

Studies on human gut microbiome have revealed that healthy individuals harbor diverse microbial populations and that the composition of the intestinal microbiota differs from individual to individual, which can have implications in health and disease. The majority of bacterial taxa belong to the *Firmicutes* and *Bacteroidetes* phyla and bacteria belonging to the

Actinobacteria, Proteobacteria, Verrucomicrobia, and *Fusobacteria* are also represented. Our knowledge of the genetic and functional diversity in gut microbes is far from being complete. Old bacterial identification methods consisting in qPCR or FISH analyses were neither comprehensive, specific nor sensitive enough. The burst of recent knowledge emerged from the targeted 16S rRNA gene analysis and far beyond by metagenomic shotgun sequencing, a costly method with heavy data mining (Nielsen *et al.*, 2014) as it allows to identify all the microbial species present (with known or unknown reference genome present in the databases) and their coding capacities. Catalogs of reference genes in the human gut microbiome have been reported, gathering data from MetaHIT, Human Microbiome Project HMP and a large Chinese diabetes study, as well as >3400 sequenced prokaryotic genomes regarded as potentially of human gut origin. This nonredundant reference catalog of 9,879,896 genes is accessible for free through the internet (Integrated Reference Catalog of the Human Gut Microbiome) and the data are available on the GigaScience Database (Li *et al.*, 2014). We may have reached saturated coverage of core gene content and functions. Of note, the number of genes present in more than 50% of the subjects remained below 300,000, pointing out the dominance of individual-specific genes. The individual-specific genes are enriched in the categories cell wall/membrane/envelope biogenesis and DNA replication, recombination and repair. The common genes are enriched in functions such as signal transduction mechanism, energy production, carbohydrate transport and metabolism, and amino acid transport and metabolism.

Perturbations of the symbiosis between the gut microbiome, the intestinal epithelium, and the host immune system are associated with various immunopathologies and chronic inflammation, including cancer. Hence, the composition of the intestinal microbiota has been associated with malnutrition, obesity, and chronic inflammatory disorders such as NASH and IBD (Pigneur and Sokol, 2016; Raoult, 2016). It has become evident that the intestinal microbiota not only mediates resistance to colonization by pathogens but also modulates immune function by promoting differentiation of different T-cell phenotypes, which may account for autoimmune or atopic disorders such as rheumatoid arthritis and asthma (Arrieta *et al.*, 2015). Previous studies from Zitvogel's group have indicated that the intestinal microbiota can influence the immune response to systemic cancer chemotherapy and disruption of the

intestinal microbiome was associated with resistance to cancer therapy (Viaud *et al.*, 2013; WO 2015/075688). Pamer *et al.* also reported that graft-versus-host disease and graft-versus leukemia effects during allogeneic hematopoietic stem cell transplantation were associated with variegated composition of the intestinal microflora with causative links between both (Shono *et al.*, 2016; Taur *et al.*, 2015).

Lung cancer (LC) remains the most common cause of death from any cancer, estimated to be responsible for nearly 1.6 million deaths worldwide and around 30 000 deaths in France each year (Fidler *et al.*, 2016). This disease condemns the young, striking at a median age of 50-55 and standing among “unmet medical needs”. In the past decade, progress in tumor biology, genomics technology, computational analysis and drug discovery has propelled advances in translational and clinical cancer research in LC. LC became the prototype for tailored targeted therapies, especially immunotherapies using Immune Checkpoint Blockers (ICB) such as monoclonal antibodies (mAb) directed against CTLA-4 and PD1/PD-L1 targeting T cell inhibitory receptors or ligands (Garon, 2015). Two mAb blocking PD1 receptors (Nivolumab and Pembrolizumab) and three mAb neutralizing PD1 ligands (PD-L1) (Atezolizumab, Durvalumab, and Avelumab) have been investigated in Phase I trials. One Phase II trial in 117 squamous non small cell LC (NSCLC) showed that Nivolumab lead to 15% objective response rates, with a median time to response of 3.3 months (Rizvi *et al.*, 2015), while two seminal Phase III randomized trials, in cisplatinum-resistant squamous (SQ) and non-squamous (NSQ) NSCLC patients, also demonstrated a benefit in overall survival (OS) in the Nivolumab arm compared to Docetaxel in second line (for SQ: median OS =9.2 months versus 6 months, for NSQ: OS rate at 18 months= 39% versus 23%). In March 2015, Nivolumab finally became the first checkpoint inhibitor approved in advanced SQ NSCLC who progressed during or after platinum-based chemotherapy. A first limit of those innovative and costly treatments is a relatively low and unpredictable efficacy. Only few parameters such as positive expression of PD-L1 on lung cancer cells (about 25% cases) as well as a high mutational load of LC appear to be linked with a high response rate to anti-PD1 mAb. Secondly, severe adverse events are usually observed (fatigue (4%), pneumonitis (3%) and diarrhea (3%)). Although co-blockade of both CTLA-4 and PD1 markedly augments objective response yields, these associations result in higher toxicities, especially immune-related adverse events (irAEs) at

sites that are exposed to commensal microbiota, e.g the gut (Berman *et al.*, 2010). IrAEs can be of grade III-IV in 20% cases, life threatening in nearly 5% cases, thereby causing premature interruption of the therapy (Champiat *et al.*, 2016). Importantly, although characterized by low regulatory Treg numbers and specific genetic traits, to date irAEs cannot be effectively predicted nor prevented. However, patients develop antibodies to gut microbiome, suggesting that intestinal commensals or bacterial antigens may contribute to those irAEs.

Therefore, predicting primary resistance to ICB and uncoupling ICB efficacy from gut toxicity represents an unmet medical need challenging the future development of immune checkpoint blockers for the treatment of LC (or any other cancers such as renal cell cancers, head and neck tumors, bladder carcinoma, and all tumors amenable to anti-PD1/PD-L1 antibodies or combinations with chemotherapy).

Zitvogel *et al.* explored how cancer therapeutics (cytotoxicants and ICB) alter such a mutualist symbiosis. Three years ago, Zitvogel's group and others reported the crucial role of gut microbiota in eliciting innate and adaptive immune responses beneficial for the host in the context of effective therapies against cancer (such as cyclophosphamide, platinum salts) (Pitt *et* al., 2016; Viaud *et al.*, 2013; WO 2015/075688; WO 2016/063263). By compromising intestinal integrity, chemotherapeutic agents enhance gut permeability, favoring the selective translocation of distinct immunogenic bacteria (namely *E. hirae*), inducing dramatic changes in the tumor microenvironment (myeloid cell oxidoreduction and accumulation of effector TH1 cells). Moreover, ICB and immunomodulators (anti-IL-10R, TLR9L) also affect the delicate symbiosis of the gut mucosa, favoring the emergence of distinct bacterial species favorably affecting the microenvironmental tone of the tumor. In fact, Zitvogel's group worked on the first-in-class immune checkpoint blocker, anti-CTLA-4 Ab/ipilimumab, showing the mandatory role of microbiota (*Bacteroidales* and *Burkholderiales*) in its antitumor immune and clinical effects and the prophylactic role of such commensals against subclinical colitis in mice (Vétizou *et al.*, 2015; WO 2016/063263). In parallel, Gajewski's group demonstrated the role of *Bifidobacterium* in maturing intratumoral dendritic cells allowing the expansion of anti-cancer T cells in the tumor beds and their activation with anti-PD-L1 Ab (Sivan *et al.*, 2015). In all these model systems, investigators show that ICB fail to mediate antitumor effects in germ free mice or after

administration of broad-spectrum antibiotics in rodents reared in specific pathogen free conditions. Therefore, a better understanding of mucosal immunity and the immunological, epithelial, microbial cross-talk at portals of entry during ICB therapy of lung cancer may open up new avenues to explain the resistance of cancer patients to ICB or chemotherapy.

In the experimental data disclosed below, the inventors showed that antibiotics intake compromised the response of individuals diagnosed with advanced cancers to PD1/PD-L1 blockade and then, identified a typical cancer-associated microbial fingerprint of the intestinal content in cancers such as lung or renal cell carcinoma, called “dysbiosis” found associated with dismal prognosis. This prognosis can be improved by allogenic fecal microbial transplantation (FMT) of feces from healthy individuals, or by administration of probiotic compositions such as a composition of bacteria selected amongst *Enterococcus hirae, Akkermansia muciniphila, Alistipes,* as well as *Bifidobacterium adolescentis, Clostridiales spp., Roseburia, Blautia, Faecalibacterium*, *Ruminococcaceae*, *Christensenella minuta*, *Eubacterium limosum* and/or immunogenic bacteria selected amongst *Methanobrevibacter smithii, Barnesiella intestinihominis, Bacteroides fragilis, Collinsella intestinalis, Dielma fastidiosa, Flavonifractor plautii, Actinotignum schalii* and *Burkholderia cepacia*.

The inventors also identified gut microbiota profiles associated with response or resistance to treatments with ICB, in particular with anti-PD1 or anti-PD-L1 or anti-PD-L2 antibodies, as well as associated blood profiles. As a result, they propose a theranostic method for identifying good responders, to whom an anti-PD1 or anti-PD-L1 or anti-PD-L2 antibodies can be administered, while a pre-treatment based on FMT and/or probiotics is recommended to bad responders exhibiting a dysbiosis.

SUMMARY OF THE INVENTION

The invention pertains to probiotic compositions designed to improve the response to an anti-PD1/PD-L1/PD-L2 Ab-based therapy, for a patient in need thereof. A probiotic composition according to the invention comprises bacteria selected from the group consisting of one or several isolates of *Enterococcus hirae, Akkermansia muciniphila, Alistipes shahii,* other *Alistipes* species such as *Alistipes indistinctus* and mixtures thereof.

According to one embodiment, the composition comprises at least two, at least three, at least four or at least five bacterial species selected from the group consisting of *Firmicutes* species, *Clostridiales* species, *Alistipes* species, *Eubacterium* species, *Bacteroidales* species, *Methanobrevibacter smithii, Akkermansia muciniphila* and *Enterococcus hirae*. A particular composition which can be used, according to the invention, to induce immunostimulation in a cancer patient receiving an anti-PD1/PD-L1/PD-L2 Ab-based therapy, is a bacterial composition comprising *Enterococcus hirae* and/or *Akkermansia muciniphila* and/or *Alistipes*.

According to one embodiment, the composition comprises at least two, at least three, at least four or at least five bacterial species selected from the group consisting of *Enterococcus hirae, Akkermansia muciniphila, Alistipes indistinctus, Eubacterium* species (such as *Eubacterium limosum*), *Firmicutes* species (such as *Christensenella minuta, Dielma fastidiosa)*, *Bacteroidesia* (such as *Bacteroides fragilis*, *Bacteroides salyersae, Barnesiella intestinihominis*), Actinobacteria (such as *Collinsella intestinalis, Collinsella tanakaei*, *Actinotignum schaalii*) and the archae *Methanobrevibacter smithii*.

A particular composition which can be used, according to the invention, to induce immunostimulation in a cancer patient receiving an anti-PD1/PD-L1/PD-L2 Ab-based therapy, is a bacterial composition comprising *Enterococcus hirae* and/or *Akkermansia muciniphila* and/or *Alistipes*.

According to another aspect, the present invention pertains to an *in vitro* theranostic method of determining if a cancer patient is likely to be a good responder to an anti-PD1/PD-L1/PD-L2 Ab-based therapy, comprising:

(i) assessing, in a feces sample from said patient, the relative abundance of at least 10 microorganism species selected from the microorganism species disclosed in Table 1 and Table 2;

(ii) for each microorganism, comparing the relative abundance measured in step (i) to a predetermined threshold,

wherein over-representation of microorganism species disclosed in Table 1 (below) and under-representation of microorganism species disclosed in Table 2 (below) are indicative that the patient is likely to be a good responder to the anti-PD1/PD-L1/PD-L2 Ab-based therapy.

Tools designed to easily perform the above method are also part of the present invention, such as a nucleic acid microarray comprising nucleic

acid probes specific for each of the microorganism species to be detected in step (i) of the method, and such as a set of primers comprising primer pairs for amplifying sequences specific for each of the microorganism species to be detected in step (i) of said method.

The present invention also relates to an immunogenic composition comprising fragments of bacteria selected from the group consisting of *Firmicutes* species, *Clostridiales* species, *Alistipes* species, *Eubacterium* species, *Bacteroidales* species, *Methanobrevibacter smithii, Akkermansia muciniphila* and *Enterococcus hirae* and mixtures thereof, for use as an adjuvant to an anti-PD1/PD-L1/PD-L2 Ab-based therapy administered to a cancer patient.

According to another important aspect, the present invention pertains to the use of an allogeneic normal volunteer or responding patient derived-fecal microbial composition, for treating a cancer in combination with an anti-PD1/PD-L1 Ab-based therapy. Fecal microbial transplantation (FMT) with such a composition is particularly useful for improving the response rate to anti-PD1/PD-L1/PD-L2 antibodies of patients being identified as poor responders by the theranostic method of the invention.

Theranostic methods for determining whether an individual needs a bacterial composition with a bacterial composition and/or by FMT before receiving an anti-PD1/PD-L1 Ab-based therapy are also part of the invention.

The present invention also pertains to a method for *ex vivo* determining whether a cancer patient is likely to benefit from a treatment with an anti-PD1/PD-L1/PD-L2 Ab-based therapy, comprising assessing the presence of memory Th1 or Tc1 cells towards *Burkholderia cepacia, Akkermansia muciniphila, Enterococcus hirae* and/or *Bacteroides fragilis* in a blood sample from said patient, wherein the presence of memory Th1 or Tc1 cells towards *Burkholderia cepacia, Akkermansia muciniphila* and *Enterococcus hirae* indicates that the patient is likely to be a good responder to said treatment, and the presence of memory Th1 cells towards *Bacteroides fragilis* indicates that the patient is likely to be a poor responder.

LEGENDS TO THE FIGURES

Figure 1: Kaplan Meier progression-free survival (PFS) curves in renal cell cancer patients. 70 metastatic renal cell carcinoma patients treated with nivolumab (ICB), according to antibiotic (ATB, upper panel) or proton pump inhibitor (PPI, bottom panel) cotreatment (both taken within one to two months prior to the first injection of ICB and up to 1 month post-initiation of PD1/PD-L1 blockade).

Figure 2: Kaplan-Meier method to assess the PFS in patients in the ATB(+) group vs ATB (-) group in bladder cancer patients. 42 metastatic bladder urothelial cell carcinoma patients treated with anti-PD-L1 Ab (ICB) according to antibiotic (ATB) cotreatment (taken within one to two months prior to the first injection of ICB and up to 1 month post-initiation of PD-L1 blockade).

Figure 3: Heatmap / hierarchical clustering of the 147 MGS contrasted in the cancer cohorts. Heatmap color code: Heatmap was performed after log10 transformation of the p.value of the 147 MGS selected on the 3 cancer cohorts. To discreminate "cancer" species from "healthy" species, "healthy" values were reverse into positive values. Healthy species thus appear on the top and cancer species on the bottom. Row color code (column labeled R): colors indicates the High and Low richness MGS identified according to 387 control individuals, with no diagnosed cancer (spearman correlation |rho| > 0.3, p.val < 2e-05). A 3 color gradient code is used for |rho| > 0.3, 0.4 and 0.5, dark colors indicating higher correlation. Cancer patients were diagnosed with either early breast, or metastatic lung or metastatic kidney cancer (3 cancer types).

Figure 4: Heatmap / hierarchical clustering of the 43 "healthy" species contrasted in the cancer cohorts. Extract from the heatmap shown in Figure 3.

Figure 5: Heatmap / hierarchical clustering of the 104 "cancer" species contrasted in the cancer cohorts. Extract from the heatmap shown in Figure 3.

Figure 6: Heatmap / hierarchical clustering of the 27 contrasted and coherent species at least in 2 different cancer cohorts (among the 3 cancer types).

Figure 7: Barcodes and p values of the MGS found contrasted between cancer patient cohorts (C) and healthy controls (H) (Gene counts (GC)*)

Figure 8: Kinetics of MGS evolution during PD1 blockade. Barcodes of the MGS contrasted between lung cancer V1 (before commencement) and pooled lung + bladder V4 (at 6 months of therapy) patients during a therapy with anti-PD1/PDL1 Abs.

Figure 9: Barcodes of the MGS contrasted between pooled lung + kidney R (responders)/NR (non responders) samples, regardless of antibiotics and at diagnosis (prior to therapy with anti-PD1 Abs)

Figure 10: Experimental setting of FMT transfer into ATB-treated tumor bearers (AVATAR mice) before PD1 blockade. FMT with lung cancer patients' feces was performed into ATB-treated mice bearing established MCA205 WT (2 experiments).

Figure 11: Typical growth curve and kinetics of MCA205 inoculated in five SPF recipients or five ATB-treated mice that received FMT from a progressor (A) or from a responding (D) NSCLC patient.

Figure 12: Concatanated data of tumor sizes at sacrifice in several independent groups of mice receiving ATB followed by no FMT (NaCl), or FMT from four different progressors (A, B, E, F) and two different responders (C, D). Each dot represents one mouse tumor size at day 12 post-treatment start. Treatment consisted in ip administrations of anti-PD1 Abs.

Figure 13: Growth curves of MCA205 inoculated in GF mice that received FMT from a progressor (A) NSCLC patient followed by oral gavage of *Akkermansia municiphila* (Akk), *Enterococcus hirae* 13144 (EH) or *Eubacterium tenue* (Euba). Two gavages were performed with 10^9 bacteria at the time of the first and second injection of anti-PD1 Abs. Three different bacteria were used, one for each group of mice (n=5 mice per group) in separated isolators.

Figure 14: Experimental setting of bacterial compensation into ATB-treated tumor bearers (AVATAR mice) before and during PD1 blockade. Bacterial gavage was performed into ATB-treated mice bearing established MCA205 WT. Five gavages were performed with 10^9 bacteria prior

to and at the time of the first, second, 3rd, 4th injection of anti-PD1 Ab. Three different bacteria were used alone (*Akkermansia muciniphila*, *Alistipes spp*. and *Enterococcus hirae* 13144) or in a combination of *Akkermansia muciniphila* + *Enterococcus hirae* 13144 for each group of mice (n=5-6 mice per group) kept in separated isolators.

Figure 15: Growth curves of MCA205 according to protocol of Figure 14 that received oral gavage of *Akkermansia muciniphila* (Akk) or *Akkermansia muciniphila* + *Enterococcus hirae* 13144 (EH). Bacteria were administered by gavage in avatar mice that were spontaneously reconstituted with the mouse microflora after stopping ATB, according to the experimental setting shown in Figure 14.

Figure 16: Growth curves of MCA205 inoculated in GF mice that received FMT from a progressor (B) NSCLC patient followed by oral gavage of *Akkermansia muciniphila* (Akk) or *Akkermansia muciniphila* + *Enterococcus hirae* 13144 (EH) or *Alistipes spp*. (Ali). Bacteria were administered by gavage in avatar mice that received FMT from a progressor (B) NSCLC patient, according to the experimental setting shown in Figure 14.

Figure 17: Evolution of tumor sizes in several independent groups of ATB-treated mice that received FMT from two different progressors (B and F) NSCLC patient followed by oral gavage of *Akkermansia muciniphila (Akk)*, *Enterococcus hirae* 13144 (*E.H.*), *Alistipes spp*. (Ali) or *Akkermansia muciniphila* + *Enterococcus hirae* 13144 *(Akk + E.H)*. Bacteria were administered by gavage in avatar mice that received FMT from a progressor (B or F) NSCLC patient, according to the experimental setting shown in Figure 14. Each dot represents one mouse tumor size at day 0, 3, 6 or 9 post-treatment start. Treatment consisted in ip administrations of anti-PD1 Abs.

Figure 18: Compensating ATB-treated dysbiotic mice which do not respond to the combination of CTLA4+PD1 coblockade with "oncobax" (immunogenic commensals). C57BL/6 mice were inoculated with MCA205 sarcoma after a 14 day broad spectrum ATB administration. Then, at day 6 of MCA205 implantation, mice were treated with iv injections of anti-CTLA4 Ab (every other 3 days for 4 injections) as well as anti-PD1 Ab (6 injections every other 3 days for 6 injections). In independent groups isolated in different cages, mice received oral gavages with distinct oncobax, such as

Bifidobacterium breve combined with *Bifidobacterium longum* or *Bacteroides fragilis alone* or combined to *Burkholderia cepacia,* or *Barnesiella intestinihominis ("rectal" for rectal route of administration).* Tumor growth kinetics were monitored in 6 mice/group, twice a week. The number of tumor free animals at sacrifice is indicated in parentheses.

Figure 19: Memory Th1/Tr1 responses predict time to progression (TTP) under PD1 blockade in stage IV NSCLC. Tc1 or TH1 memory T cell responses to indicated commensals at diagnosis or during teh first month of anti-PD1 Ab-based therapy in cancer patients segregated according to the mean value of IFNg production in the whole cohort for the calculation of TTP. Ratios between IFNg and IL-10 in memory CD4+ T cells or CD8+ T cells exposed to autologous monocytes incubated with distinct commensals in 14 advanced lung cancer patients is also shown. The Kaplan Meier curves separate the patients with high versus low ratios according to the median of the cohort.

Figure 20: Progression free survival (PFS) and overall survival (OS) of Non-Small Cell Lung Cancer patients who received ATB or not 2 months before their first injection of anti-PD1 Abs and up to month 1 after the 1st one. The Kaplan Meier survival curves of NSCLC patients treated with Nivolumab in either ATB (+)/(-) are shown for progression free survival left panel and right panel: overall survival. Log-rank (Mantel-Cox). *p<0.05, ** p<0.01,*** p<0.001, ns=not significant.

Figure 21: PFS and OS in all 3 advanced cancer types n= 175 (NSCLC, mRCC, mUC) treated with PD1/PDL1 inhibitor. Progression free survival (left panel) and survival analysis (right panel) by Kaplan-Meier curves of pooled 3 cohorts of patients with advanced cancers. N=66 Non-small cell lung cancer, n=67 metastatic renal cell carcinoma, n=42 metastatic urothelial cancer all treated with PD1/PDL1 inhibitor on ATB (+) vs ATB (-). ATB(+)/(-) groups were defined as patients treated or not with ATB before (2 months period) or within the first month of ICB. *p<0.05, ** p<0.01,*** p<0.001, ns=not significant.

Figure 22: Cancer-associated Gut fingerprint in metagenomics species (MGS): 18 contrasted MGS. Heat map of the non supervised hierarchical clustering of bacterial MGS common in two independent cohorts (1, 2) of cancer patients (Br: for breast cancer, Lu for lung cancers, Ni for kidney cancers), considering all patients (all) or high diversity subset (high)

or low diveristy subset (low). In blue, are those MGS overrepresented in healthy volunteers that are underrepresented in cancer patients while in red are those MGS overrepresented in cancer patients.

Figure 23: Cancer-associated Gut fingerprint in MG: 11 contrasted MGS (no ATB). Same analysis as in Fig. 22 but eliminating cancer patients who took any antibiotics for at least 1 week, two months prior to feces collection.

Figure 24. Higher richness of gene counts and metagenomic species in stools at diagnosis in kidney or lung cancer patients predict better progression free survival at 6 months of continuous therapy with anti-PD1 Ab. Shot gun sequencing of fecal samples at diagnosis with representation of gene and MGS counts for all cancer patients according to clinical outcome (PFS at 6 months). Means±SEM of counts are depicted for patients experiencing PFS > or < 6 months. Of note, gene or MGS richness did not predict PFS at 3 months.

Figure 25: Experimental setting of an FMT of 2 NSCLC progressors compensated by Oncobax in ATB-SPF recipients. Experimental design: Fecal microbial transplantation of two non-responders patients with NSCLC on anti-PD1 Abs in ATB treated mice. C57/BL6 SPF mice received 3 days of broad spectrum ATB: colistin, ampicillin, streptomycin. 12 hours after ATB discontinuation, FMT was performed with 200 µL of feces and 100 µL applied on the fur of each mouse (Patient feces were stored following the IHMS recommendations). Subsequently, 2 weeks later, MCA205 WT tumor were inculcated right flank and anti-PD1 Ab was started when tumor sizes reached 20-25 mm^2. Oncobax-Bacterial gavage (10^8 bacteria) was performed the day before the first intraperitoneal injection of anti-PD1 Ab and then at each anti-PD1 Ab injection.

Figure 26: Results of experiments described in Figure 25. FMT of 2 NSCLC progressors compensated by Oncobax in ATB-SPF recipients: efficacy of *Akk +/- E. hirae (oncobax)*. Tumor growth of MCA-205 WT in ATB treated mice following FMT with 2 non-responders NSCLC patients or NaCL control. Mice were treated with Iso control vs anti-PD1 Ab + different combination of Oncobax.T-test was performed. *p<0.05, ** p<0.01,*** p<0.001, ns=not significant.

Figure 27: Accumulation of effector and central memory CD8$^+$ T cells with effective oncobax in tumor draining lymph nodes. Flow cytometry analyses of CD8+ effector (CD62-CD44+) and CD8+ central memory (CD62L+CD44+) cells in draining lymph nodes of ATB treated post FMT using lung cancer feces (patient F) and treated with anti-PD1 Ab + different oncobax combinations.

Figure 28: Experimental setting in the RET melanoma tumor model in ATB-dysbiotic SPF recipients: compensation with various oncobax. RET melanoma tumors were injected in the left flank of ATB dysbiotic SPF C57/BL6 mice treated with Colistin, Streptomycin, and Ampicillin for two weeks. Then after ATB were discontinued, each group underwent bacterial gavage and mono-colonization with oncobax 10^8 bacteria or combination of two bacteria according to the detailed schedule. Each mouse received 4 intraperitoneal injections of either Iso Control or anti-PD1 Abs.

Figure 29: Results from experimental setting described in Figure 28. The combination of *Enterococcus hirae (13144)* +*Akkermansia muciniphila (Akk)* is very efficient in the RET model. Tumor growth of RET in ATB dysbiotic SPF mice and treated according to experimental setting explained in fig 19. Each line represents one mouse. Two top left panels represent control mice that only received water. Top two panels on the right depict the tumor growth in mice that received ATB. The four panels at the bottom represent tumor growth of mice that underwent oncobax monocolonization after ATB with *Akkermansia muciniphila, Enterococcus hirae 13144* + *Akkermansia muciniphila, Eubacterium tenue* + *Akkermansia muciniphila, Alistipes indistinctus* respectively. One representative experiment out of two is shown.

Figure 30: Orthotopic LLC: efficacy of PD1 blockade + radiotherapy combined with oncobax (1). Experimental settings for combined modalities Radiotherapy + PD1 in SPF mice. After, 3 days of ATB C57/BL6 mice were injected orhtotopically with Lewis Lung Cancer (LLC) cell line expressing luciferase that can be monitored using the IVIS spectrum in vivo imaging system after luciferine injection. On Day 3-4 they received 9Gy of radiotherapy targeting the thorax. Anti-PD1 Abs or Iso Control Abs were injected intraperitoneally according to the detailed schedule every 3 days. In the bacteria group: mice received orally *Enterococcus hirae* 13144 + *Akkermansia muciniphila* at 10^8 on the same day as anti-PD1 Abs injection.

Figure 31: Orthotopic LLC: efficacy of PD1 blockade + radiotherapy combined with oncobax (2). Left panel represents the tumor growth measure using the IVIS spectrum in vivo imaging system. For each group normalized total flux is represented on day#10. Right panel: Survival analysis by Kaplan-Meier curves of all mice either in the Iso-Ctrl or Radiotherapy + PD1 or Radiotherapy + anti-PD1 Ab + Bacteria group. Log-rank (Mantel-Cox). *p<0.05, ** p<0.01,*** p<0.001, ns=not significant.

Figure 32: Role of gut microbiome in the anticancer effects of anti-CTLA4+anti-PD1 Ab in a RENCA tumor model (1). Experimental setting of fecal microbial transplantation in Balb/c ATB- treated mice with feces from patients with metastatic renal cell carcinoma. After 3 days of ampicillin, colistin and streptomycin, Balb/c mice received FMT from 3 different patients with mRCC on Nivolumab. Two patients had no clinical benefit whereas the other one remained in partial response after 10 months. On day 14 post FMT, RENCA-luciferase tumors were injected orthotopically and mice received either Iso Control or PD1+CTLA4 in combination every 4 day according to the schedule. Table at the bottom shows metagenomic feces composition of the 3 donors for several bacteria.

Figure 33: Role of gut microbiome in the anticancer effects of anti-CTLA4+anti-PD1 Ab in a RENCA tumor model (2). Tumor size assessment of RENCA in Balb/c post FMT using IVIS® spectrum *in vivo* imaging system device. Total flux for each mouse is represented on Day 9-15. T-test was performed. *p<0.05, ** p<0.01, *** p<0.001, ns=not significant.

Figure 34: PD-L1 upregulation on circulating T cells is a hallmark of a favorable MG composition of feces associated with clinical benefit to PD1 blockade. Flow cytometry analyses of splenic CD4+ and CD8+ T cell expression of PDL1 molecules on the cell surface. A. Post FMT of either responders or non responders in ATB-treated mice. Panel B Post FMT of a non responder followed by treatment with oncobax.

Figure 35: Phenotypic changes post-oral gavage with oncobax in the mesenteric lymph nodes. Augmentation of CD4+ and CD8+ CCR9+ CM (and naive to some extent) with *Enterococcus hirae* alone or combined with *Akkermansia muciniphila.* Flow cytometry analyses of mesenteric lymp nodes 48 hr post gavage with oncobax and treated with 1 injection of intraperitoneal anti-PD1 Ab. Panel A: CD4+ Naïve CCR9+ and

CD4+ Central memory CCR9+. Panel B: CD8+CCR9+, CD8+ Central memory CCR9+, CD8+ CM CCR9+CXCR3+. *Enterococcus hirae* markedly increase CD8+ TCM CCR9++ and CCR9+CXCR3+ while *Akkermansia muciniphila* augments mainly CD4+ CCR9+ TCM.

Figure 36: Prediction of tumor size in Atb-treated mice post FMT using the metagenomic composition of the patients' feces. We performed FMT in ATB treated mice with feces from NSCLC (2 responders and 4 progressors) on Nivolumab (Figure 12). Spearman correlation between individual mouse tumor size and the composition of the patient's feces for different bacteria present in the metagenomic signature either in the responder or non-responder section.

Figure 37: CD4 stimulation with *Akkermansia muciniphila vs Enterococcus Faecalis*. Predictive value of CD4 IFN-g release determined by ELISA for each metastatic renal cell carcinoma patient during immunotherapy after various bacterial stimulations of monocytes/CD4+T cell co-cultures. Similar results were obtained using CD8-IFNg (Tc1) in response to restimulation with *A. muciniphila.* The Kaplan Meier curves showing PFS for each subgroups (TH1: IFNg, TR1: L-10 or ratio IFNg/IL-10) patients above/below the median of cytokine production are represented for two bacteria. Memory TH1 and Tc1 immune responses against *A. muciniphila* predict TTP during PD1 blockade in both lung and kidney cancer patients (not shown). Log-rank *$p < 0.05$, **$p < 0.01$.

Figure 38: Compensating a dysbiosis from a kidney cancer patient (RCC) with feces from a responding patient-derived stools for restoring the response to immune checkpoint inhibitors. BALB/c mice were treated with 3 days of broad spectrum antibiotics, followed by oral gavage with non responding patient- derived stools. Twenty days later, we inoculated in the kidney of BALB/c mice orthotopically the RENCA-luciferase tumor that we could follow with the reporter imaging system IVI. Eight days later, BALB/c mice received ip administration of anti-PD1 and anti-CTLA4 Ab every other 3 days for 5 injections. The day before each ip therapy with mAb, recipient tumor bearing mice received an oral gavage with feces (10^{10} cfu) from one RCC patient who responded to anti-PD1 mAb -based therapy. Three distinct RCC responding patients (R4-R5-R6) stools were utilized. Tumor growth kinetics were followed. The graph depicts tumor sizes at sacrifice, showing individual data and also pooling the data from the 3 groups R4-R6. Anova statistical analyses:*$p<0.05$.

Figure 39: Compensating a dysbiosis from a breast cancer patient (BC) with feces from a healthy volunteer (HV)-derived stools for restoring the response to metronomic cyclophosphamide (CTX). A. Experimental setting. C57BL/6 mice were treated with 3 days of broad spectrum antibiotics, followed by oral gavage (FMT) with two breast cancer patients (BC1 and BC2)- derived stools harvested post-chemotherapy when feces are dysbiostic with low diversity. BC1 is a triple negative BC with dismal prognosis while BC2 has a favorable prognosis. Twenty days later, we inoculated in the AT3 breast syngenic mouse tumor sc. Ten days later, C57BL/6 mice received ip administration of metronomic CTX every week days for 3 injections. The day before each ip therapy with CTX the first time and then the same day, recipient tumor bearing mice received an oral gavage with feces (10^{10} cfu) from one HV. Alternatively, mice (that were subjected to a FMT with HV or BC) were cohoused together for 3 weeks. **B.** Tumor growth kinetics were followed. The graph depicts tumor sizes over time and at sacrifice. Anova statistical analyses:*p<0.05; **p<0.01; ***p<0.001.

Figure 40: Compensating a dysbiosis from a non responding kidney cancer patient (RCC) with defined strains for restoring the response to immune checkpoint inhibitors. A. BALB/c mice were treated with 3 days of broad spectrum antibiotics, followed by oral gavage with non responding patient- derived stools. Twenty days later, we inoculated in the kidney of BALB/c mice orthotopically the RENCA-luciferase tumor that we could follow with the reporter imaging system IVI. Eight days later, BALB/c mice received ip administration of anti-PD1 and anti-CTLA4 Ab every other 3 days for 5 injections. The day before each ip therapy with mAb, recipient tumor bearing mice received an oral gavage with 10^{10}cfu of various strains (listed in X axis). **B.** Tumor growth kinetics were followed. The graph depicts tumor sizes at sacrifice. The percentages of tumor free mice at sacrifice is indicated below. Anova statistical analyses:*p<0.05; **p<0.01; ***p<0.001.

Figure 41: Compensating a dysbiosis from a non responding kidney cancer patient (RCC) with *Bacteroides salyersae* for restoring the response to immune checkpoint inhibitors. C57BL/6 mice were treated with 3 days of broad spectrum antibiotics, followed by oral gavage with non responding RCC patient- derived stools. Twenty days later, we inoculated in the MCA205 sarcoma sc. Eight days later, C57BL/6 mice received ip administration of anti-PD1 Ab+anti CTLA4 Ab every other 3 days for 5 injections. The day

before each ip therapy with mAb, recipient tumor bearing mice received an oral gavage with 10^{10}cfu of various strains (listed in X axis). Tumor growth kinetics were followed. The graph depicts tumor sizes at sacrifice. The percentages of tumor free mice at sacrifice is indicated below. Anova statistical analyses:*p<0.05.

Figure 42: Compensating a dysbiosis from a lung NSCLC patient with a consortium of 6 bacteria strains for restoring the response to anti-PD1 mAb. A. C57BL/6 mice were treated with 3 days of broad spectrum antibiotics, followed by oral gavage with non responding NSCLC patient-derived stools. Twenty days later, we inoculated the MCA205 sarcoma sc. Eight days later, C57BL/6 mice received ip administration of anti-PD1 Ab every other 3 days for 5 injections. The day before each ip therapy with mAb, recipient tumor bearing mice received an oral gavage with 10^{10}cfu of various strains (listed in 6A). **B.** Tumor growth kinetics (means+/- SEM or each individual curve) were followed. The graph depicts the growth kinetics over time in the best groups (mouse by mouse in upper panels, means in lower panels). **C.** All tumor sizes for all groups recapitulated at day 13. Tumor Anova statistical analyses:*p<0.05.

Figure 43: Distinct consortia of bacteria are able to ameliorate anti-PD1 efficacy. C57BL/6 mice were treated with 3 days of broad spectrum antibiotics, followed by oral gavage (FMT) with lung cancer patients-derived stools. Fifteen days later, C57BL/6 mice were inoculated with MCA205 sarcoma mouse tumor sc. 4 days later, C57BL/6 mice received *ip* administration of anti-PD1 every 3 days for a total of 4 injections eventually orally gavaged with a consortium of bacteria composed of 10^9 cfu of various strains (listed in X axis). Tumor growth kinetics were followed. Tumor growth depicted at day 8 post gavage. Each dot represents one mouse tumor size. A representative experiment out of two yielding similar results is depicted.

Figure 44: *Barnesiella intestinihominis* is contained in the hallmark signature of therapy with anti-PD1 Abs (and response to this therapy). C57BL/6 mice were inoculated with MCA205 sarcoma mouse tumor *sc*. 6 days later, C57BL/6 mice received *ip* administration of anti-PD1 or anti-PD1/anti-CTLA4 Ab combination every 3 days for a total of 6 injections. Metagenomic analysis of stools after PD1 blockade or PD1+CTLA4 co-blockade to define specific gut fingerprints associated with each mAb therapy. Lefse (A) and boxplots (B) graphs after two injections of mAb-based

immunotherapy (F3). Kruskal-Wallis analysis; $p<0.05$. MCA205 is a sarcoma while RET is a melanoma (not shown). *Barnesiella intestinihominis* is therefore selected post-PD1 blockade and associated with the clinical benefit.

Figure 45. Immunohistochemistry analyzing gut toxicity (crypts irregularities, loss of villosities, inflammatory patterns) in hematoxylin eosin or staining with anti-cleaved caspase3 Ab. Experimental settings described in Fig. 40-42. Transfer of feces from responders reduce toxicity obtained with feces from non responders. FMT compensated with oncobax reduces colitis scores.

Figure 46. Experimental setting and samples collection timing in two independent tumor mouse models. Timing of fecal samples collection from two different mouse tumor models. MCA-205 –sarcoma or RET – melanoma were inoculated in SPF mice and subsequently treated with monotherapy anti-PD-1 mAb (n=6), combination anti-PD-1 mAb + anti-CTLA-4 mAb (n=6) or Iso Ctrl (n=6). F1: before tumor inoculation. F2: 5-7 days after tumor inoculation and before the first treatment. F3: 48hr after the 2^{nd} injection. F4: 48hr after the 5^{th} injection. F5: 48hr after the 6^{th} injection.

Figure 47: Gut microbiota composition is influenced by anti-PD-1 monotherapy or in combination with anti-CTLA-4 mAb, and tumor size in MCA-205 mouse model. Left panels: Principle component analysis (PCA) graphs represent the beta-diversity of taxonomy derived from the 16 S rRNA sequencing of stools (exp setting Fig. 46), based on Bray-Curtis distance at different time points F2-F3-F4 for each group (IsoCtrl, PD-1, and combo: PD-1 + anti-CTLA-4). Right panels: R statistic (from ANOSIM) evaluating the bacterial taxonomy diversity for each treatment group with the tumor size. An R value close to "1.0" suggests difference between groups (for a definite criterion), while an R value close to "0" suggests an even distribution of high and low ranks within and between groups. R values below "0" suggest that differences are greater within groups than between groups.

Figure 48: Gut microbiota changes are influenced by the ICB combination (anti-PD-1 + anti-CTLA-4 Abs) and correlate with tumor size in RET mouse model. Left panels: Principal component analysis representing the bacterial taxonomy-beta-diversity obtained in 16 S rRNA sequencing of

stools (exp setting Fig. 46), based on Bray-Curtis distance at different time point F2-F3-F4 in each group (IsoCtrl, PD-1, and combo: anti-PD-1 + anti-CTLA-4 Abs). Right panels: R statistic (from ANOSIM) evaluating the diversity for each treatment group with the tumor size. An R value close to "1.0" suggests difference between groups (for a definite criterion), while an R value close to "0" suggests an even distribution of high and low ranks within and between groups. R values below "0" suggest that differences are greater within groups than between groups.

Figure 49: Distinctive baseline microbiota composition and bacterial spp. predicting response or resistance to ICB in Exp setting Fig. 46. 16 S rRNA pyrosequencing analysis of baseline fecal composition (F2) of mice inoculated with either RET or MCA-205. Animals were segregated according to their tumor sizes upon killing in two groups responders (R) and non-responders (NR) regardless of their treatments (Iso Control, PD-1 or PD-1+ Anti-CTLA-4). Among the responders, 78 bacteria were only found in this R group relative to the NR group.

Figure 50: Species associated with beneficial response in mice treated with the combination of immune checkpoint blockers (anti-PD-1 + anti-CTLA-4 Abs). 16 S rRNA pyrosequencing analysis of gene amplicons in baseline fecal samples at F2 in mice inoculated with MCA-205 or RET. 169 bacteria species were common in responder mice treated with anti-PD-1 + anti-CTLA-4 mAbs in both tumor models.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In the present text, the following general definitions are used:

Gut microbiota

The “gut microbiota” (formerly called gut flora or microflora) designates the population of microorganisms living in the intestine of any organism belonging to the animal kingdom (human, animal, insect, *etc.*). While each individual has a unique microbiota composition (60 to 80 bacterial species are shared by more than 50% of a sampled population on a total of 400-500 different bacterial species/individual), it always fulfils similar main physiological functions and has a direct impact on the individual’s health:

- it contributes to the digestion of certain foods that the stomach and small intestine are not able to digest (mainly non-digestible fibers);
- it contributes to the production of some vitamins (B and K);
- it protects against aggressions from other microorganisms, maintaining the integrity of the intestinal mucosa;
- it plays an important role in the development of a proper immune system;
- a healthy, diverse and balanced gut microbiota is key to ensuring proper intestinal functioning.

Taking into account the major role gut microbiota plays in the normal functioning of the body and the different functions it accomplishes, it is nowadays considered as an "organ". However, it is an "acquired" organ, as babies are born sterile; that is, intestine colonisation starts right after birth and evolves afterwards.

The development of gut microbiota starts at birth. Sterile inside the uterus, the newborn's digestive tract is quickly colonized by microorganisms from the mother (vaginal, skin, breast, *etc.*), the environment in which the delivery takes place, the air, *etc*. From the third day, the composition of the intestinal microbiota is directly dependent on how the infant is fed: breastfed babies' gut microbiota, for example, is mainly dominated by *Bifidobacteria*, compared to babies nourished with infant formulas.

The composition of the gut microbiota evolves throughout the entire life, from birth to old age, and is the result of different environmental influences. Gut microbiota's balance can be affected during the ageing process and, consequently, the elderly have substantially different microbiota than younger adults.

While the general composition of the dominant intestinal microbiota is similar in most healthy people (4 main phyla, *i.e.*, *Firmicutes, Bacteroidetes, Actinobacteria* and *Proteobacteria*), composition at a species level is highly personalised and largely determined by the individuals' genetic, environment and diet. The composition of gut microbiota may become accustomed to dietary components, either temporarily or permanently. Japanese people, for example, can digest seaweeds (part of their daily diet) thanks to specific enzymes that their microbiota has acquired from marine bacteria.

Dysbiosis

Although it can adapt to change and has a high resilience capacity, a loss of balance in gut microbiota composition may arise in some specific situations. This is called "dysbiosis", a disequilibrium between potentially "detrimental" and "beneficial" bacteria in the gut or any deviation to what is considered a "healthy" microbiota in terms of main bacterial groups composition and diversity. Dysbiosis may be linked to health problems such as functional bowel disorders, inflammatory bowel diseases, allergies, obesity and diabetes. It can also be the consequence of a treatment, such as a cytotoxic treatment or an antibiotic treatment. Here we will call "dysbiosis" any deviation of the gut composition observed in cancer patients compared to the gut composition from healthy individuals and "dysbiosis associated with lack of response to PD1/PD-L1 blockade" any under-or over-representation of distinct species described in Tables 1 and 2, respectively.

Antineoplastic treatments

"Antineoplastic treatments" herein designate any treatment for cancer except surgery. They include chemotherapy, hormonal and biological therapies, and radiotherapy.

Biological therapies

Anti-cancer "biological therapies" involve the use of living organisms, substances derived from living organisms, or laboratory-produced versions of such substances to treat cancer, by targeting either the cancer cells directly, or by stimulating the body's immune system to act against cancer cells ("immunotherapy"). Biological therapies include monoclonal antibodies (Mabs) (including those targeting cancer cell surface, *e.g.* rituximab and Alemtuzumab; anti-CTLA4 Mabs, such as Ipilimumab; targeting growth factors, *e.g.*: Bevacizumab, Cetuximab, Panitumumab and Trastuzumab; anti-PD1 Mabs, such as Nivolumab and Pembrolizumab; anti-Tim3 Mabs; anti-PD-L1 Mabs, such as Atezolizumab, Durvalumab, and Avelumab; anti-PD-L2 Mabs), agonistic antibodies (anti-ICOS Mabs, anti-OX40, anti-41BB mAbs), immunoconjugates (*e.g.*: ^{90}Y-ibritumomab tiuxetan, ^{131}I-tositumomab, and ado-trastuzumab emtansine), cytokines (including interferons such as IFNα; interleukins such as IL-2, IL-11, G-CSF, GM-CSF), therapeutic vaccines

(*e.g.*: Sipuleucel-T (Provenge®)), the bacterium bacillus Calmette-Guérin, cancer-killing viruses (oncolytic), gene therapy, and adoptive T-cell transfer.

Immune checkpoint blockers

In the present text, a "drug blocking an immune checkpoint", or "immune checkpoint blocker" or "immune checkpoint blockade drug" designates any drug, molecule or composition which blocks an immune checkpoint. In particular, it encompasses anti-CTLA-4 antibodies, anti-PD1 antibodies, anti-PD-L1 antibodies (such as Atezolizumab or Durvalumab) and anti-PD-L2 antibodies. More particularly, it can be an anti-PD1 monoclonal antibody such as Nivolumab or Pembrolizumab.

An "anti-PD1/PD-L1/PD-L2 Ab-based therapy" herein designates any drug that antagonizes PD1 or PD-L1 or PD-L2. Although the currently used drugs antagonizing PD1 or PD-L1 or PD-L2 are monoclonal antibodies, other molecules specifically binding to PD1, PD-L1 or PD-L2 could be used for the development of future ICB such as, for example, antibody fragments or specifically designed aptamers. Of course, the phrase "anti-PD1/PD-L1/PD-L2 Ab-based therapy" encompasses any therapy with active molecules that antagonize PD1 or PD-L1 or PD-L2.

Probiotics

"Probiotics" are micro-organisms that have claimed health benefits when consumed. Probiotics are commonly consumed as part of fermented foods with specially added active live cultures, such as in yogurt, soy yogurt, or as dietary supplements. Generally, probiotics help gut microbiota keep (or re-find) its balance, integrity and diversity. The effects of probiotics can be strain-dependent. Here we will use the phrase "anticancer probiotics" or the neologisms "oncobax" and "oncomicrobiotics" to designate any commensal composition that restores responsiveness to PD1/PD-L1 blockade or combination of anti-CTLA4+anti-PD1 or PD-L1 Ab. In the context of the present invention, a "probiotic composition" is thus not limited to food or food supplements, but it generally designates any bacterial composition comprising microorganisms which are beneficial to the patients. Such probiotic compositions can hence be medicaments or drugs.

Cancer, treatment, etc.

As used herein, "cancer" means all types of cancers. In particular, the cancers can be solid or non solid cancers. Non limitative examples of cancers are carcinomas or adenocarcinomas such as breast, prostate, ovary, lung, pancreas or colon cancer, sarcomas, lymphomas, melanomas, leukemias, germ cell cancers and blastomas.

The immune system plays a dual role against cancer: it prevents tumor cell outgrowth and also sculpts the immunogenicity of the tumor cells. Drugs blocking an immune checkpoint can hence be used to treat virtually any type of cancer. Thus, the methods according to the invention are potentially useful for patients having a cancer selected amongst adrenal cortical cancer, anal cancer, bile duct cancer (e.g. periphilar cancer, distal bile duct cancer, intrahepatic bile duct cancer), bladder cancer, bone cancers (e.g. osteoblastoma, osteochrondroma, hemangioma, chondromyxoid fibroma, osteosarcoma, chondrosarcoma, fibrosarcoma, malignant fibrous histiocytoma, giant cell tumor of the bone, chordoma, lymphoma, multiple myeloma), brain and central nervous system cancers (e.g. meningioma, astocytoma, oligodendrogliomas, ependymoma, gliomas, medulloblastoma, ganglioglioma, Schwannoma, germinoma, craniopharyngioma), breast cancer (e.g. ductal carcinoma *in situ*, infiltrating ductal carcinoma, infiltrating lobular carcinoma, lobular carcinoma *in situ*, gynecomastia), Castleman disease (e.g. giant lymph node hyperplasia, angiofollicular lymph node hyperplasia), cervical cancer, colorectal cancer, endometrial cancers (e.g. endometrial adenocarcinoma, adenocanthoma, papillary serous adenocarcinoma, clear cell), esophagus cancer, gallbladder cancer (mucinous adenocarcinoma, small cell carcinoma), gastrointestinal carcinoid tumors (e.g. choriocarcinoma, chorioadenoma destruens), Hodgkin's disease, non-Hodgkin's lymphoma, Kaposi's sarcoma, kidney cancer (e.g. renal cell cancer), laryngeal and hypopharyngeal cancer, liver cancers (e.g. hemangioma, hepatic-adenoma, focal nodular hyperplasia, hepatocellular carcinoma), lung cancers (e.g. small cell lung cancer, non-small cell lung cancer), mesothelioma, plasmacytoma, nasal cavity and paranasal sinus cancer (e.g. esthesioneuroblastoma, midline granuloma), nasopharyngeal cancer, neuroblastoma, oral cavity and oropharyngeal cancer, ovarian cancer, pancreatic cancer, penile cancer, pituitary cancer, prostate cancer, retinoblastoma, rhabdomyosarcoma (e.g. embryonal rhabdomyosarcoma, alveolar rhabdomyosarcoma, pleomorphic rhabdomyosarcoma), salivary gland

cancer, skin cancer (e.g. melanoma, nonmelanoma skin cancer), stomach cancer, testicular cancers (e.g. seminoma, nonseminoma germ cell cancer), thymus cancer, thyroid cancers (e.g. follicular carcinoma, anaplastic carcinoma, poorly differentiated carcinoma, medullary thyroid carcinoma, thyroid lymphoma), vaginal cancer, vulvar cancer, and uterine cancer (e.g. uterine leiomyosarcoma). More particularly, the method according to the invention can be used for predicting and optimizing a patient's response to a medicament targeting an immune checkpoint, wherein the patient has a cancer selected from the group consisting of metastatic melanoma, non-small cell lung carcinoma (NSCLC), small cell lung cancer (SCLC), mesothelioma, bladder cancer, renal cell carcinoma, head and neck cancers, oesophageal and gastric cancers, rectal cancers, hepatocarcinoma, sarcoma, Wilm's tumor, Hodgkin lymphoma, ALK-neuroblastoma, (hormone refractory) prostate cancers and GIST.

Other definitions will be specified below, when necessary.

According to a first aspect, the present invention pertains to an *in vitro* theranostic method of determining if a cancer patient is likely to be a good responder to an anti-PD1/PD-L1/PD-L2 Ab-based therapy, comprising:

(i) assessing, in a feces sample from said patient, the relative abundance of at least 10 microorganism species selected from the microorganism species disclosed in Table 1 and Table 2;

(ii) for each microorganism, comparing the relative abundance measured in step (i) to a predetermined threshold,

wherein over-representation of microorganism species disclosed in Table 1 and under-representation of microorganism species disclosed in Table 2 are indicative that the patient is likely to be a good responder to the anti-PD1/PD-L1/PD-L2 Ab-based therapy.

Bacterial species	Number of co-variant genes	Corresponding referenced species	Annotation	Sequences (SEQ ID Nos)
CAG00064	3310		*unclassified*	*1-25*
CAG00245	2277		*unclassified*	*51-75*
CAG00453	1942		*NA*	*1751-1775*
CAG00060	3391		*NA*	*1201-1225*
CAG01227	815		*NA*	*2776-2800*
CAG00774	1519	*Firmicutes*	*Firmicutes*	*2076-2100*

CAG00604	1714	*Firmicutes bacterium CAG:110*	*Firmicutes*	*401-425*
CAG01090	1026		*Firmicutes*	*776-800*
CAG00965	1210		*Firmicutes*	*751-775*
CAG00621	1695	*Firmicutes bacterium CAG:272*	*Firmicutes*	*426-450*
CAG01245	780		*Firmicutes*	*826-850*
CAG01308	666		*Firmicutes*	*851-875*
CAG00669	1649		*Firmicutes*	*526-550*
CAG00670	1648		*Firmicutes*	*551-575*
CAG00872	1359	*Firmicutes bacterium CAG:240*	*Firmicutes*	*651-675*
CAG00851	1422		*Firmicutes*	*626-650*
CAG00288	2189	*Firmicutes bacterium CAG:95*	*Firmicutes*	*76-100*
CAG1173	907	*Firmicutes bacterium CAG:321*	*Firmicutes*	*2626-2650*
CAG01223	819	*Firmicutes bacterium CAG:114*	*Firmicutes*	*2751-2775*
CAG00076	3098	*Firmicutes bacterium CAG:424*	*Firmicutes*	*1251-1275*
CAG00966	1209	*Firmicutes bacterium CAG:552*	*Firmicutes*	*2351-2375*
CAG1086	1037	*Firmicutes bacterium CAG:124*	*Firmicutes*	*2526-2550*
CAG0730	1576	*Dialister succinatiphilus*	*Firmicutes, Veillonellaceae*	*2026-2050*
CAG00901	1313	*Unclassified Erysipelotrichaeae*	*Erysipelotrichaeae*	*2251-2275*
CAG00363	2056	*Intestinimonas butyriciproducens*	*Intestinimonas butyriciproducens (closely related to Flavonifractor plautii)*	*176-200*
CAG00391	2019		*Clostridiales*	*201-225*
CAG00449	1945	*Clostridium sp. CAG:253*	*Clostridiales*	*276-300*
CAG00513	1853		*Clostridiales*	*326-350*
CAG00559	1776		*Clostridiales*	*376-400*
CAG00644	1670	*Clostridium sp. CAG:226*	*Clostridiales*	*476-500*
CAG00811	1469		*Clostridiales*	*601-625*
CAG00907	1299		*Clostridiales*	*726-750*
CAG01350	597		*Clostridiales*	*876-900*
CAG00382	2033		*Clostridiales*	*1676-1700*
CAG1075	1052		*Clostridiales*	*2501-2525*
CAG00821	1461	*Clostridiales bacterium VE202*		*2126-2150*
CAG119	2762	*Clostridium*	*Clostridiales*	*1326-1350*
CAG1158	938	*Unclassified Clostridiales*	*Clostridiales*	*2601-2625*
CAG1011	1140	*Uncultured Dore asp.*	*Clostridiales*	*2426-2450*
CAG00108	2820	*Clostridium paraputrificum*	*Clostridiales*	*1276-1300*

	3314	*Coprococcus spp. ART 55/1 And Coprococcus sp. CAG:131*	*Clostridiales, Lachnospiraceae*	
CAG00871	1162	*Lachnospiraceae*	*Lachnospiraceae*	*2226-2250*
CAG1146	956	*Oscillibacter sp. CAG:155*	*Clostridiales, Oscillospiricaeae*	*2576-2600*
CAG00006_3	785	*Oscillibacter*	*Clostridiales, Oscillospiricaeae*	*1126-1150*
CAG01047	1087	*Clostridium Incertae Sedis*	*Clostridiales Family XIII*	*2476-2500*
CAG00134	2690	*Cloacibacillus porcorum*		*1351-1375*
CAG01169	912		*Ruminococcaceae*	*801-825*
CAG00210	2389	*Ruminococcus bicirculans*	*Ruminococcaceae*	*1426-1450*
CAG00949	1224	*Ruminococcaceae bacterium D16*	*Ruminococcaceae*	*2326-2350*
CAG00558	1777	*Ruminococcus lactaris*	*Ruminococcaceae*	*351-375*
CAG01262	750	*Ruminococcus torques 2*	*Ruminococcaceae*	*2826-2850*
CAG00250	2262	*Ruminococcus sp. CAG:353*	*Ruminococcaceae*	*1476-1500*
CAG00854	2572	*Ruminococcaceae*	*Ruminococcaceae*	*2176-2200*
CAG00880	1352	*Subdoligranulum sp. CAG:314*	*Subdoligranulum sp.*	*676-700*
CAG00628	1686		Faecalibacterium	*451-475*
CAG001046	1089	*Intestinimonas butyriciproducens*	*Intestinimonas butyriciproducens (closely related to Flavonifractor plautii)*	*2451-2475*
CAG00555	1782	*Flavonifractor plautii*		*1926-1950*
CAG00469	1928	*Eubacterium sp. CAG:146*	*Eubacterium*	*301-325*
CAG00786	1484	*Eubacterium sp. CAG:202*	*Eubacterium*	
		Eubacterium sp.3_1_31	*Erysipelotrichaceae bacterium 5_2_54FAA*	
CAG00393	2011		*Eubacterium*	*226-250*
CAG00116	2783	*Bacteroides nordii*	*Bacteroidales*	*26-50*
CAG00355	2067	*Bacteroides sp. CAG:661*	*Bacteroidales*	*151-175*
CAG00440	1952	*Bacteroides sp. CAG:598*	*Bacteroidales*	*251-275*
CAG0049	3561	*Bacteroides cacae*	*Bacteroidales*	
CAG00646	1668		*Alistipes*	*501-525*
CAG00887	1332	*Alistipes sp. CAG:435*	*Alistipes sp.*	*701-725*
CAG00827	1455	*Alistipes sp. CAG:514*	*Alistipes sp.*	*2151-2175*
CAG00354	2067	*Candidatus alistipes marseilloanorexicus*	*Alistipes*	*1626-1650*
CAG1244	783	*Parabacteroides merdae*	*Bacteroidales*	*2801-2825*

CAG00062	3614	*Bacteroides salyersiae*	*Bacteroidales*	*1226-1250*
CAG00521	1835	*Prevotella sp. CAG: 1058*	*Bacteroidales*	*1851-1875*
CAG00942	1237	*Prevotella disiens*	*Bacteroidales*	*2301-2325*
CAG00301	3187	*Akkermansia muciniphila CAG:154*	*Akkermansia muciniphila*	*101-125*
CAG00313	2139	*Coraliomargarita sp. CAG:312*	*Coraliomargarita sp.*	*126-150*
CAG00721	1830	*Methanobrevibacter smithii ATCC 35061*	*Methanobrevibacter smithii*	*576-600*
CAG00302	2161	*Rhodospirillales*	*Alphaproteobacteria*	*1551-1575*
CAG00475	1917	*Eggerthellaceae*	*Actinobacteria*	*1776-1800*

Table 1: Bacterial species over-represented in cancer patients likely to be good responders to anti-PD1 or PD-L1 or anti-PD-L2 antibodies and under-represented in cancer patients likely to be resistant to anti-PD1 or PD-L1 or anti-PD-L2 antibodies, as well as corresponding referenced species (for already identified species) and sequences comprised therein. The information concerning the corresponding reference species can be found on the NCBI website.

Bacterial species	Number of co-variant genes	Corresponding referenced species	Annotation	Sequences (SEQ ID Nos)
CAG00931	*1257*		*Oscillibacter*	*901-925*
CAG00270	*2225*		*Oscillibacter*	*926-950*
CAG00702	*1609*	*Bifidobacterium adolescentis L2-32*	*Bifidobacterium adolescentis*	*951-975*
CAG00549	*1788*	*Bifidobacterium longum*	*Bifidobacteria*	*1901-1925*
CAG00720	*1590*	*Anaerotruncus colihominis DSM 17241*	*Anaerotruncus colihominis*	*976-1000*
CAG00381	*2033*	*Clostridium sp. CAG:242*	*Clostridium sp.*	*1001-1025*
CAG00365	*2055*	*Clostridium sp. CAG306*	*Clostridium sp.*	*1651-1675*
CAG01118	*994*	*Unclassified Clostridiales*	*Clostridium sp.*	*2551-2575*
CAG00048_1	*1403*	*Clostridiales*		*1151-1175*
CAG00981	*1189*	*Erysipelotrichaceae*	*Erysipelotrichaeae*	*2376-2400*
CAG00211	*2389*	*Firmicutes bacterium CAG:227*	*Firmicutes*	*1451-1475*
CAG00168	*2534*	*Clostridiales bacterium VE202-14*	*Clostridiales*	*1026-1050*
CAG00353	*2341*	*Eubacterium sp. CAG:252*	*Eubacterium sp.*	*1051-1075*
CAG01018	*1135*	*Bilophila wadsworthia*	*unclassified Bilophila*	*1076-1100*
CAG00052	*3522*	*Parabacteroides goldsteinii*		*1176-1200*
CAG00141	*2649*	*Parabacteroides distasonis*	*Bacteroidales*	*1376-1400*
CAG00492	*2012*	*Faecalibacterium*	*Firmicutes*	*1826-1850*
CAG00259	*2248*	*Unclassified firmicutes*	*Firmicutes*	*1501-1525*
CAG1214	*832*	*Blautia*	*Firmicutes*	*2701-2725*
CAG00175	*2509*	*Bacteroides clarus*	*Bacteroidales*	*1401-1425*
CAG00858	*1400*	*Streptococcus thermophilus*		*2201-2225*

Table 2: Bacterial species under-represented in cancer patients likely to be good responders to anti-PD1 or PD-L1 or anti-PD-L2 antibodies and over-represented in cancer patients likely to be resistant to anti-PD1 or PD-L1 or anti-PD-L2 antibodies, as well as corresponding referenced species (for already identified species) and sequences comprised therein. The information concerning the corresponding reference species can be found on the NCBI website.

According to a particular embodiment of the above method, the relative abundance of at least 10 microorganism species selected from the microorganism species disclosed in Table 1a and Table 2a is assessed in step (i).

Bacterial species	Number of co-variant genes	Corresponding referenced species	Annotation	Sequences (SEQ ID Nos)
CAG00064	3310		*unclassified*	*1-25*
CAG00245	2277		*unclassified*	*51-75*
CAG00604	1714	*Firmicutes bacterium CAG:110*	*Firmicutes*	*401-425*
CAG01090	1026		*Firmicutes*	*776-800*
CAG00965	1210		*Firmicutes*	*751-775*
CAG00621	1695	*Firmicutes bacterium CAG:272*	*Firmicutes*	*426-450*
CAG01245	780		*Firmicutes*	*826-850*
CAG01308	666		*Firmicutes*	*851-875*
CAG00669	1649		*Firmicutes*	*526-550*
CAG00670	1648		*Firmicutes*	*551-575*
CAG00872	1359	*Firmicutes bacterium CAG:240*	*Firmicutes*	*651-675*
CAG00851	1422		*Firmicutes*	*626-650*
CAG00288	2189	*Firmicutes bacterium CAG:95*	*Firmicutes*	*76-100*
CAG00363	2056		*Clostridiales*	*176-200*
CAG00391	2019		*Clostridiales*	*201-225*
CAG00449	1945	*Clostridium sp. CAG:253*	*Clostridiales*	*276-300*
CAG00513	1853		*Clostridiales*	*326-350*
CAG00559	1776		*Clostridiales*	*376-400*
CAG00644	1670	*Clostridium sp. CAG:226*	*Clostridiales*	*476-500*
CAG00811	1469		*Clostridiales*	*601-625*
CAG00907	1299		*Clostridiales*	*726-750*
CAG01350	597		*Clostridiales*	*876-900*
CAG01169	912		*Ruminococcaceae*	*801-825*
CAG00880	1352	*Subdoligranulum sp. CAG:314*	*Subdoligranulum sp.*	*676-700*
CAG00628	1686		*Faecalibacterium*	*451-475*
CAG00558	1777	*Ruminococcus lactaris*	*Ruminococcaceae*	*351-375*
CAG00469	1928		*Eubacterium*	*301-325*
CAG00393	2011		*Eubacterium*	*226-250*
CAG00116	2783		*Bacteroides nordii*	*26-50*
CAG00355	2067	*Bacteroides sp. CAG:661*	*Bacteroides sp.*	*151-175*
CAG00440	1952	*Bacteroides sp. CAG:598*	*Bacteroides sp.*	*251-275*
CAG00646	1668		*Alistipes*	*501-525*

CAG00887	1332	*Alistipes sp. CAG:435*	*Alistipes sp.*	*701-725*
CAG00301	3187	*Akkermansia muciniphila CAG:154*	*Akkermansia muciniphila*	*101-125*
CAG00313	2139	*Coraliomargarita sp. CAG:312*	*Coraliomargarita sp.*	*126-150*
CAG00721	1830	*Methanobrevibacter smithii ATCC 35061*	*Methanobrevibacter smithii*	*576-600*

Table 1a: subgroup of bacterial species over-represented in cancer patients likely to be good responders to anti-PD1 or PD-L1 or anti-PD-L2 antibodies and under-represented in cancer patients likely to be resistant to anti-PD1 or PD-L1 or anti-PD-L2 antibodies, as well as corresponding referenced species (for already identified species) and sequences comprised therein. The information concerning the corresponding reference species can be found on the NCBI website.

Bacterial species	Number of co-variant genes	Corresponding referenced species	Annotation	Sequences (SEQ ID Nos)
CAG00931	*1257*		*Oscillibacter*	*901-925*
CAG00270	*2225*		*Oscillibacter*	*926-950*
CAG00702	*1609*	*Bifidobacterium adolescentis L2-32*	*Bifidobacterium adolescentis*	*951-975*
CAG00720	*1590*	*Anaerotruncus colihominis DSM 17241*	*Anaerotruncus colihominis*	*976-1000*
CAG00381	*2033*	*Clostridium sp. CAG:242*	*Clostridium sp.*	*1001-1025*
CAG00168	*2534*	*Clostridiales bacterium VE202-14*	*Clostridiales*	*1026-1050*
CAG00353	*2341*	*Eubacterium sp. CAG:252*	*Eubacterium sp.*	*1051-1075*
CAG01018	*1135*	*Bilophila wadsworthia*	*unclassified Bilophila*	*1076-1100*

Table 2a: subgroup of bacterial species under-represented in cancer patients likely to be good responders to anti-PD1 or PD-L1 or anti-PD-L2 antibodies and over-represented in cancer patients likely to be resistant to anti-PD1 or PD-L1 or anti-PD-L2 antibodies, as well as corresponding referenced species (for already identified species) and sequences comprised therein. The information concerning the corresponding reference species can be found on the NCBI website.

According to another particular embodiment of the above method, the relative abundance of at least 7, 8, 9 or 10 microorganism species selected from the microorganism species disclosed in Table 1b and Table 2b is assessed in step (i).

Bacterial species	Number of co-variant genes	Corresponding referenced species	Annotation	Sequences (SEQ ID Nos)
CAG00064	3310		*unclassified*	*1-25*
CAG01245	780		*Firmicutes*	*826-850*
CAG01308	666		*Firmicutes*	*851-875*
CAG00391	2019		*Clostridiales*	*201-225*
CAG00559	1776		*Clostridiales*	*376-400*
CAG01169	912		*Ruminococcaceae*	*801-825*
CAG00210	2389	*Ruminococcus bicirculans*	*Ruminococcaceae*	*1426-1450*
CAG00949	1224	*Ruminococcaceae bacterium D16*	*Ruminococcaceae*	*2326-2350*
CAG00558	1777	*Ruminococcus lactaris*	*Ruminococcaceae*	*351-375*
CAG01262	750	*Ruminococcus torques 2*	*Ruminococcaceae*	*2826-2850*
CAG00250	2262	*Ruminococcus sp. CAG:353*	*Ruminococcaceae*	*1476-1500*
CAG00854	2572	*Ruminococcaceae*	*Ruminococcaceae*	*2176-2200*
CAG00363	2056	*Intestinimonas butyriciproducens*	*Intestinimonas butyriciproducens (closely related to Flavonifractor plautii)*	*176-200*
CAG001046	1089	*Intestinimonas butyriciproducens*	*Intestinimonas butyriciproducens (closely related to Flavonifractor plautii)*	*2451-2475*
CAG00469	1928	*Eubacterium sp. CAG:146*	*Eubacterium*	*301-325*
CAG00646	1668		*Alistipes*	*501-525*
CAG00887	1332	*Alistipes sp. CAG:435*	*Alistipes sp.*	*701-725*
CAG00827	1455	*Alistipes sp. CAG:514*	*Alistipes sp.*	*2151-2175*
CAG00301	3187	*Akkermansia muciniphila CAG:154*	*Akkermansia muciniphila*	*101-125*

Table 1b: subgroup of species from Table 1

Bacterial species	Number of co-variant genes	Corresponding referenced species	Annotation	Sequences (SEQ ID Nos)
CAG00702	*1609*	*Bifidobacterium adolescentis L2-32*	*Bifidobacterium adolescentis*	*951-975*
CAG00549	*1788*	*Bifidobacterium longum*	*Bifidobacteria*	*1901-1925*
CAG00981	*1189*	*Erysipelotrichaceae*	*Erysipelotrichaeae*	*2376-2400*
CAG01018	*1135*	*Bilophila wadsworthia*	*unclassified Bilophila*	*1076-1100*
CAG00052	*3522*	*Parabacteroides goldsteinii*		*1176-1200*
CAG00141	*2649*	*Parabacteroides distasonis*	*Bacteroidales*	*1376-1400*

Table 2b: subgroup of species from Table 2

In what precedes, “over-representation” means, for each microorganism species, that this species is present in the sample with a relative abundance that is superior to the predetermined threshold. Of course, “under-

representation" means that the species is present in the sample with a relative abundance that is inferior to the predetermined threshold.

Examples of thresholds that can be used as "predetermined thresholds" in the frame of the invention are disclosed in the experimental part below. Of course, the skilled artisan can adapt or refine these thresholds, depending on the technique used to measure the relative abundance of the microorganisms (for example, quantitative PCR, hybridization on a microarray or pyrosequencing), the specific anti-PD1/PD-L1/PD-L2 antibody, the specific pathology of the patient, the patient's food habits and other possible factors. More generally, the threshold to be considered when performing the above method is predetermined by measuring the relative abundance of the recited microorganisms in a representative cohort of individuals treated by an immune checkpoint blockade therapy, and whose response to this treatment is known.

According to one embodiment, the threshold is calculated to obtain the best predictability for the response (sensitivity and specificity). For example, the threshold is calculated to maximize the Youden index.

According to a particular embodiment of this method, the threshold used for each of the species is the detection limit of a sensitive detection method, (such as MGS anlysis). In such a case, what is assessed is not the "relative abundance" of the species but merely its presence (corresponding to the "over-representation") or absence (corresponding to the "under-representation"). This also applies to the description which follows of further methods according to the invention, where "relative abundance" can be read as "presence or absence", "presence" can be read as "over-representation" and absence can be read as "under-representation".

According to the present invention, a "bacterial species" is a group of bacterial genes from the gut microbiome (i.e., the gene repertoire of the gut microbiota), which abundance level varies in the same proportion among different individual samples. In other words, a bacterial species according to the invention is a cluster of bacterial gene sequences which abundance levels in samples from distinct subjects are statistically linked rather than being randomly distributed.

Most current approaches for analyzing metagenomic data rely on comparisons to reference genomes, but the human gut microbiota diversity extends beyond what is currently covered by reference databases. In the results disclosed herein, the inventors used a method based on binning co-abundant genes across a series of metagenomic samples, that enables comprehensive

discovery of new microorganisms without the need for reference sequences. In what follows, most of the species identified as likely to play a role in the patients' response to therapies based on antibodies against PD1, PD-L1 or PD-L2 are newly-identified species, not yet referenced in public databases. For each of the identified species (both newly-identified and species very close to already referenced species), the present application discloses a set of 25 bacterial genes which are non-redundant sequences and can be used, alone or in combination, as tracer genes to assess the presence and relative abundance to the corresponding species. Of course, once the species are identified, either by the set of non-redundant genes disclosed herein, or later on, by their further identification and/or inclusion into a data base, the skilled in the art can assess their relative abundance by any appropriate means, such as, for example, by measuring the copy number of another non-redundant gene that co-varies with the 25 sequences disclosed in the present application. Hence, the present invention is not limited to the use of the disclosed sequences to measure the relative abundance of the corresponding species.

As shown in Tables 1 and 2, some of the species which have been identified as playing an important part in the response to an anti-PD1/PD-L1/PD-L2 blockade are very close to species that are already referenced and accessible, for example, in the NCBI database. The "corresponding referenced species" indicated in Tables 1 and 2 are, for each species, the closer identified organism (strain of a given species). For each of the indicated references, the mean percentages of alignment and identity between the genes of the CAG identified by the inventors and the referenced genome are above 93% (alignment) and 98% (identity).

According to a particular embodiment of the above method, under-representation of microorganism species disclosed in Table 1 and over-representation of microorganism species disclosed in Table 2 are indicative that the patient is likely to be resistant to the anti-PD1/PD-L1/PD-L2 Ab-based therapy. In such a case, the patient can be proposed an alternative therapy, or a pre-treatment with a probiotic composition as disclosed below, to modify its microbiota and improve his/her chances of responding to the treatment.

While the relative abundance of a minimum of 10 species selected in Tables 1 and 2 are measured when performing the method of the invention, the method can be performed by measuring a higher number of species (for example 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40 species or more).

In a particular embodiment of the above methods, the measured species are chosen to optimize the relevance of the profile. For example, the relative abundances of at least 5 *Firmicutes* species, three *Clostridiales* species, one *Alistipes* species, one *Eubacterium* species, one *Bacteroidales* species and *Methanobrevibacter smithii* are measured in step (i). According to this embodiment, the relative abundance of *Akkermansia muciniphila* can measured in step (i) in place of or in addition to the relative abundance of *Methanobrevibacter smithii*.

According to another particular embodiment, the relative abundances of *Anaerotruncus colihominis* and at least one *Oscillibacter* species are also measured in step (i).

The above method can advantageously be used for any patient suffering from a cancer amenable to PD1 or PD-L1 or PD-L2 blockade. Of course, some cancers that are currently not treated with such treatments can become new applications of anti-PD1/PD-L1/PD-L2 antibodies, and patients suffering from such cancers will then benefit from the present invention. It is to be noted that the present invention is also particularly useful in the frame of clinical trials aimed at determining the efficiency of PD1 or PD-L1 or PD-L2 blockade in new indications, and/or with new molecules. According to a particular embodiment, the invention is performed to assess the responsive/resistant status of a patient who has a cancer selected from the group consisting of lung cancer (such as squamous cell lung cancer but also adenocarcinoma non small cell or small cell lung cancer), renal cell cancer, head and neck tumor, bladder carcinoma, liver cancer, mesothelioma, Merkel-cell carcinoma, esophageal cancer, stomach cancer, triple negative breast cancer, melanoma and thymoma.

The method of the invention is particularly useful for assessing the responsive/resistant status of a patient who has a locally advanced or metastatic cancer, or an operable cancer in a neoadjuvant setting, *i.e.*, in the present case, a tumor that could be surgically excised after PD1 or PD-L1 or PD-L2 blockade, for example to reduce the amount of cycles post-surgery in those patients doomed to fail therapy or to compensate them with anticancer probiotics to continue therapy.

As disclosed in the experimental part below, the method of the invention is useful for patients for whom the anti-PD1/PD-L1/PD-L2 Ab-based therapy is the second or third line therapy. According to another embodiment, the method is performed to assess the responder or resistant status of a patient before administering an anti-PD1/PD-L1/PD-L2 Ab-based therapy to said patient as first line therapy, or for assessing the responder or resistant status of a patient who already received an anti-PD1/PD-L1/PD-L2 Ab-based therapy as first line therapy.

The above method can advantageously be used when the anti-PD1/PD-L1/PD-L2 Ab-based therapy is administered alone, but also when the anti-PD1/PD-L1/PD-L2 Ab-based therapy is administered in combination with anti-CTLA4 Ab or IDO inhibitors or any other immunomodulator.

As already mentioned, the above method can be used before the beginning of a treatment with an anti-PD1/PD-L1/PD-L2 antibody, to avoid administration of such a treatment to a poor responder and/or convert this poor responder into good responder by appropriate pre-treatments, but it can also be used for assessing the responder/resistant status of a patient who already received an anti-PD1/PD-L1/PD-L2 antibody. In such a case, if the patient is identified as being resistant, the treatment can be stopped, or combined to another treatment likely to increase the patient's response. Such treatments are disclosed below.

When performing the above methods, the relative abundance of each microorganism can be assessed by measuring the number of copies of at least one, 2, 5, 10, 15, 20 or at least 25 nucleic acid sequence(s) specific for said microorganism in the sample. Any appropriate technique known by the skilled artisan can be used to measure the number of copies of the recited sequences, such as PCR-based techniques (Q-PCR, QRT-PCR etc.), hybridization (for example using a nucleic microarray), sequencing (for example by NGS) and any other appropriate method known to the person of skills in the art. Of course, any nucleic acid sequence specific for a given microorganism can be chosen to measure the relative abundance of said microorganism. By way of example, the number of copies of any sequence recited in Table 1 and Table 2 can be measured, it being understood that any sequence that co-varies with the disclosed sequences specific for a given species can also be used to

measure the relative abundance of said species. The relative abundance of species very close to (or identical to) bacteria that have already been referenced in databases can be assessed by measuring the copy number of any sequence specific for the corresponding referenced species identified in Tables 1 and 2.

In a first particular embodiment, the number of copies of sequences specific for species listed in Tables 1 and 2 is assessed by PCR-based techniques. The PCR technique used can quantitatively measure starting amounts of DNA, cDNA, or RNA. Examples of PCR-based techniques according to the invention include techniques such as, but not limited to, quantitative PCR (Q-PCR), reverse-transcriptase polymerase chain reaction (RT-PCR), quantitative reverse-transcriptase PCR (QRT-PCR), rolling circle amplification (RCA) or digital PCR. These techniques are well known and easily available and do not need a precise description. In a particular embodiment, the determination of the copy number of the bacterial genes of the invention is performed by quantitative PCR.

The PCR-based techniques are performed with amplification primers designed to be specific for the sequences which are measured. The present invention hence also pertains to a set of primers suitable for performing the above method, *i.e.*, a set of primers comprising primer pairs for amplifying sequences specific for each of the microorganism species to be detected in step (i) of said method (*i.e.*, at least 10 species selected amongst those recited in Tables 1 and 2). Such a set of primers comprises a minimum of 20 primers, but it can comprise more primers, for example 30, 40, 50, 60, 70, 80, 100, 200, 300, 500, 1000, 2000 or more primers. According to a particular embodiment, the set of primers comprises at least one primer pair specifically amplifying part of a sequence selected amongst SEQ ID Nos: 1-1125. Of course, primer sets according to the invention can advantageously comprise 2, 3, 4, 5, 10, 20, 30, 40, 50, 60, 70, 80, 100, 200, 300, 500, 1000 or more pairs of primers each specifically amplifying part of a sequence selected amongst SEQ ID Nos: 1-1125.

In another particular embodiment, the relative abundance of the selected species is assessed in step (i) by the use of a nucleic microarray. A "nucleic microarray" consists of different nucleic acid probes that are attached to a solid support, which can be a microchip, a glass slide or a microsphere-

sized bead. Probes can be nucleic acids such as cDNAs ("cDNA microarray") or oligonucleotides ("oligonucleotide microarray"), and the oligonucleotides may be about 25 to about 60 base pairs or less in length. To determine the copy number of a target nucleic acid sample, this sample is labelled and contacted with the microarray in hybridization conditions so that complexes form between probe sequences attached to the microarray surface and target nucleic acids that are complementary thereto. The presence of labelled hybridized complexes is then detected. Many variants of the microarray hybridization technology are available to the skilled artisan.

A nucleic acid microarray designed to perform the method according to the invention is hence also part of the present invention. Such a nucleic acid microarray comprises nucleic acid probes specific for each of the microorganism species to be detected in step (i) of said method (*i.e.*, at least 10 species selected amongst those recited in Tables 1 and 2). In a specific embodiment, the nucleic acid microarray is an oligonucleotide microarray comprising at least one oligonucleotide specific for at least one sequence selected from SEQ ID NOs: 1-1125. For example, the said microarray comprises at least 45 oligonucleotides, each oligonucleotide being specific for one sequence of a distinct species recited in tables 1 and 2. The microarray of the invention can of course comprise 1125 oligonucleotides specific for each of the sequences of SEQ ID NOs: 1-1125. The microarray according to the invention may further comprise at least one oligonucleotide for detecting at least one gene of at least one control bacterial species. A convenient bacterial species may be e.g. a bacterial species the abundance of which does not vary between individuals having a cancer and healthy individuals. Preferably, the oligonucleotides are about 50 bases in length. Suitable microarray oligonucleotides specific for any gene of SEQ ID NOs: 1-1125 may be designed, based on the genomic sequence of each gene, using any method of microarray oligonucleotide design known in the art. In particular, any available software developed for the design of microarray oligonucleotides may be used, such as, for instance, the OligoArray software, the GoArrays software, the Array Designer software, the Primer3 software, or the Promide software, all known by the skilled in the art.

According to yet another embodiment, determining the number of copies of at least one bacterial gene in a sample obtained from the subject is performed using sequencing. Optionally, DNA is fragmented, for example by

restriction nuclease prior to sequencing. Sequencing is done using any technique known in the state of the art, including sequencing by ligation, pyrosequencing, sequencing-by-synthesis, single-molecule sequencing or next-generation sequencing. Sequencing also includes PCR-Based techniques, such as for example quantitative PCR or emulsion PCR. A number of platforms are available for performing next-generation sequencing (NGS, also called “massive parallel DNA sequencing”), such as, but not limited to the Illumina Genome Analyzer platform, the Roche 454 platform, the ABI SOLiD platform, the Helicos single molecule sequencing platform, real-time sequencing using single polymerase molecules (Eid *et al.*, 2009), Ion Torrent sequencing (WO 2010/008480) and nanopore sequencing (Clarke *et al.*, 2009).

When the skilled person relies on sequencing methods to measure the number of copies of specific genes, bioinformatics tool are necessary to treat the collected information. Indeed, using sequencing, a searched nucleic acid sequence is identified in the global sequencing data by comparison with reference sequences. Hence, alignments are performed (between the sequenced data and the reference sequences) and the skilled artisan choses a threshold of percentage of identity above which a sequence is considered as identical to a reference sequence. For example, in an embodiment, the nucleic acid sequences of the relevant bacterial species are identified in the global sequencing data by comparison with the nucleic acid sequences comprised in the species recited indicated in Tables 1 and 2. This comparison is advantageously based on the level of sequence identity with the sequences SEQ ID NOs: 1 to 1125, or with other nucleic acid sequences comprised in the species or “corresponding referenced species” identified in Tables 1 and 2. Thus, a nucleic acid sequence exhibiting at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity with a nucleic acid sequence amongst SEQ ID NOs: 1 to 1125 is identified as a sequence comprised in one of the bacterial species identified as playing a role in the response or resistance to an anti-PD1/PD-L1/PD-L2 antibody.

The term "sequence identity" herein refers to the identity between two nucleic acids sequences. To determine the percentage of identity of two amino acids sequences, the sequences are aligned for optimal comparison. In this comparison the sequences can be the same length or can be different in length. Several alignments algorithms and softwares (such as, for example, the BLAST software) are available and can be used to assess the percentage of identity between two sequences over a window of comparison, and the skilled

artisan is free to use any appropriate algorithm and method to assess the percentage of identity between sequenced data and reference sequences.

Another aspect of the present invention is a probiotic composition.

According to one embodiment, the composition comprises bacteria selected from the group consisting of *Enterococcus hirae, Akkermansia muciniphila, Alistipes shahii,* other *Alistipes* species and mixtures thereof.

According to one embodiment, the composition is used for treating a cancer, in combination with an anti-PD1/PD-L1/PD-L2 Ab-based therapy. The basis for this therapeutical use is that such a composition induces immunostimulation in a cancer patient. The combined administration of a composition according to the invention and an anti-PD1/PD-L1/PD-L2 Ab-based therapy leads to synergistic effects, so that patients who were or would have been non-responders or poor responders to the anti-PD1/PD-L1/PD-L2 Ab-based therapy become responders. Interestingly, as illustrated in Figure 45 and Example 23 below, the compositions according to the invention also prevent some adverse effects of the anti-PD1/PD-L1/PD-L2 Ab-based therapy.

According to one embodiment, the probiotic composition according to the invention comprises bacteria selected from the group consisting of one or several isolates of *Enterococcus hirae, Akkermansia muciniphila, Alistipes indistinctus,* other *Alistipes* species and mixtures thereof. Another composition comprises at least five bacterial species selected from the group consisting of *Enterococcus hirae, Akkermansia muciniphila, Alistipes indistinctus, Eubacterium* species (such as *Eubacterium. limosum*), *Firmicutes* species (such as *Christensenella minuta, Dielma fastidiosa, Flavonifractor plautii)*, *Bacteroidia* (such as *Bacteroides fragilis*, *Bacteroides salyersae, Barnesiella intestinihominis*), Actinobacteria (such as *Collinsella intestinalis, Collinsella Tanakaei*, *Actinotignum schaalii*) and the archae *Methanobrevibacter smithii*. A particular composition which can be used, according to the invention, to induce immunostimulation in a cancer patient receiving an anti-PD1/PD-L1/PD-L2 Ab-based therapy, is a bacterial composition comprising *Enterococcus hirae* and/or *Akkermansia muciniphila* and/or *Alistipes and/or Christensenella minuta*.

In the present text, the compositions of the invention which comprise bacteria that can be advantageously administered to cancer patients are indifferently designated as “probiotic compositions”, “bacterial compositions”, “oncobax compositions” or merely “compositions” when the context makes clear that these compositions comprise bacteria.

According to a particular embodiment, the composition according to the invention comprises at least two bacterial species selected from the group consisting of *Firmicutes* species, *Clostridiales* species, *Alistipes* species, *Eubacterium* species, *Bacteroidales* species, *Methanobrevibacter smithii, Akkermansia muciniphila* and *Enterococcus hirae*, for example at least two bacterial species selected from the group consisting of *Enterococcus hirae, Akkermansia muciniphila, Alistipes* species, *Eubacterium* species, *Ruminococcacae, Clostridiales* species, *Bacteroidales* species, *Actinobacteria, Coriobacteriales* species and *Methanobrevibacter smithii*. Non-limitative examples of *Enterococococcus hirae* strains which can be used in the probiotic compositions according to the invention are the strains 13144, deposited on November 7, 2013 at the Collection Nationale de Cultures de Microorganismes (CNCM), under the number I-4815, IGR7 deposited on August 31, 2017 at the CNCM under the number I-5224, IGR4 deposited on November 27, 2017 at the CNCM under the number CNCM I-5260 and IGR11 deposited on November 27, 2017 at the CNCM under the number CNCM I-5261. Mixtures of two, three or more *E. hirae* strains (amongst those listed above and others) can also be used in the frame of the present invention.

According to one embodiment, the composition according to the invention comprises at least one or several isolates of *Enterococcus hirae, Akkermansia muciniphila, Alistipes indistinctus,* other *Alistipes* species and mixtures thereof. Another composition comprises at least five bacterial species selected from the group consisting of *Enterococcus hirae, Akkermansia muciniphila, Alistipes indistinctus, Eubacterium* species (such as *Eubacterium. limosum*), *Firmicutes* species (such as *Christensenella minuta, Dielma fastidiosa, Flavonifractor plautii)*, *Bacteroidia* (such as *Bacteroides fragilis*, *Bacteroides salyersae, Barnesiella intestinihominis*), Actinobacteria (such as *Collinsella intestinalis, Collinsella Tanakaei*, *Actinotignum schaalii*) and the archae *Methanobrevibacter smithii*. A particular composition which can be used, according to the invention, to induce immunostimulation in a cancer patient receiving an anti-PD1/PD-L1/PD-L2 Ab-based therapy, is a bacterial composition comprising at best two bacteria of *Enterococcus hirae* and/or *Akkermansia muciniphila* and/or *Alistipes and/or Christensenella minuta*.

According to one embodiment, the composition further comprises bacteria selected from the group consisting of *Bifidobacterium adolescentis, Clostridiales spp., Roseburia, Blautia, Faecalibacterium*, *Ruminococcaceae*, *Flavonifractor plautii* and *Burkholderia cepacia*.

According to a preferred embodiment of the probiotic composition of the invention, said composition comprises *Enterococcus hirae* and *Akkermansia muciniphila.*

According to a preferred embodiment of the probiotic composition of the invention, said composition comprises *Alistipes shahii or other Alistipes spp*. with or without *Enterococcus hirae* and *Akkermansia muciniphila.*

Such a composition can advantageously further comprise *Burkholderia cepacia* and/or *Bacteroides fragilis* and/or *Actinotignum schaalii* and/or *Alistipes indistinctus* and/or *Alistipes onderdonkii*.

According to a particular embodiment, the composition of the invention comprises:

- *Clostridiales* bacteria of the species *Christensenella minuta*; and/or
- *Erisipelotrichia* (*Dielma fastidiosa, Erysipelatoclostridium ramosum*); and/or
- *Alistipes* bacteria of species selected from the group consisting of *Alistipes shahii, Alistipes indistinctus, Alistipes onderdonkii* and *Alistipes finegoldii*; and/or
- *Eubacterium* bacteria of the species *Eubacterium limosum*; and/or
- *Bacteroidales* bacteria of species selected from the group consisting of *Bacteroides fragilis, Bacteroides salyersiae* and *Barnesiella intestinihominis*, especially *Bacteroides salyersiae* and/or *Barnesiella intestinihominis;* and/or
- *Actinobacteria* of the species *Actinotignum schaalii* ; and/or
- *Coriobacteriales bacteria* of the species *Collinsella intestinalis* and/or *Collinsella tanakaei;* and/or
- *Firmicutes such as Flavonifractor plautii;* and/or
- Archae bacteria of the species *Methanobrevibacter smithii.*

Several bacterial consortia which proved particularly efficient for compensating dysbiosis and retoring a response to an anti-PD1/PD-L1/PD-L2 Ab-based therapy are illustrated in the examples below (see at least Fig. 42 and 43). The present invention thus pertains to compositions comprising specific bacterium consortia.

According to a particular embodiment of the present invention, the composition comprises:

(i) *Enterococcus hirae* selected from the group consisting of strain 13144 (CNCM I-4815), strain IGR7 (CNCM I-5224), strain IGR4 (CNCM I-5260), strain IGR11 (CNCM I-5261) and mixtures thereof; and

(ii) *Akkermansia muciniphila,* for example bacteria of the strains p2261 and/or p3415, both deposited at the Collection de souches de l'Unité des Rickettsies (CSUR); and

(iii) *Eubacterium limosum*.

According to another particular embodiment of the present invention, the composition comprises:

(i) *Enterococcus hirae* selected from the group consisting of strain 13144 (CNCM I-4815), strain IGR7 (CNCM I-5224), strain IGR4 (CNCM I-5260), strain IGR11 (CNCM I-5261) and mixtures thereof; and

(ii) *Barnesiella intestinihominis*.

According to yet another particular embodiment of the present invention, the composition comprises:

(i) *Enterococcus hirae* selected from the group consisting of strain 13144 (CNCM I-4815), strain IGR7 (CNCM I-5224), strain IGR4 (CNCM I-5260), strain IGR11 (CNCM I-5261) and mixtures thereof; and

(ii) *Christensenella minuta*.

According to a further particular embodiment of the present invention, the composition comprises:

(i) *Enterococcus hirae* selected from the group consisting of strain 13144 (CNCM I-4815), strain IGR7 (CNCM I-5224), strain IGR4 (CNCM I-5260), strain IGR11 (CNCM I-5261) and mixtures thereof; and

(ii) *Actinotignum schaalii*.

According to the invention, a composition as above-described is advantageously used as a medicament for compensating dysbiosis in a cancer patient. In particular, the composition can be used as an adjuvant to an anti-PD1/PD-L1/PD-L2 Ab-based treatment administered to a cancer patient.

The compositions of the invention are also efficacious as medicaments for decreasing or preventing gut toxicity of an anti-PD1/PD-L1/PD-L2 Ab-based therapy and/or an anti-CTLA-4 Ab-based therapy, for example crypts irregularities, loss of villosities and inflammatory patterns frequently associated with treatments by immune checkpoint blockers.

The above probiotic compositions can advantageously be formulated for oral administration and administered either as food supplements or as functional food. The skilled artisan knows a variety of formulas which can encompass living or killed microorganisms and which can present as food supplements (*e.g.*, pills, tablets and the like) or as functional food such as drinks, fermented yoghurts, *etc*. The compositions according to the present invention can also be formulated as medicaments, in capsules, pills, liquid solution, for example as encapsulated lyophylized bacteria *etc*..

As already mentioned, the above probiotic composition according to the invention can advantageously be administered to a patient in need thereof in combination with an anti-PD1/PD-L1/PD-L2 Ab-based therapy. In such a combination, the probiotic bacterial composition advantageously induces immunostimulation.

As used herein, the term "in combination" refers to the use of more than one agent (*e.g.*, a probiotic composition strain and an anti-PD1/PD-L1/PD-L2 antibody). The use of the term "in combination" does not restrict the order in which therapies are administered to the patient, although it is preferable to administer the probiotic strain prior to or simultaneously with the antineoplastic treatment. For example, the probiotic strain can be administered prior to the anti-PD1/PD-L1/PD-L2 antibody (*e. g.*, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks before), either punctually or several times (for example, each day) before the anti-PD1/PD-L1/PD-L2 Ab-based treatment is administered. A method for treating a cancer patient, comprising administering a composition as above-described, for example a probiotic bacterial composition comprising at least two bacterial species selected from the group consisting of *Firmicutes* species, *Clostridiales* species, *Alistipes* species, *Eubacterium* species, *Bacteroidales* species, *Methanobrevibacter smithii, Akkermansia muciniphila* and *Enterococcus hirae*, prior to administering a drug blocking PD1, PD-L1 or PD-L2 to said patient, is hence also part of the present invention.

The above probiotic composition is particularly useful in the context of anti-PD1 alone or combined with anti-CTLA4 coblockade.

According to a particular aspect, the present invention pertains to a probiotic composition comprising bacteria selected from the group consisting of *Enterococcus hirae, Akkermansia muciniphila, Alistipes spp.* and mixtures thereof, for use for treating a cancer, in combination with an anti-PD1/PD-L1/PD-L2 Ab-based therapy, wherein the probiotic bacterial composition induces immunostimulation in a cancer patient.

The probiotic compositions according to the invention are particularly useful as adjuvant therapy for treating a cancer patient who has been identified as likely to be resistant to an anti-PD1/PD-L1/PD-L2 Ab-based treatment.

According to a particular embodiment, the probiotic composition of the invention is administered to the patient before and during the anti-PD1/PD-

L1/PD-L2 Ab-based therapy, for example every 3 days during the whole anti-PD1/PD-L1/PD-L2 Ab-based therapy, or at least the day before and the day after each intravenous administration of the anti-PD1/PD-L1/PD-L2 antibody (which is typically given twice a month during 1.5 year).

According to another particular embodiment, the patient receives a treatment that will kill or remove at least part of the bacteria present in his/her intestines, before administration of a probiotic composition of the invention. Such a treatment aims at favoring the niching of the "favorable" bacteria present in the probiotic composition. Non-limitative examples of such pre-treatments are administration of polyethylene glycol and/or short duration (typically one to three days) broad spectrum antibiotics (e.g. ampicilline or cephalosporines). A method for treating a cancer patient, comprising administering (i) polyethylene glycol and/or short duration broad spectrum antibiotics, then (ii) a probiotic bacterial composition as above-described, and (iii) a drug blocking PD1, PD-L1 or PD-L2 to said patient, is hence also part of the present invention.

According to another aspect, the compositions according to the invention are used as an immunostimulating therapy for preventing a cancer relapse. A method of preventing cancer relapse in an individual who has been treated for a cancer, for example with a drug blocking PD1, PD-L1 or PD-L2, wherein a composition as above-described is administered to the individual as an immunostimulating treatment, is hence also part of the present invention.

The present invention also pertains to a theranostic method for determining if an individual in need an anti-PD1/PD-L1/PD-L2 Ab-based therapy, whether this individual needs a bacterial compensation before administration of this therapy. Indeed, anti-PD1/PD-L1/PD-L2 Ab-based are heavy, both in terms of possible side effects and in terms of financial cost, and everything should be done to ensure that the treatment will be beneficial to the patient.

The present invention thus pertains to a theranostic method for determining if a cancer patient needs a bacterial compensation before administration of an anti-PD1/PD-L1/PD-L2 Ab-based therapy comprising assessing, in a feces sample from said patient, the presence or absence of *Akkermansia muciniphila*, wherein if *Akkermansia muciniphila* is absent from said feces sample, the patient needs a bacterial compensation with a bacterial composition or a fecal microbial composition as above-described.

Another theranostic method of the invention, for determining if a cancer patient needs a bacterial compensation before administration of an anti-PD1/PD-L1/PD-L2 Ab-based therapy, comprises assessing, in a feces sample from said patient, the presence or absence of *Enterococcus hirae*, wherein if *Enterococcus hirae* is absent from said feces sample, the patient needs a bacterial compensation with a bacterial composition or a fecal microbial composition as above-described.

Another theranostic method of the invention, for determining if a cancer patient needs a bacterial compensation before administration of an anti-PD1/PD-L1/PD-L2 Ab-based therapy, comprises assessing, in a feces sample from said patient, the presence or absence of *Ruminococcus* sp. CAG:353, *Ruminococcus* bacterium LM158, *Ruminococcus torques* 2 and *Ruminococcaceae* bacterium D16, wherein if none of *Ruminococcus* sp. CAG:353, *Ruminococcus* bacterium LM158, *Ruminococcus torques* 2 and *Ruminococcaceae* bacterium D16 is present in said feces sample, the patient needs a bacterial compensation with a bacterial composition or a fecal microbial composition as above-described.

Another theranostic method of the invention, for determining if a cancer patient needs a bacterial compensation before administration of an anti-PD1/PD-L1/PD-L2 Ab-based therapy, comprises assessing, in a feces sample from said patient, the presence or absence of *Alistipes* sp. CAG:435, *Alistipes* sp. CAG:514, *Alistipes indistinctus* CAG328 and *Alistipes* sp. CAG/268, wherein if none of *Alistipes* sp. CAG:435, *Alistipes* sp. CAG:514, *Alistipes indistinctus* CAG328 and *Alistipes* sp. CAG/268 is present in said feces sample, the patient needs a bacterial compensation with a bacterial composition or a fecal microbial composition as above-described.

Another theranostic method of the invention, for determining if a cancer patient needs a bacterial compensation before administration of an anti-PD1/PD-L1/PD-L2 Ab-based therapy, comprises assessing, in a feces sample from said patient, the presence or absence of *Bacteroides xylanosolvens CAG945, Bacteroides ovatus CAG1165, Prevotella CAG :255, CAG163, Ruminococcus bromii CAG611, Roseburia intestinalis CAG291* and *Eubacterium CAG :38, CAG 629*, wherein if *Bacteroides xylanosolvens CAG945, Bacteroides uniformis CAG159, Bacteroides ovatus CAG1165, Prevotella CAG :255, CAG163, Ruminococcus bromii CAG611, Roseburia intestinalis CAG291* or *Eubacterium CAG :38, CAG 629* are present in said

feces sample, the patient needs a bacterial compensation with a bacterial composition or a fecal microbial composition as above-described.

Another theranostic method of the invention, for determining if a cancer patient needs a bacterial compensation before administration of an anti-PD1/PD-L1/PD-L2 Ab-based therapy, comprises assessing, in a feces sample from said patient, the presence or absence of short chain fatty acid producing Clostridiaceae selected from the group consisting of *Firmicutes bacterium, Eubacterium, Blautia* and *Roseburia*, wherein the absence of *Firmicutes bacterium, Eubacterium, Blautia* and *Roseburia* from said feces sample indicates that the patient needs a bacterial compensation with *Enterococcus hirae* and/or *Akkermansia muciniphila* or with another bacterial composition as above-described, or with a fecal microbial composition as above-described.

Another theranostic method of the invention, for determining if a cancer patient needs a bacterial compensation before administration of an anti-PD1/PD-L1/PD-L2 Ab-based therapy, comprises assessing, in a feces sample from said patient, the presence or absence of *Dielma fastidiosa*, wherein the absence of *Dielma fastidiosa* from said feces sample indicates that the patient needs a bacterial compensation with *Enterococcus hirae* and/or *Akkermansia muciniphila* or with another bacterial composition as above-described, or with a fecal microbial composition as above-described.

According to another of its aspects, illustrated in the experimental part below, the present invention pertains to an *in vitro* method for assessing whether a cancer patient has a dysbiosis associated with cancer, comprising assessing, in a feces sample from said patient, the presence of at least 10, 11, 12, 13, 14, 15, 16, 17, 18 or 19 microorganism species selected from the group consisting of *Faecalibacterium* CAG297, *Blautia* CAG179, *Roseburia* CAG55, *Haemophila parainfluenzae* CAG1056, *Clostridiales* CAG1132, *Bifidobacterium adolescentis*, *Firmicutes* CAG1308, *Firmicutes bacterium* CAG713, *Bifidobacterium dentium*, *Enterococus faecalis*, *Subdoligranulum* CAG140, *Lachnospiricaeae bacterium* CAG14, *Clostridium innocuum* CAG36, *Ruminococcus torques 1*, *Hungatella hathewayi 1* CAG25, *E.coli* CAG 11, *E.coli* CAG 371, *Clostridiales* CAG533 and *Tyzzerella nexilis* CAG311, wherein:

- the presence of microorganism species selected from the group consisting of *Bifidobacterium dentium*, *Enterococus faecalis*, *Subdoligranulum* CAG140, *Lachnospiricaeae bacterium* CAG14, *Clostridium innocuum* CAG36, *Ruminococcus torques 1*, *Hungatella hathewayi 1* CAG25, *E.coli*

CAG 11, *E.coli* CAG 371, *Clostridiales* CAG533 and *Tyzzerella nexilis* CAG311 and

- the absence of microorganism species selected from the group consisting of *Faecalibacterium* CAG297, *Blautia* CAG179, *Roseburia* CAG55, *Haemophila parainfluenzae* CAG1056, *Clostridiales* CAG1132, *Bifidobacterium adolescentis*, *Firmicutes* CAG1308 and *Firmicutes bacterium* CAG713

are indicative that the patient has a dysbiosis associated with cancer.

The present invention also pertains to an *in vitro* method for predicting relapse in a patient who is treated or who has been treated for a cancer, comprising assessing, in feces samples from said patient obtained at different time-points, the presence of at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 or 19 microorganism species selected from the group consisting of *Faecalibacterium* CAG297, *Blautia* CAG179, *Roseburia* CAG55, *Haemophila parainfluenzae* CAG1056, *Clostridiales* CAG1132, *Bifidobacterium adolescentis*, *Firmicutes* CAG1308, *Firmicutes bacterium* CAG713, *Bifidobacterium dentium*, *Enterococus faecalis*, *Subdoligranulum* CAG140, *Lachnospiricaeae bacterium* CAG14, *Clostridium innocuum* CAG36, *Ruminococcus torques 1*, *Hungatella hathewayi 1* CAG25, *E.coli* CAG 11, *E.coli* CAG 371, *Clostridiales* CAG533 and *Tyzzerella nexilis* CAG311, wherein:

- an increase of microorganism species selected from the group consisting of *Bifidobacterium dentium*, *Enterococus faecalis*, *Subdoligranulum* CAG140, *Lachnospiricaeae bacterium* CAG14, *Clostridium innocuum* CAG36, *Ruminococcus torques 1*, *Hungatella hathewayi 1* CAG25, *E.coli* CAG 11, *E.coli* CAG 371, *Clostridiales* CAG533 and *Tyzzerella nexilis* CAG311 and
- a decrease of microorganism species selected from the group consisting of *Faecalibacterium* CAG297, *Blautia* CAG179, *Roseburia* CAG55, *Haemophila parainfluenzae* CAG1056, *Clostridiales* CAG1132, *Bifidobacterium adolescentis*, *Firmicutes* CAG1308 and *Firmicutes bacterium* CAG713

are indicative that the patient is likely to relapse.

The present invention also pertains to methods for *ex vivo* determining whether a cancer patient is likely to benefit from a treatment with an anti-PD1/PD-L1/PD-L2 Ab-based therapy, based on the analysis of memory T

cells of said patient. According to one of these methods, the presence of memory Th1 or Tc1 cells towards *Burkholderia cepacia, Enterococcus hirae* and/or *Bacteroides fragilis* in a blood sample from said patient is assessed, wherein the presence of memory Th1 or Tc1 cells towards *Burkholderia cepacia* and *Enterococcus hirae* indicates that the patient is likely to be a good responder to said treatment, and the presence of memory Th1 cells towards *Bacteroides fragilis* indicates that the patient is likely to be a poor responder.

Another method according to the invention for *ex vivo* determining whether a cancer patient is likely to benefit from a treatment with an anti-PD1/PD-L1/PD-L2 Ab-based therapy, illustrated in the experimental part below, comprises assessing the presence of memory Th1 or Tr1 cells towards *Akkermansia muciniphila* in a blood sample from said patient, wherein the presence of memory CD4+ Th1 or CD8+ Tc1 (IFNg producing) or CD4+ Tr1 cells (IL-10 producing) towards *Akkermansia muciniphila* indicates that the patient is likely to be a good responder to said treatment, and the presence of only memory Tr1 cells towards *Akkermansia muciniphila* indicates that the patient is likely to be a poor responder.

The present invention also provides means to follow-up the response of a patient treated with an anti-PD1/PD-L1/PD-L2 Ab-based therapy. The present invention thus pertains to a method for assessing if a cancer patient who is treated with an anti-PD1/PD-L1/PD-L2 Ab-based therapy is a good responder to said treatment, comprising the following steps:

(i) assessing, in a feces sample from said patient obtained at the beginning of the treatment, the presence or the relative abundance of at least 3, 4, 5, 6, 7, 8, 9 or 10 microorganism species selected from the group consisting of *Bacteroides xylanosolvens CAG945, Bacteroides uniformis CAG159, Bacteroides ovatus CAG1165, Prevotella CAG :255, CAG163, Ruminococcus bromii CAG611, Roseburia intestinalis CAG291, Eubacterium CAG :38, CAG 629, Ruminococaeae CAG1003, Flavonifractor plautii CAG 439* and *Firmicutes bacterium CAG : 124, CAG 629*;

(ii) assessing, in a feces sample from said patient obtained after three or four administrations of the anti-PD1/PD-L1/PD-L2 Ab, the presence or the relative abundance of the same species as in step (i),

wherein:
- a decrease or complete loss of *Bacteroides xylanosolvens CAG945, Bacteroides uniformis CAG159, Bacteroides ovatus CAG1165, Prevotella CAG :255, CAG163, Ruminococcus bromii CAG611, Roseburia intestinalis CAG291* and *Eubacterium CAG :38, CAG 629*
- and/or in increase or gain of *Ruminococaeae CAG1003, Flavonifractor plautii CAG 439* and *Firmicutes bacterium CAG : 124, CAG 629*
indicate that the patient is a good responder to the treatment.

In the probiotic compositions according to the invention, the probiotic bacteria are preferentially alive. However, compositions comprising dead bacteria, such as autoclaved, pasteurized, irradiated or fragmented bacteria, are also part of the present invention, as well as compositions only comprising certain bacterial antigens. Indeed, it has been shown that isolated bacterial components can exert the same effects as live bacteria, or even enhanced effects (Plovier *et al*., 2016). In the above compositions, live bacteria can hence be replaced by suitable bacterial products for triggering innate or acquired immune responses that favor the elimination of tumor cells from the organism.

According to another aspect, the present invention thus pertains to an immunogenic composition comprising fragments of bacteria selected from the group consisting of *Firmicutes* species, *Clostridiales* species, *Alistipes* species, *Eubacterium* species, *Bacteroidales* species, *Methanobrevibacter smithii, Akkermansia muciniphila* and *Enterococcus hirae* and mixtures thereof, for use as an adjuvant to an anti-PD1/PD-L1/PD-L2 Ab-based therapy administered to a cancer patient. According to a preferred embodiment, the immunogenic composition comprises fragments of *Enterococcus hirae (its TLR2 or a NOD2 agonists for instance)*, for example fragments of the strain CNCM I-4815, together with fragments of *Akkermansia muciniphila*. An immunogenic composition as above-described can further comprise fragments of *Bacteroides fragilis* and/or *Burkholderia cepacia.*

In what precedes, the term "fragment" may refer to cellular components, metabolites, secreted molecules and compounds resulting from the metabolism of the recited bacteria. Fragments may be obtained, for example, by recovering the supernatant of a culture of one or several bacterial species or by extracting cell components or cell fractions, metabolites or secreted compounds from such a culture. The term “fragment” may also refer to

a degradation product. A fragment may correspond to a component in the isolated form or to any mixture of one or more components derived from the considered bacterial species. Non-limitative examples of bacterial components that can advantageously be ingredients of the immunogenic compositions as above-described include Amuc_1100, a specific protein isolated from the outer membrane of *Akkermansia muciniphila* (Plovier *et al.*, supra), capsular polysaccharides A (PSA), which are required by *B. fragilis* to occupy a mucosal niche in the colon, zwitterionic polysaccharide (ZPS) repeated motifs from *Bacteroides* species (for example from *B. fragilis*) and lysine-aspartic acid (KD) peptides with >15 repetitive units.

The immunogenic compositions according to the invention are preferably formulated for intradermal, subcutaneous, intravenous or intramuscular or oral administration. They can advantageously be administered before, at the same time and/or after administration of an anti-PD1/PD-L1/PD-L2 Ab-based therapy, in order to induce an immune response which will have an adjuvant effect to the treatment.

According to another aspect of the present invention, an individual in need of a treatment with an anti-PD1/PD-L1/PD-L2 Ab-based therapy is treated by fecal microbiota transplantation (FMT), using to this aim fecal microbiota from healthy individual(s), and/or fecal microbiota from one or several individual(s) treated with an anti-PD1/PD-L1/PD-L2 Ab-based therapy and who proved to respond to this therapy, and/or fecal microbiota from one or several individual(s) exhibiting a gut microbiota profile that identifies him/her/them as likely to respond to the envisioned treatment or from a responding patient. The theranostic method of the present invention can of course be used to select appropriate donors. This can be done for any patient, but of course such an FMT is particularly useful for an individual who has been identified as likely to be a poor responder to a treatment with said anti-PD1/PD-L1/PD-L2 Ab-based therapy.

The present invention hence also pertains to a fecal microbial composition, for use for treating a cancer, in combination with an anti-PD1/PD-L1/PD-L2 Ab-based therapy. As mentioned above, the fecal microbial composition is preferably obtained (directly or indirectly) from a stool sample from (a) healthy individual(s) or (a) responder(s) to a treatment with an anti-PD1/PD-L1/PD-L2 Ab-based therapy, or at least from an individual exhibiting a gut microbiota profile that identifies him/her as likely to respond to the

envisioned treatment. The fact that the fecal microbial composition can be obtained indirectly from a healthy individual's stool sample means that banks of fecal microbial material may be created, with possible mixes of stool samples, and possible creation of "standard healthy fecal microbial compositions", possibly adapted to certain conditions requiring FMT (a fecal microbial composition for treating a *Clostridium* infection may be different from a fecal microbial composition for use in a cancer context) and/or to other characteristics of patients (age, ethnic origin, food regimen *etc.*). Several ways of conditioning fecal microbial material and conducting FMT have been described and are currently developed, and the skilled artisan is free to choose appropriate techniques for preparing the fecal microbial composition according to the invention, which can be freshly-prepared liquid, freeze-dried material or any other conditioning.

According to a particular embodiment, the fecal microbial composition of the invention comprises at least 10 bacterial species selected from the group consisting of at *Firmicutes* species, *Clostridiales* species, *Alistipes* species, *Eubacterium* species, *Bacteroidales* species, *Methanobrevibacter smithii, Akkermansia muciniphila* and *Enterococcus hirae* and, more preferably, at least 5 different *Firmicutes* species, 3 different *Clostridiales* species, one *Alistipes* species, one *Eubacterium* species, one *Bacteroidales* species, *Methanobrevibacter smithii, Akkermansia muciniphila* and *Enterococcus hirae.*

In a particular embodiment, the composition allows the niching of at least 10 bacterial species selected from the group consisting of at *Firmicutes* species, *Clostridiales* species, *Alistipes* species, *Eubacterium* species, *Bacteroidales* species, *Methanobrevibacter smithii, Akkermansia muciniphila* and *Enterococcus hirae* upon transplant into a germ-free animal used as a MCA205 sarcoma bearing animal model to validate the composition and its antitumor efficacy.

According to another embodiment, the fecal microbiota composition allows the expansion of at least 10 bacterial species selected from the group consisting of at *Firmicutes* species, *Clostridiales* species, *Alistipes* species, *Eubacterium* species, *Bacteroidales* species, *Methanobrevibacter smithii, Akkermansia muciniphila* and *Enterococcus hirae* upon transplant into a germ-free tumor-bearing host after treatment of said host with an anti-PD1/PD-L1/PD-L2 antibody. In what precedes, the host can be a cancer patient having received a pre-treatment for eliminating germs from his/her intestines, such as

those described above, but it can also be an animal used as a preclinical model suitable for screening the fecal microbial material to select appropriate fecal microbiota compositions.

According to another embodiment, the fecal microbiota composition has been enriched with a bacterial composition as above-described.

A fecal microbial composition of the invention is advantageously used for allogeneic healthy fecal microbial transplantation in a cancer patient who has been identified as likely to be resistant to the anti-PD1/PD-L1/PD-L2 Ab-based therapy by the theranostic method described above.

FMT with a composition of the invention is preferably performed before the beginning of the treatment, for example a few days before, but it can also be performed in the course of said treatment.

As described in the experimental part below (Example 8), the inventors also showed that a Th1 or Tc1 response towards certain bacteria is indicative that a patient treated with an anti-PD1 antibody is a good responder to the treatment. Indeed, patients who developed a Th1 or Tc1 immune response towards *Enterococcus hirae* or *Akkermansia muciniphila* or *Burkholderia cepacia* after the 2-4 administrations of anti-PD1 Abs have a long term benefit to PD1 blockade a memory, contrary to those who developed a Th1 or Tc1 immune response towards *Bacteroides fragilis*. Accordingly, the present invention also pertains to a method for *ex vivo* determining whether a cancer patient is likely to benefit from a treatment with an anti-PD1/PD-L1/PD-L2 Ab-based therapy, comprising assessing the presence of memory Th1 or Tc1 cells towards *Enterococcus hirae* and/or *Burkholderia cepacia* and/or *Bacteroides fragilis* in a blood sample from said patient, wherein the presence of such memory Th1 or Tc1 cells indicates that the patient is likely to be a good responder to said treatment, and the presence of memory Th1 or Tc1 cells towards *Bacteroides fragilis* indicates that the patient is likely to be a poor responder.

This method can be performed with a blood sample obtained before the beginning of any treatment with an anti-PD1/PD-L1/PD-L2 Ab-based drug. However, the sensitivity of the test may be insufficient to provide any clear result. This is not a problem anymore after one or two administrations of said drug, which is another moment when this method can be performed.

One particularly advantageous aspect of this method is that it can be performed using a blood sample. The Th1 or Tc1 response of the patient can be assessed at different times during the treatment and lead to a decision to stop the treatment or to adjuvant it with FMT or administration of a probiotic or immunogenic composition as above described if the patient is or becomes a poor responder. Of course, this method can be done for patients having any kind of cancers, including lung cancer, renal cell cancer, head and neck tumor, bladder carcinoma, liver cancer, mesothelioma, Merkel-cell carcinoma, esophageal cancer, stomach cancer, melanoma and thymoma. The results of this method are particularly relevant for patients having an advanced non-small cell lung cancer.

Other characteristics of the invention will also become apparent in the course of the description which follows of the biological assays which have been performed in the framework of the invention and which provide it with the required experimental support, without limiting its scope.

EXAMPLES

Materials and Methods

In the absence of any indications to the contrary, Examples 1 to 8 were performed using the following materials and methods. Additional materials and methods are described in the examples, when necessary.

Dataset

127 samples were sequenced, corresponding to 3 distinct cohorts designed to test Nivolumab in three different cancers with several timepoints: before (V1) or with ongoing treatment (V2 - V4).

- 54 samples from 28 patients with lung cancer
- 16 samples / patients with kidney cancer (Nivoren cohort)
- 4 samples / patients with bladder cancer

Patients bearing either a lung or a kidney or a bladder cancer (first tables 3-5) were all advanced or non operable or metastatic patients enrolled in a PD1 or PD-L1 Ab-based monotherapy after progression with conventional treatments in second or third line. They were enrolled at Gustave Roussy in the

context of the EMA approval of the drug and according to ethical guidelines allowing harvesting of stools at different time points of PD1 blockade to analyze the MG composition. The endpoint of this ancillary study was to establish correlates between feces composition (at species and genes levels) and clinical response to PD1 blockade (response rates and progression-free survival (PFS)).

Characteristics-Lung cancer	**n=28**
Median age - yr	65 (48-76)
Sex - no.(%) Male Female	 16 (57) 12 (43)
Histology - no.(%) Adenocarcinoma Squamous cell carcinoma Other	 21 (75) 3 (11) 4 (14)
Clinical Stage - no.(%) II IV	 1 (4) 27 (96)
Metastasis - no.(%) Brain Liver Bone	 14 (50) 7 (25) 10 (36)
Mutations - no.(%) EGFR ALK KRAS	 3 (11) 1 (4) 7 (25)
Clinical response - no.(%) Complete response Partial response Stable disease Progression	 0 (0) 4 (14) 5 (18) 19 (68)
Survival outcome- no.(%) Dead Alive	 8 (29) 20 (71)

Table 3: Patient characteristics in the lung cancer cohort (batch 1)

Characteristics-Renal cell cancer	**n=16**
Median age - yr	60 (46-77)
Sex - no.(%) Male Female	 13 (81) 3 (19)
Histology - no.(%) Clear cell renal cell carcinoma Non-clear cell renal cell carcinoma	 15 (94) 1 (6)
Fuhrmann score - no.(%) II III IV N/A	 5 (31) 5 (31) 1 (6) 5 (31)
Clinical Stage - no.(%) IV	 16 (100)
Median tumor burden	82 (30-197)
IMDC risk group - no.(%) Favorable Intermediate Poor	 3 (19) 8 (50 5 (31)
Karnofsky performance status - no.(%) ≤70 ≤80 ≤90	 6 (38) 5 (31) 5 (31)
Clinical response - no.(%) Complete response Partial response Stable disease Progression	 0 (0) 3 (19) 7 (44) 6 (38)

Table 4: Patient characteristics in the renal cell cancer cohort (batch 1)

Characteristics-Bladder cancer	**n=4**
Median age - yr	52 (49-62)
Sex - no.(%) Male Female	 3 (75) 1 (25)
Histology - no.(%) Transitional Urothelial carcinoma	 1 (25) 3 (75)
Clinical Stage - no.(%) IV	 4 (100)
Median tumor burden	42 (32-68)
Karnofsky performance status - no.(%) ≤70 ≤80 ≤90	 0 1 (25) 3 (75)
Clinical response - no.(%) Complete response Partial response Stable disease Progression	 2 (50) 0 1 (25) 1 (25)
Survival outcome - no.(%) Dead Alive	 1 (25) 3 (75)

Table 5: Patient characteristics in the bladder cancer cohort (batch 1)

In other larger retrospective analyses (featuring below, tables 6 and 8) of the same trials encompassing at least >60 patients, in which feces were not collected, we analyzed the impact of antibiotics use on clinical outcome.

Analysis pipeline and methodology

Gut microbiota analysis was performed using the quantitative metagenomics pipeline developed at MetaGenoPolis (MGP). This approach allows the analysis of the microbiota at the gene and species levels.

Total DNA has been extracted from the 127 stool samples (SAMBO platform) and subjected to shotgun sequencing using Ion Proton sequencer to reach > 20 million short DNA sequence reads (MetaQuant platform). High quality reads were selected and cleaned to eliminate possible contaminants as human reads. The HQ clean reads were then mapped (shared procedure) and counted using the MetaHIT hs_9.9M genes catalogue (Li *et al.*, 2014) using the METEOR Studio in house pipeline using a two steps procedure: first using uniquely mapping reads, then attributing shared reads (mapping different genes from the catalogue) according to their mapping ratio using unique reads. Mapping was performed using a > 95% identity threshold to account gene variability and the no redundant nature of the catalogue.

After a downsizing step (to correct for the different sequencing depth) and normalization (RPKM), a gene frequency profile matrix was obtained which is used as the starting point to perform the analysis using MetaOMineR, a suite of R packages developped at MGP, dedicated to the analysis of large quantitative metagenomics datasets.

MGS

The hs_9.9M gene catalogue has been clustered into 1438 MGS (MetaGenomic Species, groups of >500 genes that covary in abundance among hundreds samples and thus belong to the same microbial species). The taxonomical annotation of the MGS was performed using the homology of its genes with previously sequenced organisms (using blastN against nt and wgs databanks).

MGS signal among samples was calculated as the mean signal of 50 marker genes.

A MGS frequency profile matrix was constructed using the MGS signals and after normalization (sum of the MGS frequency of a sample = 1).

MGS barcode: MGS occurrence and abundance within samples is visualized using "barcodes", heatmap of a frequency abundance table of 50 marker genes with samples in columns and genes in rows. A heat color code is used (white for 0, lightblue < blue < green < yellow < orange < red for

increasing abundance, each color change corresponding to a 4-fold abundance change). In these barcodes, MGS appear as vertical lines (co-abundant genes in the sample) colored according to gene abundance.

Richness

The richness of the samples can be evaluated at the gene or MGS level.

GC: gene count, number of genes seen in a sample.
MGS count: number of MGS seen in a sample

Similarity

Similarity between samples was evaluated at the gene and MGS levels using spearman correlation.

Microbiota analysis

Microbiota analysis was performed at the MGS level.

First, we analysed the microbiota of the different cohorts of cancer patients compared to that of healthy "controls".

Second, exploratory microbiota analyses were performed according to several hot questions.

Contrasted or correlated MGS were searched according to sample classes or clinical parameters using Wilcoxon test or Spearman correlation and the MGS mean signal, and selected according to p-value.

Statistical analysis

Determination of bacterial cut-off: Two-sided p-values were calculated from the Wilcoxon rank-sum test. Biomarker effectiveness was determined from the area under the receiver operating characteristic curve (AUC) that describes the relationship between the sensitivity and the complement of the specificity for each possible value taken by the biomarker as a discrimination threshold. Optimal cut-off corresponds to the most effective biomarker discrimination threshold. It corresponds to the value that maximises the Youden index as defined as the sum of the sensitivity and specificity for each possible value of the biomarker. 95% confidence intervals for the AUC and the optimal cut-off were determined by bootstrapping (B=1999).

Example 1: The efficacy of PD1 blockade is significantly decreased by oral antibiotics in renal cell carcinoma and urothelial cancer patients

First, a cohort of 70 metastatic RCC patients enrolled in the phase II NIVOREN protocol in second or third line therapy with anti-PD1 or anti-PD-L1 Ab were retrospectively analyzed for the effects of oral administration antibiotics (ATB, at least 7 days of β-lactamines) or proton pump inhibitors (PPI) taken 60 to 30 days prior to enrollment in NIVOREN. 14 and 21 patients out of 70 received β-lactamines and PPI respectively. In univariate analysis of all clinical parameters associated with accelerated time to progression (PFS), Karnofsky performance status (KPS) and ATB uptake were the two significant variables retained in the model, predicting shorter PFS. In multivariate Cox regression analysis, ATB (and to a lesser extent PPI) administration remained significant factors associated with reduced benefit from PD1 blockade (reduced PFS, $p<0.01$ and $p<0.03$ respectively) (Fig. 1, Tables 6, 7).

Characteristics-Renal cell cancer	**n=70**
Age-yr-no.(%)	
<60	32 (46)
≥60	38 (54)
Sex-no.(%)	
Male	48 (69)
Female	22 (32)
Nephrectomy-no. (%)	
Yes	63 (90)
No	7 (10)
Tumour Histology-no. (%)	
Clear cell	68 (97)
Non-clear cell	2 (3)
Fuhrmann Grade-no. (%)	
I	4 (8)
II	14 (29)
III	22 (46)
IV	8 (17)
N/A	22
Median Tumor Burden-median (range)	84 (48-271)

Karnofsky performance status-no.(%)	
≤70	10 (14)
≤80	23 (33)
≤90	37 (53)
IMDC Risk Group-no.(%)	
Good	15 (21)
Intermediate	41 (59)
Poor	14 (20)
ATB-no. (%)	
No	56 (80)
Yes	14 (20)

Table 6: Patient characteristics in the mRCC cohort

			Univariate		Multivariate	
Parameter	Levels	Group	HR [95 %CI]	LRT	HR [95 %CI].adj	LRT.adj
ATB	NoATB	56/29	1	7.34, p<0.0067	1	6.07, p<0.0138
	ATB	14/11	2.89 [1.4;5.99], p<0.0067		2.89 [1.25;6.66],p<0.0138	
AgeDiagCl	(25,60]	32/18	1	0.24, p<0.6271		0.20, p<0.6535
	(60,85]	38/22	0.86 [0.46;1.6], p<0.6271		0.85 [0.41;1.77],p<0.6535	
Gender	F	22/15	1	0, p<0.9663		0.00, p<0.9661
	M	48/25	1.01 [0.53;1.93], p<0.9663		1.02 [0.49;2.12],p<0.9661	
KPSBis	70	10/8	1	8.99, p<0.0111		7.64, p<0.0219
	80	23/11	0.27 [0.1;0.68], p<0.0071		0.20 [0.06;0.63],p<0.0059	
	90	37/21	0.25 [0.11;0.59], p<0.0032		0.21 [0.06;0.73],p<0.0191	
IMDC	Poor	14/10	1	1.85, p<0.3961		0.65, p<0.7219
	Inter	41/22	0.59 [0.28;1.26], p<0.1778		1.53 [0.47;4.97],p<0.4612	
	Good	15/8	0.73 [0.29;1.88], p<0.5105		1.75 [0.41;7.51],p<0.4386	
TBCl	Lo	23/10	1	4.64, p<0.0981		1.11, p<0.5738
	Mi	22/11	1.04 [0.44;2.47], p<0.9237		1.09 [0.42;2.82],p<0.8483	
	Hi	25/19	2.03 [0.94;4.38], p<0.0612		1.58 [0.62;4.04],p<0.3284	
PPI	NoPPI	48/27	1	2.12, p<0.1454		4.24, p<0.0396
	PPI	22/13	1.66 [0.85;3.27], p<0.1454		2.20 [1.04;4.66],p<0.0396	

Table 7: Multivariate analysis of clinical parameters affecting progression free survival in 70 RCC from NIVOREN protocol. ATB: uptake of antibiotics. KPS: Karnofsky performance status. IMDC: International metastatic renal cell carcinoma database consortium-prognosis index, TBCI: Tumor burden classification (lo:low, Mi: Middle, Hi-High) PPI: uptake of a proton pump inhibitor.

Secondly, a similar analysis was performed in patients with urothelial carcinoma (UC) treated with PD-L1 inhibitors (anti-PD-L1 Ab). We conducted a retrospective study of UC patients treated at Gustave Roussy with PD-L1 inhibitors and available data on ATB. ATB (+)/(-) group were defined as patients treated or not with ATB (2 months before the first injection and thereafter up to 1 month). Progression-Free survival (PFS), Response Rate

(RR) and Overall Survival (OS) were compared between ATB (+) and ATB (-). Statistical analyses were performed using the Kaplan-Meier method and Cox regression adjusted for risk factors. 42 patients were included in this study, 12 patients (29%) received ATB (beta-lactamases and fluoroquinolones most frequently). ATB (+) group had decrease PFS when compared to ATB (-) group (1.8 *vs*. 4.3 months, p<0.04). This statistical association was maintained after multivariate analysis adjusted for age, gender, Karnofsky Performance Status, hemoglobin and presence of liver metastases. In ATB (+) group compared to ATB (-) group, the RR was lower (4 pts (33%) *vs* 21 pts (70%)) (p<0.03). After a median follow-up of 15 months, there was a negative trend on the OS driven by ATB, but the median OS was still unreached in the ATB(-) group. (Table 8, 8a and Figure 2).

Characteristics - Bladder cancer	**n=42**
Age-yr no.(%)	
<65	21 (50)
≥65	21 (50)
Sex-no.(%)	
Male	30 (71)
Female	12 (29)
Histology-no. (%)	
Urothelial carcinoma	42 (100)
Median Tumor Burden-median (range)	54 (15-157)
Karnofsky performance status-no.(%)	
≤70	2 (5)
≤80	14 (33)
≤90	26 (62)
Hemoglobin-no. (%)	
>10 mg/dL	32 (76)
< 10 mg/dL	10 (24)
Liver metastasis-no. (%)	
No	26 (62)
Yes	16 (38)
ATB-no. (%)	
No	30 (71)
Yes	12 (29)

Table 8: Patients' characteristics in the urothelial cancer cohort.

	Multivariate p-value
ATB	0.026*
KPS	0.046*
Hb	0.565
Liver Met	0.765
Sex	0.138

Table 8a: Multivariate analysis of known clinical risk factors to determine the influence of ATB on PFS in 42 patients with urothelial cancer. ATB: uptake of antibiotics. KPS: Karnofsky performance status. Hb: hemoglobin

In conclusion, these findings indicate that ATB hamper the clinical benefit expected from anti-PD1 or anti-PD-L1 Abs, suggesting that an intact intestinal microbiota might be required for the bioactivity of nivolumab or atezolizumab. This data prompted us to investigate potential deviations of the gut microbiome in cancer patients (versus normal individuals) and their consequences on prognosis and responses to chemotherapy or ICB.

Additional data regarding the impact of ATB uptake on the bioactivity and clinical effects of anti-PD1 or anti-PD-L1 Abs are disclosed in Example 9 below (Figure 20-21).

Example 2: Cancer affects the richness of the intestinal microbiota

The quality control of the reads was satisfactory and indicated an homogeneity among all cancer patients. Interestingly, a contamination with human reads was observed in cancer patients that progressed post-nivolumab (not shown), suggesting that intestinal apoptosis might precede or be concomitant to dissemination of tumor cells. Similar richness (GC and MGS count) was observed whatever the cohort or the outcome (not shown).

Comparisons of cancer patients with "healthy controls" (selected among the samples sequenced using Ion Torrent with >= 13M mapped reads onto the hs_9.9M gene catalogue: 85 samples) revealed that there was only a tendency for lower richness in cancer patients compared with healthy controls both in gene or MGS level (not shown).

Despite the lack of significance of richness contrast between cancer and healthy (H) controls, a deep loss of richness appears in ~ half of

cancer patients when performing a gene (but not MGS) based-hierarchical clustering. Indeed, 54% (68/127) cancer (C) samples and 9% (8/85) healthy (H) samples belong to the "low" group (chi.test p.value = 1.4 e-10), suggesting that genes relevant for functions outside species taxonomy are separating cancer bearers from healthy individuals (not shown).

Example 3: Phenotypic traits of the cancer-associated microbiota compared with healthy microbiota : 19 markers associated with cancer dysbiosis

We aim to identify microbiota contrast between the different Cancer (C) patients' cohorts and Healthy (H) cohorts. For each patient, the first sample (at diagnosis prior to therapy) was chosen for the analysis.

For this example, an additional cohort was added, comprising 53 breast cancer patients (Table 9).

Characteristics-Breast cancer	n=53
Median age-yr	50 (35-74)
Histology -no.(%) Ductal carcinoma Lobular carcinoma Other	 43 (81) 6 (11) 4 (8)
Ellis-Elston 1 2 3 N/A	 2 (52) 22 (42) 28 (53) 1 (2)
Clinical Stage T-no.(%) 1 2 3 N/A	 22 (42) 22 (42) 8 (15) 1 (2)
Clinical Stage N-no.(%) 0 1 2 3	 30 (57) 16 (30) 3 (6) 4 (8)

Clinical Stage-no.(%) I II A II B III A III C	 16 (30) 20 (38) 5 (9) 8 (15) 4 (8)
Hormonal receptor-no.(%) Luminal Non Her2 Luminal Her 2 Non luminal Her2 Triple Negative	 26(49) 13 (25) 4 (8) 10 (19)

Table 9

Samples :

- Patients with Cancer: 101
  - Breast: 53 patients
  - Kidney: 16 patients
  - Lung: 28 patients
  - Bladder: 4 patients
- Healthy controls: 85 individuals.

Microbiota :

The following strategy was used to identify contrasted MGS between cancer patients and healthy controls:

- ✓ based on the 2 clusters obtained using correlation of the samples according to their gene content, the whole cohort was separated in "high" and "low" richness samples
  - Breast : 28 "high" and 25 "low"
  - Kidney : 11 "high" and 5 "low"
  - Lung : 14 "high" and 14 "low"
  - Healthy : 77 "high" and 8 "low"
- ✓ for each cancer cohort, MGS contrast with the whole healthy cohort was searched using :
  - all the individuals
  - the "high" richness samples

- the "low" richness samples

✓ contrasted MGS were selected if they met the following criteria:

- being both significantly contrasted for all samples and high richness samples (p.val < 0.05)
- same status in both analysis
- significance ≤ 0.001 for at least one of the tests

Using this strategy, the following contrasted MGS were found:

✓ Breast : 90 contrasted MGS using both p.val < 0.05 and 40 MGS using best p.val < 0.001

✓ Kidney : 157 contrasted MGS using both p.val < 0.05 and 42 MGS using best p.val < 0.001

✓ Lung : 160 contrasted MGS using both p.val < 0.05 and 100 MGS using best p.val < 0.001

147 MGS were found significantly contrasted (p < 0.001) in at least one of the former analyses, some of them being common as judged by the heatmaps / hierarchical clusterings presented in Figures 3 to 5. Despite the lack of significance in richness loss upon cancer, a great correlation was observed between bacterial species enriched in Healthy controls & High richness species vs Cancer species & Low richness species.

29% of the contrasted MGS appear to be high or low richness MGS with a very strong association between richness MGS and phenotype: high richness MGS / healthy subjects vs low richness MGS / cancer cohorts. Among the "healthy MGS" are found many potential short-chain fatty acids (SCFA) producing bacteria (as *Faecalibacterium*, *Roseburia*, *Blautia*, species) (Figure 4).

27 MGS were found contrasted in at least 2 cancer cohorts (Figures 6-7).

In conclusion, a typical cancer-associated microbial fingerprint of intestinal content would be based on the 19 markers defined as follows:

Over-representation of *Bifidobacterium dentium, Cryptobacterium sp. CAG:338, unclassified Eggerthella, Phascolarctobacterium sp. CAG:266, Clostridium sp. CAG:169, Enterococcus faecalis, Faecalitalea*

cylindroides, Suddoligranulum sp. 4_3_54A2FAA, Ruminococcus torques, Hungatella hathewayi, Clostridium sp. CAG:242, Escherichia coli CAG00011; CAG00815 Lactococcus lactis.

CAG	**Corresponding referenced species**	**Number of co-variant genes**	**Threshold maximizing the Youden index**
CAG00456	*Bifidobacterium dentium*	1968	2.082182e-05 [2.082182e-05;5.039616e-05]
CAG00998	*Cryptobacterium sp. CAG:338*	1546	7.378485e-05 [7.378485e-05;0.0004056758]
CAG00905	*unclassified Eggerthella*	1302	4.286171e-05 [4.286171e-05;0.0001209414]
CAG00885	*Phascolarctobacterium sp. CAG:266*	1336	9.264798e-05 [9.264798e-05;0.005263659]
CAG00672	*Clostridium sp. CAG:169*	1645	0.0001620558 [0.0001620558;0.000452764]
CAG00257	*Enterococcus faecalis*	2253	0.0001263241 [0.0001263241;0.0001986582]
CAG00681	*Faecalitalea cylindroides*	1635	5.495815e-05 [3.013049e-05;0.0002242631]
CAG00140	*Subdoligranulum sp. 4_3_54A2FAA*	2650	0.001252674 [0.000247579;0.001681001]
CAG00243	*Ruminococcus torques*	2625	0.0004426992 [0.0002992785;0.001181286]
CAG00025	*Hungatella hathewayi*	4407	3.731168e-05 [9.958701e-06;5.823888e-05]
CAG00381	*Clostridium sp. CAG:242*	2033	5.09195e-05 [5.09195e-05;9.205743e-05]
CAG00011	*Escherichia coli*	5523	0.0003582595 [7.379654e-05;0.001757729]
CAG00815	*Lactococcus lactis*	1464	4.785713e-05 [4.785713e-05;0.0001031883]

Table 10

Under-representation of *Bifidobacterium adolescentis, unclassified Clostridiales, Roseburia sp. CAG:182, unclassified Blautia, Blautia sp. CAG:237, unclassified Faecalibacterium*

CAG	Corresponding referenced species	Number of co-variant genes	Threshold maximizing the Youden index
CAG00702	*Bifidobacterium adolescentis*	1609	0.002773964 [0.0006811621;0.003241451]
CAG01132	*unclassified Clostridiales*	976	0.0005991194 [0.0002469458;0.0007978176]
CAG00055	*Roseburia sp. CAG:182*	3490	5.27458e-05 [5.27458e-05;0.0002543267]
CAG01239	*unclassified Blautia*	796	8.193166e-05 [5.975853e-05;0.0001334845]
CAG00179	*Blautia sp. CAG:237*	2495	2.026261e-05 [1.20318e-05;2.713887e-05]
CAG00297	*unclassified Faecalibacterium*	2174	0.0001188712 [0.0001188712;0.0009145373]

Table 11

This observation of a typical cancer-associated microbial fingerprint of intestinal content led the inventors to propose allogeneic fecal microbial transplantation of healthy individual feces to cancer patients in need thereof and/or administration of a composition aimed at favoring the patient's response to an antineoplastic treatment, such as (i) a composition admixing *Bifidobacterium adolescentis, unclassified Clostridiales, Roseburia sp. CAG:182, unclassified Blautia, Blautia sp. CAG:237, unclassified Faecalibacterium,* or (ii) a probiotic composition of immunogenic bacteria such as *Enterococcus hirae or Akkermansia muciniphila or Alistipes shahii or other Alistipes spp. or Barnesiella intestinihominis or Bacteroides fragilis and Burkholderia cepacia.*

Additional data regarding the cancer-associated microbial fingerprint of intestinal content are disclosed in Example 10 below (Figure 22-23).

Example 4: Gut fingerprints predicting clinical benefit with antibodies blocking the anti-PD1/PD-L1-PD-L2 axis

First, we examined the microbiome profile before the first administration of anti-PD1 Ab (V1) and compared it with that observed after 6 months (6 months of bimonthly injections, V4). All V4 were responders (since these therapeutic administrations were stopped at 3 months in non-responders). We pooled bladder and lung cancer bearing patients for this analysis since results were quite similar in lung cancers taken separately.

Samples :

- lung: 16 samples; 11 V1 / 5 V4
- bladder ; 3 more responders V4 samples

Results

There were significant shifts in the microbiome composition over the course of the treatment in responders to PD1 blockade who tend to manifest the following profile (Fig. 8):

Over-representation of *Blautia sp. KLE 1732, Alistipes shahii, Firmicutes bacterium CAG:114, Clostridium sp. CAG:265, unclassified Firmicutes (CAG00618), unclassified Clostridiales, Bifidobacterium adolescentis*

Under-representation of *unclassified Clostridiales, Clostridiales bacterium 1_7_47FAA, Dorea formicigenerans, Eubacterium sp. CAG:38.*

CAG	Corresponding referenced species	Number of co-variant genes	Threshold maximizing the Youden index
	Over-representation		
CAG00482	*Blautia sp. KLE 1732*	1904	0.000170535 [0.000170535;0.00329442]
CAG00384	*Alistipes shahii*	2666	0.005322663[0.005322663;0.007488159]
CAG00603	*Firmicutes bacterium CAG:114*	1715	0.0001692879 [0.0001692879;0.001029164]
CAG00306	*Clostridium sp. CAG:221 - Clostridium sp. CAG:265*	2154	2.168856e-05 [2.168856e-05;5.477953e-05]
CAG00618	*unclassified Firmicutes*	1699	0.0001257995 [0.0001257995;0.01956294]
CAG01268	*unclassified Clostridiales*	742	9.118905e-05 [9.118905e-05;0.0004603249]
CAG00702	*Bifidobacterium adolescentis*	1609	0.0002381096 [0.0002381096;0.003149554]
	Under-representation		
CAG50003	*unclassified Clostridiales*	699	0.0006258979 [0.0006258979;0.0006736022]
CAG01230	*Clostridiales bacterium 1_7_47FAA & VE202-28*	814	5.596526e-05 [5.596526e-05;0.0002626544]
CAG00650	*Dorea formicigenerans*	1667	0.001599624 [0.0003350047;0.002411789]
CAG00178	*Eubacterium sp. CAG:38*	2496	0.002371692 [7.063138e-05;0.003622146]

Table 12

Secondly, we aimed at predicting the clinical benefit to anti-PD1 Ab (nivolumab) by analyzing the specimen before onset of PD1 blockade, by identifying microbiota contrast between Responders / Non Responders at time 0 (sample V1, before treatment). As no clear difference could be detected between paired V1 and V2 lung samples (not shown), we used V2 samples when V1 samples were not available to increase cohort size.

Samples:

- Lung: 6 R (3 V1 and 3 V2) / 19 NR (8 V1 and 11 V2),
  NB: excluded from contrasts 3 R (1 V3 and 2 V4)
  1 R was only followed for 2.4 months

- Kidney: 9 R V1 / 6 NR V1

Richness

No significant richness difference between responders and non responders was observed but a tendancy of lower richness in lung NR (not shown).

We pooled lung + kidney patients as such:

Since outcome was evaluated after ~3 months in kidney patients, the pooled analysis was performed using R + NR > 3 months: 13 lung + 9 kidney patients / NR < 3 months: 12 lung + 6 kidney

We found 36 MGS more abundant in Responders, 11 MGS more abundant in Progressors (Figure 9).

In conclusion a microbial fingerprint of intestinal content prone to respond to PD1 blockade, is the following profile, based on approximately 45 markers, enriched in *Firmicutes, Clostridiales, Alistipes and Eubacterium* as well as interesting species such as *Methanobrevibacter smithii, and Akkermansia muciniphila,* but poor in *Oscillibacter* and defined precisely as such:

Over-representation of

- *unclassified species:*
  - CAG00064
  - CAG00245
- *Firmicutes species:*
  - CAG00604, 1714
  - CAG01090, 1026
  - CAG00965, 1210
  - CAG00621, 1695
  - CAG01245, 780
  - CAG01308, 606
  - CAG00669, 1649
  - CAG00670, 1648
  - CAG00872, 1359
  - CAG00851, 1422
  - CAG00288, 2189

Clostridiales species:

- CAG00363, 2056
- CAG00391,2019

- CAG00449, 1945
- CAG00513, 1853
- CAG00559,1776
- CAG00644, 1670
- CAG00811
- CAG00907, 1299
- CAG01350

Ruminococcaceae species:

- unclassified *Ruminococcaceae*, CAG01169, 912
- *Subdoligranulum sp*. CAG:314, CAG00880, 1352
- *unclassified Faecalibacterium*, CAG00628, 1686
- *Ruminococcus lactaris CAG00558, 1777*

Eubacterium species:

-CAG00469, 1928
-CAG00393, 2011

- *Bacteroidales species*
  - *Bacteroides nordii,* CAG00116, 2783
  - *Bacteroides sp. CAG:661, CAG00355, 2067*
  - *Bacteroides sp. CAG:598, CAG00440, 1952*
  - *unclassified Alistipes, CAG00646, 1668*
  - *Alistipes sp. CAG:435, CAG00887, 1332*
- *Verrucomicrobia species*
  - *Akkermansia muciniphila, CAG00301, 3187*
  - *Coraliomargarita sp. CAG:312, CAG00313, 2139*
- *Archaea species*
  - *Methanobrevibacter smithii CAG00721, 1830*

Under-representation of:

Oscillibacter (CAG00931, 1257, and CAG00270, 2225)
Bifidobacterium adolescentis (CAG00702, 1609)
Anaerotruncus colihominis (CAG00720, 1590)
Clostridium sp. CAG:242, CAG00381, 2033
Clostridiales bacterium VE202-14, CAG00168, 2534
Eubacterium sp. CAG:252, CAG00353, 2341
unclassified Bilophila, CAG01018, 1135

CAG	Corresponding referenced species	Number of co-variant genes	Threshold maximizing the Youden index
Unclassified species			
CAG00064	*unclassified species*	3310	0.0003947016 [7.891756e-05;0.001301043]
CAG00245	*unclassified species*	2277	0.0001214906 [0;0.006284153]
Firmicutes species			
CAG00604	*Firmicutes bacterium CAG:110*	1714	7.147811e-05 [7.147811e-05;0.006567028]
CAG01090	*unclassified Firmicutes*	1026	0.0003618442 [2.265689e-05;0.002130399]
CAG00965	*Firmicutes*	1210	3.247673e-05 [3.247673e-05;0.003148149]
CAG00621	*Firmicutes bacterium CAG:272*	1695	0.000151022 [0.000151022;0.00308653]
CAG01245	*Firmicutes*	780	0.0002287271 [0;0.002195563]
CAG01308	*unclassified Firmicutes*	666	4.033813e-05 [4.033813e-05;0.0006620213]
CAG00669	*Firmicutes*	1649	0.0001890876 [0.0001890876;0.0107959]
CAG00670	*unclassified Firmicutes*	1648	0.0002932635 [0.0001187004;0.003081848]
CAG00872	*Firmicutes bacterium CAG:240*	1359	0.0008436382 [5.18632e-05;0.005900856]
CAG00851	*unclassified Firmicutes*	1422	0.0003027565 [0;0.004660334]
CAG00288	*Firmicutes bacterium CAG:95*	2189	0.001213057 [6.712724e-05;0.001438314]
Clostridiales species			
CAG00363	*unclassified Clostridiales*	2056	5.290508e-05 [0;9.015663e-05]
CAG00391	*unclassified Clostridiales*	2019	0.001109153 [0;0.01542335]
CAG00449	*Clostridium sp. CAG:253*	1945	8.256957e-05 [8.256957e-05;0.000333628]
CAG00513	*unclassified Clostridiales*	1853	0.0007258855 [0.0007258855;0.003000979]
CAG00559	*unclassified Clostridiales*	1776	0.0008511848 [0.00028557;0.008890015]
CAG00644	*Clostridium sp. CAG:226*	1670	0.0001241694 [0.0001241694;0.04165556]
CAG00811	*unclassified Clostridiales*	1469	3.800777e-05 [0;0.0008164238]
CAG00907	*unclassified Clostridiales*	1299	0.002714397 [0;0.008836334]
CAG01350	*unclassified Clostridiales*	597	0.0002351261 [0;0.00110505]

Ruminococcaceae species			
CAG01169	*unclassified Ruminococcaceae*	912	6.304785e-05 [6.304785e-05;0.0007941971]
CAG00880	*Subdoligranulum sp. CAG:314*	1352	0.006182719 [0;0.0202024]
CAG00628	*unclassified Faecalibacterium*	1686	0.0009421326 [0.0003118048;0.001708944]
CAG00558	*Ruminococcus lactaris*	1777	0.006544936 [2.066638e-05;0.008354078]
Eubacterium species			
CAG00469	*Eubacterium sp. CAG:146*	1928	4.815212e-05 [1.161598e-05;0.00016962]
CAG00393	*Eubacterium sp. CAG:86*	2011	0.0006926536 [4.554846e-05;0.001057236]
Bacteroidales species			
CAG00116	*Bacteroides nordii*	2783	1.220512e-05 [1.220512e-05;0.001216345]
CAG00355	*Bacteroides sp. CAG:661*	2067	0.0001412803 [0.0001412803;0.08686365]
CAG00440	*Bacteroides sp. CAG:598*	1952	0.004614811 [0;0.01166431]
CAG00646	*Alistipes*	1668	0.001840316 [0;0.01288615]
CAG00887	*Alistipes sp. CAG:435*	*1332*	0.017485 [0;0.09121542]
Verrumicrobia species			
CAG00301	*Akkermansia muciniphila*	3187	0.01598361 [0.0002493237;0.01987093]
CAG00313	*Coraliomargarita sp. CAG:312*	2139	0.02535175 [0;0.02535175]
Archaea species			
CAG00721	*Methanobrevibacter smithii*	1830	0.006472178 [0;0.008177933]
Under-representation			
CAG00931	*Oscillibacter*	1257	0.0003035762 [0.0001338014;0.0005623075]
CAG00270	*Oscillibacter sp. KLE 1728 / KLE 1745 / VE202-24*	2225	0.001063076 [0.0008154304;0.005943308]
CAG00702	*Bifidobacterium adolescentis*	1609	0.0003875448 [0.0003420176;0.00213834]
CAG00720	*Anaerotruncus colihominis*	1590	0.0003752534 [1.467377e-05;0.0004653186]
CAG00381	*Clostridium sp. CAG:242*	2033	7.505283e-05 [2.96228e-05;0.003922126]
CAG00168	*Clostridiales bacterium VE202-14*	2534	6.323201e-05 [6.323201e-05;0.0005837004]
CAG00353	*Eubacterium sp. CAG:252*	2341	0.0001766626 [0.0001766626;0.002312927]
CAG01018	*unclassified Bilophila*	1135	0.0002772262 [0.0002772262;0.004455528]

Table 13

Example 5: Fecal microbial transplantation of human feces from lung (NSCLC) cancer patients confer sensitivity or resistance to PD1 blockade in germ free tumor bearing mice

We selected four stage IV NSCLC patients (A, B, E, F) who failed to respond to 4 months of nivolumab as well as two stage IV NSCLC patients who exhibited partial response to nivolumab to transfer their stools into ATB-treated recipient animals. In parallel, control specific pathogen free (SPF) mice were given ip administrations of nivolumab and responded by decreased tumor outgrowth as expected (experimental setting described in Fig. 10). However, ATB-treated animals transferred with progressors-derived feces failed to respond while ATB-treated littermates transferred with responders-derived stools did (Figure 11-12).

Prior to perform FMT, the mice received 3 days of ATB. Then the FMT was performed using feces from different NSCLC patients treated with anti-PD1 Ab. Subsequently, MCA205 WT tumor was inoculated and the mice received four injections of anti-PD1 or isotype control Abs.

There was no difference in tumor size in the Isotype groups from mice that underwent FMT from either responders or not. However, significant smaller final tumor sizes in mice treated with anti-PD1 Abs were observed in animals that received FMT from Responders (RES) compared to that which Progressed (PRO).

This experiment was duplicated in germ free animals as recipients instead of ATB-treated C57BL/6 mice, yielding similar results.

Example 6: Compensatory anticancer probiotics also called "oncomicrobiotics" or "oncobax" (such as *Akkermansia muciniphila* or *Enterococcus hirae or Alistipes* or combinations)

To establish the proof-of-concept that oral feeding with immunogenic bacteria that are absent in non-responders patients can be beneficial, we performed FMT in avatar model (described in Figure 10-11-12) followed by bacterial compensation. We could have access to the metagenomic data of Patient A, B (both experiencing Progression) whose feces had a low level of *Akkermansia municiphila* CAG00301: 0 and 0,00344667 respectively (cut-off 0,01598361). *Bacteroides nordii* CAG00116 values were also below the cut-off of 1,22 E-05 (0 & 0 respectively).

Based on the experience presented in figure 10-12, we performed FMT of feces from patient A (clinically non responder, metagenomic profile: low *Akkermansia*) in GF mice. Then, 2 gavages were performed with 10^9 bacteria at the time of the first and second injection of anti-PD1 Abs. Three different bacteria were used, one for each group of mice (n=5 mice per group) in separated isolators.

The oral gavage of *Akkermansia muciniphila (Akk)* or *Enterococcus hirae* 13144 (EH) significantly decreased the final tumor size of avatar mice that received FMT from a patient A who progressed. On the other hand, *Eubacterium tenue* did not modify the tumor size post FMT (Figure 13).

This experiment was repeated with FMT of feces from other patients (patients B and F), with the same and other bacteria and with a higher number of probiotic administrations.

We could have access to the metagenomic data of Patients B and F (both experiencing progression), whose feces had a low level of *Akkermansia muciniphila* CAG00301 and *Alistipes spp.* Then, 5 gavages were performed with 10^9 bacteria prior to and at the time of the first, second, 3^{rd}, 4^{th} injection of anti-PD1 Ab. Three different bacteria were used alone (*Akkermansia muciniphila*, *Alistipes spp*. and *Enterococcus hirae* 13144), as well as the combination of Akk+EH 13144 for each group of mice (n=5-6 mice per group) kept in separated isolators (Figure 14).

The best responding group was the one with oral gavage of *Akkermansia muciniphila* + *Enterococcus hirae* 13144, which significantly decreased the final tumor size of avatar mice that were either spontaneously reconstituted with the mouse microflora (after stopping ATB, Figure 15) or that received FMT from patient B (Figure 16) or F. Figure 17 shows the results for all mice in this experiment, in which the combination of *Akk+EH* was more effective than each commensal alone.

Example 7: The efficacy of PD1 and CTLA4 coblockade depends on gut microbiota and in dysbiotic mice, the compromised tumoricidal activity of the combination can be restored by the combination of *B. fragilis and B. cepacia or Barnesiella intestinihominis*

C57BL/6 mice were inoculated with MCA205 sarcoma after a 14 day broad spectrum ATB administration. Then, at day 6 of MCA205 implantation, mice were treated with iv injections of anti-CTLA4 Ab (every other

3 days for 4 injections) as well as anti-PD1 Ab (6 injections every other 3 days for 6 injections). In independent groups isolated in different cages, mice received oral gavages with distinct "oncobax", such as *Bifidobacterium breve* combined with *Bifidobacterium longum* or *Bacteroides fragilis* alone or combined to *Burkholderia cepacia* or *Barnesiella intestinihominis*. Tumor growth kinetics were monitored in 6 mice/group, twice a week.

The best result in two independent experiments was obtained with the combination of *Bacteroides fragilis* + *Burkholderia cepacia* or *Barnesiella intestinihominis*. The percentages of tumor free mice which rejected the tumor was 3/6 (50%) in this group (Figure 18).

Conclusive remarks to Examples 1-7

We aimed at better defining the intestinal microbiome of cancer patients at very different stages of their disease: breast cancer at diagnosis, cis-platinum resistant bladder cancers responding at 6 months post-PD1 blockade, second or third line metastatic kidney cancer resistant to tyrosine kinase inhibitors and mTOR inhibitors, cis-platinum-resistant advanced lung cancers. None, except one patient, had taken antibiotics in the last month before feces collection.

Despite this broad heterogeneity of disease histology and stage, we found very homogenous sequenced samples according to reads number and high mapping %.

Similar richness of the cancer samples (GC and MGS count) was found, whatever the cohort or the outcome.

According to gene and MGS count, cancer patients tend to have a lower richness but the difference was not significant. However, hierarchical clustering of the samples gave 2 big clusters driven by microbiota richness, the high richness samples encompassing most (90%) of the healthy samples and only half of the cancer samples, which suggests a richness deficiency in half of the cancer associated microbiomes.

Microbial species associated to cancer were contrasted to that of healthy subjects. 138 MGS were found significantly contrasted in at least one cancer cohort, 40% of them appearing to be high or low richness MGS with a very strong association between richness MGS and phenotype: high richness MGS / healthy subjects *vs* low richness MGS / cancer cohorts. We thus

stratified all samples according to gene richness clusters and focused on MGS significantly contrasted using both the full cohort and the high richness samples. 147 MGS were found significantly contrasted in at least one cancer cohort and 27 were contrasted in at least 2 different cohorts. 30% of the contrasted MGS still appear to be high or low richness MGS but the richness/phenotype association is less clear. Among the "healthy MGS", many potential SCFA producing bacteria (as *Faecalibacterium* and *Roseburia* species) are found as well as *Bifidobacterium adolescentis* that appears to be highly depleted in cancer patients.

In particular, we propose a typical cancer-associated microbial fingerprint of intestinal content based on 19 markers defined as follows:

Over-representation of *Bifidobacterium dentium, Cryptobacterium sp. CAG:338, unclassified Eggerthella, Phascolarctobacterium sp. CAG:266, Clostridium sp. CAG:169, Enterococcus faecalis, Faecalitalea cylindroides, Suddoligranulum sp. 4_3_54A2FAA, Ruminococcus torques, Hungatella hathewayi, Clostridium sp. CAG:242, Escherichia coli, Lactococcus lactis*.

Under-representation of *Bifidobacterium adolescentis, unclassified Clostridiales, Roseburia sp. CAG:182, unclassified Blautia, Blautia sp. CAG:237, unclassified Faecalibacterium*

Accordingly, lung and bladder cancer patients maintained at least 6 months on prolonged PD1 blockade (and responding to this therapy) manifested a microbial shift, resembling that of healthy volunteers, with decreased abundance of *Clostridium leptum* (considering lung patients only) and enrichment of *Bifidobacterium adolescentis* (highly enriched in healthy controls *vs* all cancer cohorts).

A microbial fingerprint of intestinal content associated with responses to PD1 blockade is based on 16 MGS, enriched in *Firmicutes, Clostridiales, Ruminococcaceae, Alistipes* and *Eubacterium* as well as interesting species such as *Methanobrevibacter smithii* and *Akkermansia muciniphila*, but poor in *Oscillibacter*. *Akkermansia muciniphila* has been found in anorexia, as well as in metformin-treated individuals, ameliorating diabetes and insulinodependence. This *Verrucomicrobiaceae*-belonging *Akkermansia muciniphila* was also overrepresented in cancer patients responding to PD1

blockade and converted non responding avatar mice into responders in our FMT experiments. Interestingly, an *enterococcus* of high immunogenicity (*Enterococcus hirae*) in the context of cyclophosphamide was also very efficient in converting progressors into responders in avatar mice. Moreover, culturomics analyses revealed higher numbers of detectable colonies of *Enterococcus hirae* in stools at diagnosis in NSCLC patients proned to respond compared to stools of patients doomed to fail therapy with anti-PD1/PDL1 Abs.

In summary, cancer patients display a dysbiotic fecal microbial composition, with low gene diversity, low representation of "health associated MGS" such as SCFA-making bacteria which reduce their immune tone and their propensity and likelihood to respond to immunomodulation such as PD1 blockade. They could benefit from allogeneic fecal microbial transplantation of healthy individual feces or appropriate probiotic compositions, such as a composition admixing *Bifidobacterium adolescentis, unclassified Clostridiales, Roseburia sp. CAG:182, unclassified Blautia, Blautia sp. CAG:237,* and *unclassified Faecalibacterium,* or a probiotic composition of immunogenic bacteria such as *Enterococcus hirae* or *Akkermansia muciniphila* or *Barnesiella intestinihominis* or *Bacteroides fragilis* and *Burkholderia cepacia*.

Example 8: Stage IV NSCLC patients who develop a TH1 immune response towards *Enterococcus hirae* or *Burkholderia cepacia or best Akkermansia muciniphila* after the 2-4 first administrations of anti-PD1 Abs have a long term benefit to PD1 blockade.

Fourteen NSCLC patients (Table 14 below) treated with anti-PD1 Abs (Nivolumab) were followed up for their memory TH1 and TC1 or Tr1 immune responses against a variety of different commensals as described in Vétizou *et al.* Science 2015, after the 1st or 2nd or third injection.

Patient ID	Outcome	PFS in month
1-DAM-GU	Dead	7.495069034
5-DAN-TH	PRO	5.489809336
6-MOU-GI	PRO	6.47600263
11-FAY-JU	Partial response	8.777120316
13-MAI-CH	Partial response	8.777120316
15-BRU-JA	Stable disease	9.204470743

16-ALL-JE	Stable disease	10.09204471
19-MAT-HE	PRO	7.232084155
20-BEN-TR	PRO	4.109138725
22-RIF-JE	PRO	5.029585799
23-VIA-JM	Partial response	9.105851414
26-BEN-KA	Stable disease	2.399737015
27-SET-JA	Stable disease	9.007232084
29-MAB-PA	PRO	2.399737015

Table 14

Briefly, autologous monocytes were incubated with live bacteria at a 1:10 ratio, then bacteria were inactivated by appropriate ATB prior to addition of autologous CD4+ or CD8+ T cells (sorted from patients' blood using magnetic beads). The 48 hr supernatants of these cocultures were tested in ELISA for the concentration of IFNγ or IL-10. The median of IFNγ or IL-10 production or their ratio for each bug in the whole cohort was used to segregate the patients and calculate the time to progression during PD1 blockade. The memory TH1/Tr1 CD4+ T cell responses directed toward *Enterococcus hirae* 13144 (MH13144) was associated with long term time to progression (TTP) in these 14 patients treated with anti-PD1 Ab, while those directed toward *Enterococcus faecalis* (EF) or TCR cross linking (beads) failed to predict TTP (Fig.19 A, B, C).

To confirm the relevance of the immunogenicity of *Enterococcus hirae* in this cohort of NSCLC patients for the response to PD1 blockade, we also exemplified that $CD8^+$ Tc1 immune response to the other isolates of *Enterococcus hirae* (EH17/13344) are also critical to predict TTP (Fig. 19 D-E). High Tc1 immune responses to *Enterococcus hirae* are a favourable immunodynamic parameter predicting TTP under anti-PD1 Ab-based therapy. In Fig. 37, we also demonstrate that CD4+ TH1 (Fig. 37) and $CD8^+$ Tc1 immune responses (Routy et al Science 2017) to the Verrucomicrobiaceae *A. muciniphila* are also critical to predict TTP.

Among 8 other commensals, only TH1 responses to *Burkholderia cepacia* (B) were also relevant to predict TTP in this cohort of patients, high TH1 or TH1/Tr1 ratios being associated with prolonged TTP (Fig. 19 F and 19H).

Example 9: The efficacy of PD1 blockade is significantly decreased by oral antibiotics in non small lung cancer patients

We have extended the deleterious role of antibiotics (ATB) for the response to PD1 blockade to non small cell lung cancer patients. 66 NSCLC patients (characteristics detailed in Table 15) in second or third line therapy enrolled in a treatment using anti-PD1 Ab (ICB) were followed up for 18 months. Twenty patients received antibiotics (detailed in Table 16), either up to 2 months before the start of ICB (V1) or during the first month post-1st inoculation of ICB (V2). ATB (+) group: V1 (before the 1st injection of ICB) + V2 (within the 1st month of ICB).

Characteristics - Lung cancer		ATB (+) group (N=20)	ATB (-) group (N=46)	P-value	Total (N=66)
Age-yr	Median Range	63 46-77	62 32-77	<0.308	63 33-77
Age-yr-no.(%)	<65 ≥65	11 (55) 9 (45)	26 (57) 20 (43)	<0.901	37 (56) 29 (44)
Gender-no.(%)	Male Female	8 (40) 12 (60)	33 (72) 13 (28)	<0.015	41 (62) 25 (38)
Smoking status-no.(%)	Current Former Never	7 (35) 12 (60) 2 (5)	13 (28) 28 (61) 7 (15)	<0.570	20 (30) 40 (61) 6 (9)
ECOG PS-no.(%)	0-1 ≥2 Not reported	12 (60) 3 (15) 5 (25)	14 (70) 5 (11) 9 (19)	<0.860	40 (60) 11 (17) 15 (23)
Histology-no.(%)	Squamous Non-squamous	5 (25) 15 (75)	4 (9) 42 (91)	<0.475	13 (20) 53 (80)
Mutation status-no.(%)	EGFR positive KRAS positive ALK positive	1 (5) 4 (10) 2 (5)	1 (2) 7 (15) 0 (0)	*	5 (7) 13 (20) 2 (3)
Brain metastasis-no.(%)		5 (25)	7 (15)	<0.723	13 (20)
Number of prior systemic regimens-no.(%)	≥2	14 (70)	16 (35)	<0.596	30 (45)
List of ATBs-no.(%)	Macrolides Beta Lactamases Fluoroquinolones Sulfonamides Tetracycline Not reported	2 (10) 9 (45) 4 (20) 2 (10) 2 (10) 1 (5)	- - - - - -		2 (3) 9 (14) 4 (6) 2 (3) 2 (3) 1 (1)

Table 15: Baseline characteristics of 66 patients with NSCLC treated with Nivolumab as 2nd- 3rd line treatment. ATB(+)/(-) groups were defined as patients treated or not with ATB before (2 months period) or within the first month of Nivolumb. Beside the mutational status, no significant difference in clinical characteristics between ATB (+) and ATB (–) group. ATB (+) group: V1 (before the 1st injection of ICB) + V2 (within the 1st month of ICB).
* Not calculated due to small numbers

List of ATBs	ATB (+) group (N=20)	ATB (V2) group (N=7)
Macrolides	2	1
Beta Lactamases	9	4
Fluoroquinolones	4	2
Sulfonamides	2	-
Tetracycline	2	-
Not reported	1	-

Table 16: ATB specificities. V2 patient charts were reviewed and V2 ATB were prescribed for urinary tract infection, cough or fever (based on CTCAE v4). None was in ICU or actively dying from sepsis.

The Kaplan Meier survival curves are shown for progression free survival (no significant difference between the groups treated or not with ATB, Fig. 20 left panel) or overall survival (significant difference, Fig. 20, right panel). The Cox regression multivariate analyses show that ATB administration remains an independent predictor of resistance to ICB in NSCLC patients (Table 17).

			Multivariate (1)	
Parameter	Levels	N/Nevent	HR [95%CI].adj (n=66)	LRT.adj (n=66)
Antibiotics	NoATB	46/17	1	6.10, p<0.0136
	ATB	20/11	3.15 [1.28;7.80],p<0.0136	
Age at diagnostic	<65	37/18		2.76, p<0.0967
	>=65	29/10	0.51 [0.22;1.18],p<0.0967	
Gender	F	25/12		0.00, p<0.9985
	M	41/16	1.00 [0.35;2.85],p<0.9985	
Histology	NonSq	53/20		10.58, p<0.0011
	Squamous	13/8	5.80 [2.00;16.81],p<0.0011	
Smoking	NonS	9/6		3.31, p<0.0691
	Smoker	57/22	0.31 [0.09;1.09],p<0.0691	
Num. prior therapies (2)	0-2	50/21		0.28, p<0.5944
	3-5	16/7	1.27 [0.52;3.11],p<0.5944	
Num. met. site (3)	0-2	50/21		1.31, p<0.2529
	3-4	16/7	1.81 [0.65;5.04],p<0.2529	
ECOG (2)	0-1	55/22		3.59, p<0.0580
	2	11/6	2.78 [0.99;7.79],p<0.0580	

Table 17: Multivariate analysis for overall survival in NSCLC. Multivariate analysis to determine hazard ratio calculated adjusting for known prognostic risk factors: ATB, Age, Gender, Histology (Non-squamous vs squamous), Smoking History (non-smoker vs smoker), Number of prior therapies, Number of metastatic sites, ECOG scale of performance status (0=fully active, 1=restricted in physically strenuous activity, 2=confined to bed or chair <50%, 3= confined to bed or chair>50%, 4= completely disable). *p<0.05, ** p<0.01,*** p<0.001, ns=not significant.

We gathered the cancer patients suffering from lung cancer (N=66, cohort described in Table 15), bladder cancer (N=42, cohort described in Table 5) and kidney cancer (N=67, described in Table 18 below). In this total of N=175 patients amenable to an ICB-based therapy in 2nd or 3rd line therapy, we showed that ATB induce resistance to PD-1 blockade (PFS and OS, Fig. 21).

Characteristics - Renal cell carcinoma		ATB (+) group (N=20)	ATB (-) group (N=47)	P-value	Total (N=67)
Age-yr	Median Range	60 40-83	61 30-82	<0.322	61 30-83
Gender-no.(%)	Male Female	11 (55) 9 (45)	34 (72) 13 (28)	<0.170	45 (67) 22 (33)
Histology-no. (%)	Clear cell Non-clear cell	17 (85) 3 (15)	44 (94) 3 (6)	<0.877	61 (91) 6 (9)
Fuhrman Grade-no.(%)	I-II III-IV Not reported	9 (45) 7 (35) 4 (20)	9 (19) 22 (47) 16 (34)	<0.055	18 (27) 29 (43) 20 (30)
Tumor Burden-mm	<100 ≥100	11 (55) 9 (45)	36 (77) 11 (23)	<0.077	47 (70) 20 (30)
KPS-no.(%)	≤70 ≤80 ≤90	4 (20) 7 (35) 9 (45)	6 (13) 15 (32) 26 (55)	<0.890	10 (15) 22 (33) 35 (52)
IMDC Risk Group-no.(%)	Good Intermediate Poor	5 (25) 12 (60) 3 (15)	9 (19) 27 (58) 11 (23)	<0.697	14 (21) 39 (58) 14 (21)
Number of prior lines-no.(%)	≥2	18 (90)	20 (42)	<0.001	38 (57)
Concomitant drugs-no.(%)	Corticosteroids Proton pump inhibitors	4 (20) 6 (30)	1 (5) 14 (70)	*	5 (7) 20 (30)
Antibiotics	Beta-lactamase Fluroquinolone Tetracycline Aminoglycosides Nitrofurantoin N/A	12 (60) 2 (10) 1 (5) 1 (5) 1 (5) 3 (15)			

Table 18: Update on patient baseline characteristics with mRCC treated with PD1 included in the analysis on the influence of ATB in PFS and OS.

Example 10: Gut fingerprint associated with cancer (with or without ATB)

Materials

292 healthy controls

Cancer patients:

Breast cancer: batch 1 described in Table 9 and batch 2 described in Table 19.

Lung cancer : batch 1 described in Table 3 and batch 2 described in Table 20.

Kidney cancer: batch 1 described in Table 4 and batch 2 described in Table 21.

30 samples come from patients treated using antibiotics (AB).

- 3: Augmentin (beta-lactamine + penicilline),
- 1: Ofloxacine (fluoroquinolone)
- 1: Ceftriaxone (beta-lactamine)
- 25: NA

With only partial information from the AB prescribed, the AB samples were treated as a whole.

2 analyses were performed:

- all samples
- paired samples +/- AB (12 Lung + 5 Kidney paired samples)

AB treated samples have a tendency of lower richness (not significant).

Characteristics-Breast cancer	N=30
Median age-yr	60 (38-75)
Histology -no. (%) Ductal carcinoma Lobular carcinoma	 29 (97) 1 (3)
Ellis-Elston 1 2 3 N/A	 5 (17) 11 (37) 13 (43) 1 (3)
Clinical Stage T-no.(%) 1 2 3	 13 (43) 10 (33) 7 (21)
Clinical Stage N-no.(%) 0 1 2 3	 17 () 10 () 2 (7) 1(3)
Clinical Stage-no-(%) 1 2 3	 14 (47) 10 (33) 6 (20)
Hormonal receptor-no.(%) Luminal Non Her2 Luminal Her2 Non luminal Her2 Triple Negative	 11 (37) 5 (17) 5 (17) 9 (30)

Table 19: Breast cancer patient characteristics included in the second batch.

Characteristics - NSCLC		Total (N=38)
Age-yr	Median Range	62 32-77
Gender-no.(%)	Male Female	21 (55) 17 (45)
Histology-no.(%)	Squamous Non-squamous	10 (26) 28 (74)
ECOG-no.(%)	0-1 ≥2	30 (79) 8 (21)
Mutation-no.(%)	EGFR positive KRAS positive ALK positive	3 (7) 2 (5) 1 (2)
Smoking -no.(%)	Current Former Never	12 (32) 24 (63) 2 (5)

Table 20: Lung cancer patient characteristics included in batch 2.

Characteristics - mRCC		Total (N=25)
Age-yr	Median Median	63 50-75
Gender-no. (%)	Male Female	20 (80) 5 (20)
Histology-no. (%)	Clear cell Non-Clear cell	25 (100) -
ECOG-no.(%)	0-1 ≥2 NA	23 (92) - 2 (3)
IMDC -no.(%)	Good Intermediate Poor NA	8 (32) 13 (52) 2 (8) 2 (8)

Table 21: Metastatic renal cell carcinoma patient characteristics included in batch 2.

Results

Eighteen metagenomic species (MGS) were found contrasted with the 2 batches in at least 2 cancer cohorts. Of these, fifteen were in the list of the 27 species initially identified (Fig. 6 and 7).

Eleven metagenomic species are maintained if we take out the ATB-treated patients.

We hence propose a gut fingerprint of cancer patients validated in 2 batches with or without (*) ATB, which comprises 19 MGS.

<u>*8 MGS underexpressed in cancer versus healthy volunteers:*</u>

Faecalibacterium CAG297 *
Blautia CAG179
Roseburia CAG55 *
Haemophila parainfluenzae CAG1056
Clostridiales CAG1132
Bifidobacterium adolescentis (CAG702)
Firmicutes CAG1308 *
Firmicutes bacterium CAG713 *

<u>*11 MGS overexpressed in cancer versus HV :*</u>

Bifidobacterium dentium (CAG456) *
Enterococus faecalis (CAG257)
Subdoligranulum CAG140 *
Lachnospiricaeae bacterium CAG14
Clostridium innocuum CAG36
Ruminococcus torques 1 (CAG243) *
Hungatella hathewayi 1 CAG25 *
2 E.coli (CAG 11* et 371*)
Clostridiales CAG533 *
Tyzzerella nexilis CAG311

18 of them are contrasted in 2 (out of 3) cancer cohorts at least (Fig. 22, data not shown).

If one considers only those patients who did not take antibiotics (no ATB), there are only 11 MGS contrasted in at least 2 (out of 3) cancer cohorts (Fig. 23).

The direct effects of ATB on the cancer cohorts are apparent upon MGS analysis: 9 MGS are enriched post-ATB, while 6 are eliminated. ATB tend to enrich in deleterious bacteria blunting the efficacy of PD1 blockade or other immunogenic chemotherapy such as *Bacteroides uniformis, Bacteroides cellulosilyticus, Coprobacter fastidiosus, Lachnospiracae bacterium*. Moreover, ATB tend to deplete the host flora from important protective anticancer bacteria such as *Roseburia CAG100, Blautia CAG01214, Eubacterium CAG38 and CAG115.*

Example 11: Gut fingerprint predicting clinical benefit with antibodies blocking the anti-PD1/PD-L1-PD-L2 axis

The same experiments as disclosed in Example 4 above were conducted on a pool of patients suffering from kidney or lung cancer (N=100 patients).
The results confirm the favorable role of several species, such as:

1. Signature at diagnosis: favorable and dominant role of *Akkermansia muciniphila*, *Ruminococcaceae* (*CAG 210 bicirculans, CAG854, torques 2 CAG1262, 250, bacterium D16 CAG949*)
2. Signature at diagnosis: favorable role of *Intestinimonas butyriciproducens, Oscillibacter, Collinsella spp. (tanakaei* and *intestinihominis)* (data not shown + table 27)
3. Signature at diagnosis: deleterious roles of *Bifidobacteria adolescentis, longum*, as well as *Parabacteroides distasonis and goldsteinii, Bilophila wadsworthia, Erysipelotricaceae, Bacteroides uniformis.*

Example 12: Gut fingerprint associated with response to PD1 blockade in lung cancers (with or without ATB)

A gut fingerprint specific for end stage lung carcinoma associated with responses or resistance to anti-PD1 Ab was identified for time to progression at 3 months.

Bacterial species	Number of co-variant genes	Corresponding referenced species	Annotation	Sequences (SEQ ID Nos)
CAG00363	2056	*Intestinimonas butyriciproducens*	*Intestinimonas butyriciproducens (closely related to Flavonifractor plautii)*	*176-200*

CAG00134	2690	*Cloacibacillus porcorum*		*1351-1375*
CAG00278	2203	*Roseburia sp. CAG: 380*		*1526-1550*
CAG00469	1928	*Eubacterium sp. CAG:146*	*Eubacterium*	*301-325*
		Eubacterium sp.3_1_31	*Erysipelotrichacea e bacterium 5_2_54FAA*	
CAG00676	1643	*Firmicutes bacterium CAG:176*	*Firmicutes*	*1976-2000*
CAG01188	880	*Firmicutes bacterium CAG:321*		*2651-2675*
CAG01090	1026	*Firmicutes bacterium CAG:110*	*Firmicutes*	*776-800*
CAG0994	1171	*Firmicutes bacterium CAG:124*	*Firmicutes*	2401-2425
CAG1205	855	*Phascolarctobacterium succintulens*		*2676-2700*
CAG1220	823	*Phascolarctobacterium succintulens*		*2726-2750*
CAG1399	524	*Oscillibacter sp. CAG:155*	*Clostridiales, Oscillospiricaeae*	*2901-2925*
CAG1086	1037	*Firmicutes bacterium CAG:124*	*Firmicutes*	*2526-2550*
CAG01245	780	*Firmicutes*	*Firmicutes*	*826-850*
CAG1011	1140	*Uncultured Dore asp.*	*Clostridiales*	*2426-2450*
CAG00064	3310		*NA*	*1-25*
	3561	*Bacteroides caccae*	*Bacteroidales*	
CAG00112	3092	*Blautia sp CAG:52*		*1301-1325*
CAG1386	546	*Blautia sp. KLE 1732*		*2876-2900*
CAG00453	1942		*NA*	*1751-1775*
CAG00674	1643	*Dore asp. CAG:317*		*1951-1975*
CAG00328	2113	*Alistipes indistinctus*	*Alistipes*	*1601-1625*
CAG00646	1668	*Alistipes sp. CAG:268*	*Alistipes*	*501-525*
CAG00301	3187	*Akkermansia muciniphila CAG:154*	*Akkermansia muciniphila*	*101-125*
CAG00731	1576	*Proteobacteria bacterium CAG:139*	*Parasutterella*	*2051-2075*
CAG0903	2783	*Desulfovibrio piger*		*2276-2300*
CAG00250	2262	*Ruminococcus sp. CAG:353*	*Ruminococcaceae*	*1476-1500*
CAG001342	613	*Ruminococcus bacterium LM158*	*Ruminococcaceae*	*2851-2875*
CAG01262	750	*Ruminococcus torques 2*	*Ruminococcaceae*	*2826-2850*

Table 22: bacterial species **over**-represented in lung cancer patients likely to be good responders at 3 months to anti-PD1 or PD-L1 or anti-PD-L2 antibodies and under-represented in lung cancer patients likely to be resistant at 3 months to anti-PD1 or PD-L1 or anti-PD-L2 antibodies, as well as corresponding referenced species (for already identified species) and sequences comprised therein.

Bacterial species	Number of co-variant genes	Corresponding referenced species	Annotation	Sequences (SEQ ID Nos)
CAG 00048_1	*1403*	*Clostridiales*		*1151-1175*
CAG00211	*2389*	*Firmicutes bacterium CAG:227*	*Firmicutes*	*1451-1475*
CAG01629	*505*	*Firmicutes bacterium CAG:466*	*Firmicutes*	
CAG01018	*1135*	*Bilophila wadsworthia*	*unclassified Bilophila*	*1076-1100*
CAG00702	*1609*	*Bifidobacterium adolescentis L2-32*	*Bifidobacterium adolescentis*	*951-975*
CAG00549	*1788*	*Bifidobacterium longum*	*Bifidobacteria*	*1901-1925*
CAG430	*1970*	*Lactobacillus salivarius*		*1726-1750*
CAG00389	*2021*	*Fusobacterium sp. CAG:439*		*1701-1725*
CAG00981	*1189*	*Erysipelotrichaceae*	*Erysipelotrichaeae*	*2376-2400*
CAG00005_4	*503*	*Clostridiales*	*Clostridiales*	*1101-1125*

Table 23: bacterial species over-represented in lung cancer patients likely to be resistant at 3 months to anti-PD1 or PD-L1 or anti-PD-L2 antibodies and under-represented in lung cancer patients likely to be good responders at 3 months to anti-PD1 or PD-L1 or anti-PD-L2 antibodies, as well as corresponding referenced species (for already identified species) and sequences comprised therein.

Hence, in addition to the MGS searched for generally determining if the patient is likely to respond to a treatment with anti-PD1 or PD-L1 or anti-PD-L2 antibodies, the relative abundance of following MGS can be measured in a lung cancer patient:

- Additional bacterial species **over**-represented in lung cancer patients likely to be good responders at 3 months to anti-PD1 or PD-L1 or anti-PD-L2 antibodies: CAG00278, CAG00676 (*Firmicutes*), CAG01188, CAG0994 (*Firmicutes*), CAG1205, CAG1220, CAG1399 (*Clostridiales, Oscillospiricaeae*), CAG00112, CAG1386, CAG00674, CAG00328 (*Alistipes*), CAG00731 (*Parasutterella*), CAG0903, CAG001342 (*Ruminococcaceae*), CAG01262 (*Ruminococcaceae*).
- Additional bacterial species over-represented in lung cancer patients likely to be resistant at 3 months to anti-PD1 or PD-L1

or anti-PD-L2 antibodies: CAG01629 (*Firmicutes*), CAG430, CAG00389, CAG00005_4 (*Clostridiales*).

Example 13: Gut fingerprint associated with response to PD1 blockade in kidney cancers (with or without ATB)

In addition to MGS outlined in Tables 1 and 2, common to lung and kidney cancers, there are some additional MGS that could participate to early relapse or response to PD1-blockade in kidney cancer patients (tables 24-25).

Bacterial species	Number of co-variant genes	Corresponding referenced species	Sequences (SEQ ID Nos)
CAG452	1942	*Clostridium sp CAG167*	
CAG00317	2130	*Clostridium sp CAG 230*	
CAG00062	3614	*Bacteroides salyersiae*	
CAG 00510	*1860*	*Alistipes timonensis*	
CAG00816	*1464*	*Candidatus stoquefichus massilensis*	
CAG00215	*2381*	*Anaerotruncus*	
CAG001401	*522*	*Lachnospiracaeae*	
CAG00774	*1519*	*Firmicutes*	*2076-2100*

Table 24: bacterial species over-represented in kidney cancer patients likely to be responders at 3 months of PD1 blockade (the first two bacteria *Clostridium* spp. being reproducibly found in 2 independent cohorts of advanced kidney cancers proned to respond to PD1 blockade in second line)

Bacterial species	Number of co-variant genes	Corresponding referenced species	Sequences (SEQ ID Nos)
CAG00481	*1908*	*Blautia sp. CAG: 237*	
CAG00541	*1799*	*Dorea sp CAG: 105*	
CAG00448	*1945*	*Clostridium sp. CAG: 122*	
CAG 00650	*1667*	*Dorea formicigenerans*	
CAG01141	*964*	*Holdemania biformis*	
CAG00719	*1591*	*Ruminococcus*	
CAG01208	*844*	*Coprococcus catus*	3226-3250
CAG00079	*3078*	*Clostridium sp. CAG:7*	
CAG00722	*1587*	*Acidaminococcus fermentans*	
CAG00943	*1236*	*Firmicutes bacterium CAG:212*	
CAG1004	*1161*	*Prevotella copri*	
CAG01039	*1098*	*Faecalibacterium prausnitzii*	
CAG00903	*1305*	*Desulfovibrio piger*	2276-2300
CAG01399	*524*	*Oscillibacter sp. CAG:155*	2901-2925

Table 25: bacterial species over-represented in kidney cancer patients likely to be progressors (poor responders) at 3 months of PD1 blockade.

Example 14: Gut fingerprint associated with response to PD1 blockade in a validation cohort of non-small cell lung cancer and kidney cancers (without ATB)

Table 26 below shows the clinical data relative to the validation cohorts. Bacteria present in this validation cohort (group without ATB) are listed in Table 27.

NSCLC	n=27	RCC	n=26
Histology		**Histology**	
Adenocarcinoma no. (%)	17 (63)	Clear cell no. (%)	25 (96)
Squamous no. (%)	7 (26)	Non clear cell no. (%)	1 (4)
Undifferentiated no. (%)	3 (11)		
Smoking Status		**IMDC**	
Former no. (%)	19 (70)	Good no. (%)	5 (19)
Current no. (%)	6 (22)	Intermediate no. (%)	17 (65)
Non-smoker no. (%)	2 (7)	Poor no. (%)	4 (15)
Outcome		**Outcome**	
Partial Response no. (%)	4 (15)	Complete response no. (%)	1 (4)
Stable Disease no. (%)	5 (19)	Partial Response no (%)	7 (27)
Progression/Death no. (%)	18 (67)	Stable disease no. (%)	12 (46)
		Progression/Death no. (%)	6 (23)

Table 26: baseline characteristics of the validation cohorts

Bacterial species	**Number of co-variant genes**	**Corresponding referenced species**
CAG00301	*3187*	*Akkermansia muciniphila*
CAG00487	*2806*	*Prevotella sp. CAG:279*
CAG00417	*1981*	*Megamonas funiformis*
CAG01551	*508*	*Oscillibacter*
CAG01018	*1135*	*Bilophila wadsworthia*
CAG00534	*2650*	*Prevotella sp. CAG:386*
CAG00472	*1921*	*Azospirillum*
CAG00591	*1728*	*Collinsella tanakaei*
CAG01066	*1068*	*Succinatimonas hippei*
CAG00986	*1179*	*Collinsella intestinalis*
CAG00048_1	*1403*	*Clostridiales*
CAG01075	*1052*	*Clostridiales*

Table 27: bacterial species over-represented in non-small cell lung cancer and kidney cancer patients with the best clinical outcome based on the RECIST criteria: PR > SD > PD assessed by MG and analyzed by Cochran-Armitage test.

Example 15: GS and MGS Diversity at diagnosis dictates response to PD1 blockade at 6 months in kidney+lung cancers (with or without ATB)

Another index to predict patients proned to respond at 6 months of therapy with anti-PD1 Ab is to analyze the diversity index for gene counts (GC) and metagenomic species (MGS) (Fig. 24).

When GS >6x106 or MGS>250, cancer patients will be proned to respond.

Example 16: Dynamic parameters based on MGS to predict long term responses to PD1 blockade at 6 months in kidney+lung cancers (with or without ATB)

All the parameters stated above pertained to values calculated at baseline, at diagnosis, prior to initiation of therapy.

Here we outlined some MGS for which significant changes over the course of therapy will indicate the propensity of a patient to respond or relapse.

No significant changes appear significant between V1 (prior to therapy) and V2 (after 2 injections of mAb, 15 days apart).

Modifications observed at V3 (time of the first computerized tomography scan) are the most critical ones to predict clinical benefit (MGS data not shown). Modifications associated with a clinical benefit are:

(i) a loss of:

- *Bacteroides xylanosolvens CAG945*
- *Bacteroides uniformis CAG159 (common in 2 cancers)*
- *Bacteroides ovatus CAG1165*
- *Prevotella CAG :255, CAG163*
- *Ruminococcus bromii CAG611*
- *Roseburia intestinalis CAG291*
- *Eubacterium CAG :38, CAG 629*

and

(ii) a gain of:

- *Ruminococaeae CAG1003*

- *Flavonifractor plautii CAG 439*
- *Firmicutes bacterium CAG : 124, CAG 629.*

Example 17: Efficacy of oncobax across several tumor models and histological types in preclinical models

The following bacterial strains were used:

- *Enterococcus hirae*: strain deposited on November 7, 2013 at the *Collection Nationale de Cultures de Microorganismes* (CNCM), under the number I-4815
- *Akkermansia muciniphila*: strain deposited at the *Collection de souches de l'Unité des Rickettsies* (CSUR) under the reference CSUR P2261
- *Alistipes indistinctus*: strain deposited at the Collection de souches de l'Unité des Rickettsies (CSUR) under the reference CSUR P723.

Akkermansia muciniphila was grown on 5% sheep blood enriched Columbia agar (bioMerieux, Marcy l'Etoile, France) in an anaerobic atmosphere created using 3 anaerobic generators (bioMerieux) at 37°C for 72 hrs. *Alistipes indistinctus*, was also grown on 5% sheep blood enriched Columbia agar (bioMerieux, Marcy l'Etoile, France) in an anaerobic atmosphere created using a single anaerobic generator at 37°C for 48 hrs. *Enterococcus hirae* was grown on 5% sheep blood enriched Columbia agar (bioMerieux, Marcy l'Etoile, France) in an aerobic atmosphere at 37°C for 48 hrs.

For bacteria gavage: Suspensions of 10^9 cfu/mL were obtained using a fluorescence spectrophotometer (Eppendorf) at a wave length of 600 nm in PBS. Then mice orally received 100 µL of each bacterial suspension.

A. Model MCA205 sarcoma: the oncobax capable of compensating a dysbiosis (Fig. 25-26) are *Enterococcus. hirae, Akkermansia muciniphila* alone or admixed together simultaneously or sequentially, as well as *Alistipes indistinctus. Akkermansia muciniphila* + *Enterococcus hirae* and *Alistipes indistinctus* augmented effector and central memory CD8+ T cells in the tumor microenvironment (tumor draining lymph node at day 12 at sacrifice) (Fig. 27).

B. Model RET melanoma: As shown in Fig. 28-29, the combination of *Enterococcus hirae* + *Akkermansia. muciniphila* is very efficient in this model.

C. Model Lewis Lung orthotopic carcinoma: Fig. 30-31. This model shows that i) our conclusions hold true in the context of PD1 blockade in conjunction with radiotherapy, ii) in the absence of a dysbiosis, oncobax can still ameliorate the antitumor effects of the combination of ICB.

D. RENCA-luciferase tumor model where the combination of ICB is anti-CTLA4+anti-PD1 Ab: FMT was performed with feces from 2 dysbiotic patients. No *Akkermansia muciniphila/Ruminococcus bacterium D16 CAG949* were detectable in the stool composition in metagenomics. The FMT did not promote the efficacy of the combined ICB therapy (Fig. 32-33).

We conclude that administration of anticancer probiotics, or "oncobax", can improve the efficacy of anti-PD1 or anti-PDL1 or anti-PDL2 Abs in dysbiotic patients, dysbiosis being based on a MG analysis focusing on *Akkermansia muciniphila*, distinct CAG of *Ruminococcus*, *Alistipes*, *Eubacterium*, *Intestinihominas butyriciproducens*, *Bifidobacteria* (*adolescentis*, *longum*), and *Bacteroides* (*nordii, goldsteinii*), *Bilophila wadsworthia*…

Importantly, these anticancer probiotics also potentially improve the response in eubiotic patients and they boost the efficacy of not only anti-PD1 Ab alone, but also combined with anti-CTLA4 Ab or radiotherapy.

Example 18: Blood marker associated with bioactivity of « favorable feces composition or eubiosis » during PD-1 blockade

One of the best surrogate markers of efficacy or bioactivity of oncobax or FMT of a favorable stool (good MG composition) is the upregulation of PD-L1 expression on CD4+ or CD8+ circulating (or splenic) T cells (Fig. 34-37) or the upregulation of CCR9 on circulating T cells.

Example 19: FMT with feces from a responder patient can compensate a dysbiosis in a non-responder patient and restore the responsiveness to immune checkpoint inhibitors

In an orthotopic kidney cancer model (RENCA), we have shown that FMT from a dysbiotic non-responding RCC cancer patient (escaping from PD1 blockade) was able to abolish anti-PD1 efficacy in avatar mice. Oral gavage with feces from a responding RCC patient (who received anti -PD1 or

PD-L1 Ab and exhibited a clinical response according to RECIST criteria) was able to rescue the antitumoral efficacy of anti-PD1 lost in a dysbiotic avatar recipient. Hence, it is conceivable to treat a dysbiotic NSCLC/RCC or breast cancer patient with a FMT from a responder patient (feces at diagnosis or after 6 months of therapy), as an alternative to FMT from normal volunteers (Figure 38).

Example 20: FMT with feces from a healthy volunteer can compensate a dysbiosis in a non-responder patient and restore the response to metronomic cyclophosphamide (CTX)

Using a murine breast tumor model (AT3), we have shown that FMT from a dysbiotic breast cancer patient (non-responding to chemotherapy, triple negative, Ki67 high, who had a reduced alpha and beta diversity in MG analyses) reduced CTX efficacy in avatar AT3 bearing mice (Fig. 39). Compensation with feces from a healthy volunteer HV (either by oral gavage or cohousing) compensates for the loss of efficacy of CTX by restoring some kind of eubiosis (HV derived microflora).

Example 21: Dysbiosis from patients with distinct cancers can be compensated with defined strains and bacterial consortia

In an orthotopic kidney cancer model (RENCA), we have shown that FMT from a dysbiotic non-responding RCC cancer patient (escaping from PD1 blockade) was able to abolish anti-PD1 efficacy in avatar mice. Oral gavage with *Akkermansia muciniphila*, *Bacteroides salyersiae* or *Akkermansia muciniphila* and *Enterococcus hirae* was able to rescue the antitumoral efficacy of anti-PD1.

In parallel, in a different tumor model, a sarcoma in avatar mice, (as depicted in Fig. 40 to 43), we were able to ameliorate anti-PD1 efficacy that was compromised in FMT-treated mice bearing MCA205 sarcoma injected with anti-PD1 alone. Indeed, oral gavage with *Akkermansia muciniphila*, *Bacteroides salyersiae* or *Akkermansia muciniphila* and *Enterococcus hirae* was able to rescue the antitumoral efficacy of anti-PD1. Moreover, a combination of *Enterococcus hirae* (clones 13144=CNCM I-4815, IGR7=CNCM I-5224 and IGR4=CNCM I-5260) and *Barnesiella intestinihominis* or a combination of *Enterococcus hirae* (clones IGR7, IGR4, 13144) and *Christensenella minuta* or a combination of *Enterococcus hirae* (clones IGR7, IGR4, 13144) and

Actinobaculum schaalii or a combination of *Enterococcus hirae* (clones IGR7, IGR4, 13144) and *Akkermansia muciniphila* (p2261 and p3415) and *Eubacterium limosum* was efficient in doing so as well.

These consortia of commensals depicted here can be effective in the context of any tumor type amenable to a PD1 blockade, most specifically NSCLC, RCC and breast cancers.

Example 22: *Barnesiella intestinihominis* is associated with anti-PD1 efficacy

By performing metagenomic analysis of the stools of MCA205 tumor-bearing mice treated with anti-PD1 versus isotype control Ab, we were able to demonstrate the presence of *Barnesiella intestinihominis* in mice treated with anti-PD1 correlating with the treatment efficacy (Fig. 44). Indeed, oral gavages with *Barnesiella intestinihominis* are able to ameliorate anti-PD1 efficacy alone or combined with anti-CTLA4 Ab (Fig. 43).

We conclude that compensation with oncobax *Barnesiella intestinihominis* (strains derived from patients or mice) alone or in consortia comprising *Enterococcus hirae* isolates +/- *Akkermansia muciniphila* can restore efficacy to PD1 blockade or PD1/CTLA4 coblockade in kidney or lung cancer patients or any cancer patient amenable to a treatment with immune checkpoint inhibitors, specifically in those patients who received antibiotics or those patients diagnosed with a gut dysbiosis.

Example 23: Protecting oncobax decrease the toxicity of anti-PD1 treatment (autoimmune disorders generated with the combo anti-CTLA4+anti PD1 Ab)

In the same tumor model as that used in Example 21, the inventors demonstrated that it is possible to uncouple the efficacy and toxicity of immune checkpoint blockers. Indeed, as allustrated in Fig. 45, the transfer of feces from responders reduced the toxicity obtained with feces from non responders. FMT compensated with oncobax reduced colitis scores.

Example 24: Immune checkpoint blockers induce significant changes in the intestinal microbiome composition that participate to treatment efficacy (reduction of tumor size).

We have performed a serial analyses of microbiome changes over time in two different mouse tumor models (MCA205 sarcoma and RET melanoma) located in the skin by analyzing the significant modulation of bacterial composition using 16S rRNA sequencing of gene amplicons of mice stools, in 6 mice/group, prior to, after 2 and 5 injections of anti-PD1 Ab alone or combined to anti-CTLA4 Abs (Fig. 46). Principle component analyses of the beta diversity show significant differences overtime, meaning that the combination of ICB induce shifts in the microbiome composition that are significantly correlated with tumor sizes (Fig. 47, Fig. 48).

The 78 bacterial species found in common in both tumor models in responders (but not in non responders) belong to SCFA producing bacteria (health-realted bacteria such as *Clostridiacae, Blautia, Faecalibacterium prausnitzii*) (Fig. 49). We confirm the dominance of certain members of the Bacteroidetes genus (*Bacteroides finegoldii, Bacteroides Intestinalis, Bacteroides mediterraneensis*), as well as *Alistipes onderdonkii, Bifidobacteria* and *Eubacterium* found in the human lung/kidney cancer patients responding to PD1 blockade. Based on other data from a previous patent application EP 17306509.5 (colon cancer and oxaliplatin, where *Erysipelotrichaceae Erysipelatoclostridium ramosum* were found immunogenic), we extrapolate the potential beneficial role of *Dielma fastidiosa (Firmicutes/Erysipelotrichia*) in the efficacy of immune cehckpoint blockers (ICB) (Figure 49).

Interestingly, common bacteria shared in responders to the combination of anti-PD1+anti-CTLA4 comprise *Akkermansia muciniphila, Alistipes indistinctus,* several *Bacteroides* spp., *Barnesiella intestinihominis*, *Erysipelatoclostridium ramosum* and *Flavonifractor plautii*, all found in humans (NSCLC/Kidney cancer) responding to PD1 blockade and in our list of immunogenic probiotics “oncobax” (Figure 50). The association of these bacteria provides an ecosystem associated with clinical response to combination of ICB.

REFERENCES

Arrieta, M.-C., Stiemsma, L.T., Dimitriu, P.A., Thorson, L., Russell, S., Yurist-Doutsch, S., Kuzeljevic, B., Gold, M.J., Britton, H.M., Lefebvre, D.L., *et al.* (2015). Early infancy microbial and metabolic alterations affect risk of childhood asthma. Sci. Transl. Med. *7*, 307ra152.

Berman, D., Parker, S.M., Siegel, J., Chasalow, S.D., Weber, J., Galbraith, S., Targan, S.R., and Wang, H.L. (2010). Blockade of cytotoxic T-lymphocyte antigen-4 by ipilimumab results in dysregulation of gastrointestinal immunity in patients with advanced melanoma. Cancer Immun. *10*, 11.

Blaser, M.J. (2016). Antibiotic use and its consequences for the normal microbiome. Science *352*, 544–545.

Champiat, S., Lambotte, O., Barreau, E., Belkhir, R., Berdelou, A., Carbonnel, F., Cauquil, C., Chanson, P., Collins, M., Durrbach, A., *et al.* (2016). Management of immune checkpoint blockade dysimmune toxicities: a collaborative position paper. Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. *27*, 559–574.

Clarke, J., Wu, H.-C., Jayasinghe, L., Patel, A., Reid, S., and Bayley, H. (2009). Continuous base identification for single-molecule nanopore DNA sequencing. Nat. Nanotechnol. *4*, 265–270.

Eid, J., Fehr, A., Gray, J., Luong, K., Lyle, J., Otto, G., Peluso, P., Rank, D., Baybayan, P., Bettman, B., *et al.* (2009). Real-time DNA sequencing from single polymerase molecules. Science *323*, 133–138.

Fidler, M.M., Soerjomataram, I., and Bray, F. (2016). A global view on cancer incidence and national levels of the human development index. Int. J. Cancer *139*, 2436–2446.

Garon, E.B. (2015). Current Perspectives in Immunotherapy for Non-Small Cell Lung Cancer. Semin. Oncol. *42 Suppl 2*, S11-18.

Gensollen, T., Iyer, S.S., Kasper, D.L., and Blumberg, R.S. (2016). How colonization by microbiota in early life shapes the immune system. Science *352*, 539–544.

Goodrich, J.K., Davenport, E.R., Waters, J.L., Clark, A.G., and Ley, R.E. (2016). Cross-species comparisons of host genetic associations with the microbiome. Science *352*, 532–535.

Hugon, P., Lagier, J.-C., Colson, P., Bittar, F., and Raoult, D. (2016). Repertoire of human gut microbes. Microb. Pathog.

Li, J., Jia, H., Cai, X., Zhong, H., Feng, Q., Sunagawa, S., Arumugam, M., Kultima, J.R., Prifti, E., Nielsen, T., *et al.* (2014). An integrated catalog of reference genes in the human gut microbiome. Nat. Biotechnol. *32*, 834–841.

Nielsen, H.B., Almeida, M., Juncker, A.S., Rasmussen, S., Li, J., Sunagawa, S., Plichta, D.R., Gautier, L., Pedersen, A.G., Le Chatelier, E., *et al.* (2014). Identification and assembly of genomes and genetic elements in complex metagenomic samples without using reference genomes. Nat. Biotechnol. *32*, 822–828.

Pigneur, B., and Sokol, H. (2016). Fecal microbiota transplantation in inflammatory bowel disease: the quest for the holy grail. Mucosal Immunol. *9*, 1360–1365.

Pitt, J.M., Vétizou, M., Daillère, R., Roberti, M.P., Yamazaki, T., Routy, B., Lepage, P., Boneca, I.G., Chamaillard, M., Kroemer, G., *et al.* (2016). Resistance Mechanisms to Immune-Checkpoint Blockade in Cancer: Tumor-Intrinsic and -Extrinsic Factors. Immunity *44*, 1255–1269.

Plovier, H., Everard, A., Druart, C., Depommier, C., Van Hul, M., Geurts, L., Chilloux, J., Ottman, N., Duparc, T., Lichtenstein, L., *et al.* (2016). A purified membrane protein from Akkermansia muciniphila or the pasteurized bacterium improves metabolism in obese and diabetic mice. Nat. Med.

Raoult, D. (2016). Microbiota, obesity and malnutrition. Microb. Pathog.

Rizvi, N.A., Mazières, J., Planchard, D., Stinchcombe, T.E., Dy, G.K., Antonia, S.J., Horn, L., Lena, H., Minenza, E., Mennecier, B., *et al.* (2015). Activity and safety of nivolumab, an anti-PD-1 immune checkpoint inhibitor, for patients with advanced, refractory squamous non-small-cell lung cancer (CheckMate 063): a phase 2, single-arm trial. Lancet Oncol. *16*, 257–265.

Rooks, M.G., and Garrett, W.S. (2016). Gut microbiota, metabolites and host immunity. Nat. Rev. Immunol. *16*, 341–352.

Shono, Y., Docampo, M.D., Peled, J.U., Perobelli, S.M., Velardi, E., Tsai, J.J., Slingerland, A.E., Smith, O.M., Young, L.F., Gupta, J., *et al.* (2016). Increased GVHD-related mortality with broad-spectrum antibiotic use after allogeneic hematopoietic stem cell transplantation in human patients and mice. Sci. Transl. Med. *8*, 339ra71.

Sivan, A., Corrales, L., Hubert, N., Williams, J.B., Aquino-Michaels, K., Earley, Z.M., Benyamin, F.W., Lei, Y.M., Jabri, B., Alegre, M.-L., *et al.* (2015). Commensal Bifidobacterium promotes antitumor immunity and facilitates anti-PD-L1 efficacy. Science *350*, 1084–1089.

Taur, Y., Jenq, R.R., Ubeda, C., van den Brink, M., and Pamer, E.G. (2015). Role of intestinal microbiota in transplantation outcomes. Best Pract. Res. Clin. Haematol. *28*, 155–161.

Vétizou, M., Pitt, J.M., Daillère, R., Lepage, P., Waldschmitt, N., Flament, C., Rusakiewicz, S., Routy, B., Roberti, M.P., Duong, C.P.M., *et al*. (2015). Anticancer immunotherapy by CTLA-4 blockade relies on the gut microbiota. Science *350*, 1079–1084.

Viaud, S., Saccheri, F., Mignot, G., Yamazaki, T., Daillère, R., Hannani, D., Enot, D.P., Pfirschke, C., Engblom, C., Pittet, M.J., *et al*. (2013). The intestinal microbiota modulates the anticancer immune effects of cyclophosphamide. Science *342*, 971–976.

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0-1	**Form PCT/RO/134 Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis)**	
0-1-1	Prepared Using	PCT Online Filing Version 3.5.000.251e MT/FOP 20141031/0.20.5.20
0-2	**International Application No.**	EP2017084552
0-3	**Applicant's or agent's file reference**	B160458QTA

1	**The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:**	
1-1	**page**	40
1-2	**line**	13
1-3	**Identification of deposit**	
1-3-1	Name of depositary institution	CNCM Collection nationale de cultures de micro-organismes (CNCM)
1-3-2	Address of depositary institution	Institut Pasteur, 25 Rue du Dr. Roux, 75724 Paris Cedex 15, France
1-3-3	Date of deposit	07 November 2013 (07.11.2013)
1-3-4	Accession Number	CNCM I-4815
1-4	**Additional Indications**	Enterococcus hirae 13144
1-5	**Designated States for Which Indications are Made**	All designations
2	**The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:**	
2-1	**page**	40
2-2	**line**	14
2-3	**Identification of deposit**	
2-3-1	Name of depositary institution	CNCM Collection nationale de cultures de micro-organismes (CNCM)
2-3-2	Address of depositary institution	Institut Pasteur, 25 Rue du Dr. Roux, 75724 Paris Cedex 15, France
2-3-3	Date of deposit	31 August 2017 (31.08.2017)
2-3-4	Accession Number	CNCM I-5224
2-4	**Additional Indications**	Enterococcus hirae IGR7
2-5	**Designated States for Which Indications are Made**	All designations

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3	**The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:**	
3-1	**page**	40
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3-3	**Identification of deposit**	
3-3-1	Name of depositary institution	CNCM Collection nationale de cultures de micro-organismes (CNCM)
3-3-2	Address of depositary institution	Institut Pasteur, 25 Rue du Dr. Roux, 75724 Paris Cedex 15, France
3-3-3	Date of deposit	27 November 2017 (27.11.2017)
3-3-4	Accession Number	CNCM I-5260
3-4	**Additional Indications**	Enterococcus hirae IGR4
3-5	**Designated States for Which Indications are Made**	All designations
4	**The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:**	
4-1	**page**	41
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4-3	**Identification of deposit**	
4-3-1	Name of depositary institution	CNCM Collection nationale de cultures de micro-organismes (CNCM)
4-3-2	Address of depositary institution	Institut Pasteur, 25 Rue du Dr. Roux, 75724 Paris Cedex 15, France
4-3-3	Date of deposit	27 November 2017 (27.11.2017)
4-3-4	Accession Number	CNCM I-5261
4-4	**Additional Indications**	Enterococcus hirae IGR11
4-5	**Designated States for Which Indications are Made**	All designations

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CLAIMS

1. Use of a composition comprising bacteria of the species *Enterococcus hirae* and bacteria selected from the group consisting of *Akkermansia muciniphila, Christensenella minuta* and mixtures thereof, in the manufacture of a medicament in the form of an immunostimulating anti-cancer agent, for the treatment of cancer amenable to treatment by PD1 blockade, in combination with an anti-PD1/PD-L1/PD-L2 Ab-based therapy.

2. The use of claim 1, wherein the composition further comprises bacteria of at least one species selected from the group consisting of *Barnesiella intestinihominis, Alistipes shahii*, *Eubacterium* species, *Ruminococcacae, Clostridiales* species, *Bacteroidales* species*, Actinobacteria, Coriobacteriales* species and *Methanobrevibacter smithii*.

3. The use of claim 1 or claim 2, wherein the composition comprises bacteria of the species *Enterococcus hirae* and *Akkermansia muciniphila.*

4. The use of any of claims 1 to 3, wherein said composition further comprises bacteria of the species *Burkholderia cepacia* and/or *Bacteroides fragilis* and/or *Actinotignum schaalii* and/or *Alistipes indistinctus* and/or *onderdonkii.*

5. The use of any of claims 1 to 4, wherein said composition further comprises:

- *Erysipelotrichia* of the species *Dielma fastidiosa or Erysipelatoclostridium ramosum*; and/or
- *Alistipes* bacteria of species selected from the group consisting of *Alistipes shahii, Alistipes indistinctus, Alistipes onderdonkii* and *Alistipes finegoldii*; and/or
- *Eubacterium* bacteria of the species *Eubacterium limosum*; and/or
- *Bacteroidales* bacteria of the species *Bacteroides salyersiae* and/or *Barnesiella intestinihominis*; and/or
- *Actinobacteria* of the species *Actinotignum schaalii;* and/or
- *Coriobacteriales bacteria* of the species *Collinsella intestinalis* and/or *Collinsella tanakaei*; and/or
- *Firmicutes bacteria* of the species *Flavonifractor plautii.*

6. The use of any of claims 1 to 4, wherein said composition comprises:

(i) *Enterococcus hirae* selected from the group consisting of *Enterococcus hirae* strain 13144 deposited on November 7, 2013 at the *Collection Nationale de Cultures de Microorganismes* (CNCM) under the number I-4815, *Enterococcus hirae* strain IGR7 deposited on August 31, 2017 at the CNCM under the number I-5224, *Enterococcus hirae* strain IGR4 deposited on November 27, 2017 at the CNCM under the number CNCM I-5260, *Enterococcus hirae* strain IGR11 deposited on November 27, 2017 at the CNCM under the number CNCM I-5261 and mixtures thereof; and

(ii) *Akkermansia muciniphila* selected from the group consisting of *Akkermansia muciniphila* strains p2261 and p3415, both deposited at the *Collection de souches de l'Unité des Rickettsies* (CSUR) and mixtures thereof; and

(iii) *Eubacterium limosum.*

7. The use of any of claims 1 to 4, wherein said composition further comprises:

(i) *Enterococcus hirae* selected from the group consisting of *Enterococcus hirae* strain 13144 deposited on November 7, 2013 at the *Collection Nationale de Cultures de Microorganismes* (CNCM) under the number I-4815, *Enterococcus hirae* strain IGR7 deposited on August 31, 2017 at the CNCM under the number I-5224, *Enterococcus hirae* strain IGR4 deposited on November 27, 2017 at the CNCM under the number CNCM I-5260, *Enterococcus hirae* strain IGR11 deposited on November 27, 2017 at the CNCM under the number CNCM I-5261 and mixtures thereof; and

(ii) *Barnesiella intestinihominis.*

8. The use of any of claims 1 to 4, wherein said composition comprises:

(i) *Enterococcus hirae* selected from the group consisting of *Enterococcus hirae* strain 13144 deposited on November 7, 2013 at the *Collection Nationale de Cultures de Microorganismes* (CNCM) under the number I-4815, *Enterococcus hirae* strain IGR7 deposited on August 31, 2017 at the CNCM under the number I-5224, *Enterococcus hirae* strain IGR4 deposited on November 27, 2017 at the CNCM under the number CNCM I-5260, *Enterococcus hirae* strain IGR11

deposited on November 27, 2017 at the CNCM under the number CNCM I-5261 and mixtures thereof; and

(ii) *Christensenella minuta.*

9. The use of any of claims 1 to 8, wherein the medicament is for compensating dysbiosis in a cancer patient.

10. The use of any of claim 1 to 9, wherein the medicament is for an individual who is to be treated with anti-PD1 and anti-CTLA4 coblockade or with anti-PD-L2 and anti-CTLA4 coblockade.

11. Use of a composition as described in claim 3, for the manufacture of a medicament for decreasing or preventing gut toxicity of an anti-PD1/PD-L1/PD-L2 Ab-based therapy and/or an anti-CTLA-4 Ab-based therapy.

12. Use of a fecal microbial composition comprising *Akkermansia muciniphila* and *Enterococcus hirae* and further comprising bacterial species selected from the group consisting of *Clostridiales* species, *Alistipes shahii*, *Eubacterium* species, *Bacteroidales* species and *Methanobrevibacter smithii,* in the manufacture of an agent for treating a cancer amenable to treatment by PD1 blockade, in combination with an anti-PD1/PD-L1/PD-L2 Ab-based therapy.

13. The use of claim 12, wherein said composition has been enriched with a composition as described in any of claims 1 to 9.