

# Novel insights on gut microbiota manipulation and immune checkpoint inhibition in cancer (Review)

SILVIA VIVARELLI<sup>1</sup>, LUCA FALZONE<sup>2</sup>, GIULIA COSTANZA LEONARDI<sup>1</sup>,  
MARIO SALMERI<sup>1,3\*</sup> and MASSIMO LIBRA<sup>1,3\*</sup>

<sup>1</sup>Department of Biomedical and Biotechnological Sciences, University of Catania, I-95123 Catania;

<sup>2</sup>Epidemiology and Biostatistics Unit, IRCCS Istituto Nazionale Tumori 'Fondazione G. Pascale', I-80131 Naples;

<sup>3</sup>Research Centre for Prevention, Diagnosis and Treatment of Cancer, University of Catania, I-95123 Catania, Italy

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**Abstract.** Cancer affects millions of individuals worldwide. Thus, there is an increased need for the development of novel effective therapeutic approaches. Tumorigenesis is often coupled with immunosuppression which defeats the anticancer immune defense mechanisms activated by the host. Novel anticancer therapies based on the use of immune checkpoint inhibitors (ICIs) are very promising against both solid and hematological tumors, although still exhibiting heterogeneous efficacy, as well as tolerability. Such a differential response seems to derive from individual diversity, including the gut microbiota (GM) composition of specific patients. Experimental evidence supports the key role played by the GM in the activation of the immune system response against malignancies. This observation suggests to aim for patient-tailored complementary therapies able to modulate the GM, enabling the selective enrichment in microbial species, which can improve the positive outcome of ICI-based immunotherapy. Moreover, the research of GM-derived predictive biomarkers may help to identify the selected cancer population, which can benefit from ICI-based therapy, without the occurrence of adverse reactions and/or cancer relapse. The present review summarizes the landmark studies published to date, which have contributed to uncovering the tight link existing between GM composition, cancer development and the host immune

system. Bridging this triangle of interactions may ultimately guide towards the identification of novel biomarkers, as well as integrated and patient-tailored anticancer approaches with greater efficacy.

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## 1. Introduction

The gut microbiota (GM) is composed of >100 trillion of microorganisms (including bacteria, viruses, protozoa and fungi) resident in the gastro-intestinal lumen, mainly the large intestine (1). Among all, bacteria represent the broader category, with thousands of different species, belonging to the Firmicutes and Bacteroidetes phyla in particular (2). Gut microbes have been widely studied given their key role in the modulation of both the host homeostasis and pathology (3). The main functions of the GM include the following: i) The maintenance of the host's gut health; ii) gastrointestinal barrier function; and iii) the neo-synthesis or transformation of dietary compounds and essential nutrients (4-6). The fulfillment of all the aforementioned activities suggests the establishment of a functional two-way association between GM and the host immune system (7). This interrelation guarantees the preservation of a microbial balance or eubiosis (7). However, the absence of such an equilibrium (with the concurrent depletion of the gut microflora) is termed dysbiosis and is associated with a number of pathologies, including diabetes, inflammation, autoimmune disorders and cancer (8).

In order to examine the impact of GM on human health, it is important to characterize what is known as the gut microbiome, corresponding to the entire genome of the whole GM. The gut microbiome accounts for 100-fold more genes than the entire human genome (9). Presently, the advent of

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*Correspondence to:* Dr Luca Falzone, Epidemiology and Biostatistics Unit, IRCCS Istituto Nazionale Tumori 'Fondazione G. Pascale', Via Mariano Semmola 53, I-80131 Naples, Italy  
E-mail: l.falzone@istitutotumori.na.it

Professor Massimo Libra, Department of Biomedical and Biotechnological Sciences, University of Catania, Via Santa Sofia 97, I-95123 Catania, Italy  
E-mail: m.libra@unict.it

\*Contributed equally

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metagenomics allows the transition from merely depicting the microbiome composition to functionally analyzing the impact of the microbiome balance vs. imbalance in human health (10-12). The computational analysis of the 16S rRNA amplicons coupled with next-generation sequencing (NGS) allows the characterization of both the abundance and diversity of the gut microbiome. Overall, metagenomics, together with metatranscriptomics, metabolomics and proteomics help to quantify the impact of specific bacterial species on human health (13-15).

The reconstruction of the whole bacterial genomes beginning from metagenomic datasets is currently helping to identify uncharacterized bacterial species, both from the gut and other body sites, thus expanding the known phylogenetic diversity. The recent study from Almeida *et al.* (16) established the ultimately most comprehensive collection of microbial genomes composing the human gut microbiome, comprised of >200,000 of non-redundant genomes from 4,644 gut prokaryotes. This will allow their use as a reference in future metagenomics studies (16). Given the tight interconnection occurring between the gut microbiome and the human host, it is of pivotal importance to identify all the host-dependent variables (e.g., physiology, lifestyle habits and diet), which alter the GM to further increase both robustness and the reproducibility of metadata analyses (17). This will help to identify the members of the GM that are directly associated with human diseases, including cancer, and ultimately with the response to anticancer therapy (7).

Cancer is a leading cause of mortality worldwide, second only to cardiovascular diseases, accounting for almost 10 million deaths in 2020 (18). Over the past 10 years, anticancer immunotherapy has taken the central stage in the treatment of a variety of tumors (19). In general, immunotherapy targets immune cells in order to activate or boost their capacity of eliminating cancerous cells. Malignant cells are surrounded by a variety non-cancer cells, including stromal cells and immune cells [e.g., macrophages, dendritic cells (DCs), natural killer (NK) cells, T-cells and B-cells], together forming the so-called tumor microenvironment (20,21). The 'immune contexture' of a given tumor plays a key role in both the prognosis and treatment of cancer patients. Despite the presence of cancer immune-evasion mechanisms, any residual anticancer immune response may suggest a better prognosis. Moreover, anticancer therapies triggering the 'immune contexture' may produce a more durable anticancer efficacy (22).

Among the immunotherapies which are currently tested in clinical practice, immune checkpoint inhibitors (ICIs) have been shown to efficiently reshape the host immune response against cancer (23). The discovery of immune checkpoints led to the designation of the 2018 Nobel Prize in Physiology or Medicine to the two scientists, James Allison and Tasuku Honjo (24).

ICIs are monoclonal antibodies designed to inhibit the immune checkpoint pathway, thereby boosting the host immune system to efficiently eliminate cancer cells (25). In detail, ICIs trigger cytotoxic CD8<sup>+</sup> T-cells to destroy target malignant cells, thereby reactivating the cancer immunity cycle (26). In fact, the immune checkpoints are co-receptors of the T receptor signaling complex, which overall prevent T-cell overactivation and establish the tolerance against

self-antigens (27). The suppression of T-cell activation via these co-receptors constitutes the main immune escape mechanism carried out by neoplastic cells. In fact, when cancer cells efficiently activate the immune checkpoint, they may evade the immune system and overgrow (28). However, the administration of ICIs blocks these inhibitory co-receptors and positively modulates CD8<sup>+</sup> T-cell cytotoxicity, directing it to effectively eliminating malignant cells (28).

Approved ICIs can target two main co-inhibitory routes: Either the programmed cell death protein 1 (PD-1) or the cytotoxic T-lymphocyte antigen-4 (CTLA-4) pathway (29). PD-1 is expressed principally by T-cells and other immune cells (including NK cells, DCs and B-cells), whereas its ligand, programmed cell death ligand 1 (PD-L1) is expressed by the antigen-presenting cells (APCs), including malignant cells (30). Thus, antibodies directed against PD-1 or PD-L1 block the inhibitory immune checkpoint interaction between CD8<sup>+</sup> T-cells and tumor cells, rehabilitating cytotoxic T-cells to efficiently eliminate target cancer cells (31). Instead, CTLA4 is a receptor expressed by T- and B-cells, and inhibits the binding of CD28 receptor with its B7 ligand, expressed by APCs at the earlier phases of antigen presentation (32). Consequently, ICIs targeting CTLA4 receptor reactivate the cellular-mediated immune response earlier in the cancer immunity cycle (33).

Since the first ICI was authorized by the US Food and Drug Administration (FDA) in 2011, a wide range of ICIs has been further approved (34). ICIs have been presently employed for the treatment of ~50 cancer types either as late-line, first-line, or as neoadjuvant therapies. They are administered either as single agents or in combination (with chemotherapy or with another ICI) (34). ICIs are currently under study in >60% of all ongoing oncology clinical trials (for some examples on the specific use of ICIs in oncology in relation to the gut microbiome, please see the studies listed in Tables I and II) (35,36). Impressively, for a number of recalcitrant and otherwise incurable tumors, such as advanced melanoma (AM), metastatic melanoma (MM) or advanced non-small cell lung cancer (NSCLC), the use of ICIs has led to a substantial long-term remission (37,38).

Despite this substantial clinical success, the administration of ICIs is accompanied by some limitations. Amongst the reported issues, there is the modification of the main clinical endpoints due to the often-associated delay in the appearance of positive effects mediated by ICI-based immunotherapy (21,39). Additionally, in the majority of cases, patients with advanced disease finally develop resistance to ICIs, mainly due to the development of innate and adaptive immune-resistance to the checkpoint blockade (40,41). Finally, ICI blockage may be associated with the occurrence of a broad range of immune-related adverse events (irAEs), caused by the potential immune and pro-inflammatory overactivation of the host's immune system. The reported irAEs are several, and include: Colitis, intestinal mucositis, diarrhea, thyroiditis, hepatitis, dermatological manifestations, pneumonitis, myocarditis and others. The outcome of irAEs can range from mild to severe and in some cases, fatal events occur (42,43).

The differential response of patients observed with the use of ICIs, in terms of both efficacy and tolerability, can be linked to the intrinsic individual diversity of the immune system and other host-related factors (44). Thus, identifying effective

Table I. Current ongoing clinical trials registered at *clinicaltrials.gov* that analyze the intestinal microbiota through metagenomics during immune-checkpoint immunotherapy (alone or in combination with other anti-cancer therapies).

Tim	Condition(s)	Anticancer therapy	Enrollment	Start date	(Refs.)
NCT02600143	Melanoma	ICIs	123	2013	n.a.
NCT01896999	Hodgkin lymphoma	Ipilimumab; nivolumab; brentuximab	126	2014	(146)
NCT02478099	Advanced solid tumors	MPDL3280A	98	2016	(147)
NCT02681302	Multiple myeloma; lymphoma	Ipilimumab; nivolumab	42	2016	n.a.
NCT04204434	Advanced solid tumors	ICIs	150	2016	n.a.
NCT02858921	Melanoma	Dabrafenib; trametinib; pembrolizumab	60	2017	n.a.
NCT03083691	Non-small cell lung cancer	Ipilimumab; nivolumab	106	2017	n.a.
NCT03161756	Melanoma	Ipilimumab; nivolumab; denosumab	72	2017	n.a.
NCT03164993	Breast cancer	Atezolizumab; doxorubicin; cyclophosphamide	75	2017	(148)
NCT03168464	Non-small cell lung cancer	Ipilimumab; nivolumab; radiotherapy	45	2017	n.a.
NCT03331562	Pancreatic cancer	Pembrolizumab	24	2017	n.a.
NCT03289819	Breast cancer	Pembrolizumab; paclitaxel; epirubicin; cyclophosphamide	50	2018	n.a.
NCT03688347	Lung cancer	ICIs	60	2018	n.a.
NCT04054908	Gastrointestinal cancer	SOC, ICIs	60	2018	n.a.
NCT04169867	Melanoma	Nivolumab; ipilimumab; atezolizumab	1160	2018	n.a.
NCT04579978	Advanced solid tumors	ICIs	60	2018	n.a.
NCT03694834	Endometrial cancer	Pembrolizumab	20	2019	n.a.
NCT03799744	Head and neck cancer	VCN-01; durvalumab	20	2019	n.a.
NCT03818061	Head and neck cancer	Atezolizumab; bevacizumab	110	2019	n.a.
NCT03894007	Breast cancer	Docetaxel; carboplatin; trastuzumab; pertuzumab; epirubicin; cyclophosphamide; atezolizumab	190	2019	n.a.
NCT04006262	Oeso-gastric cancer	Ipilimumab; nivolumab	32	2019	(149)
NCT04013542	Lung cancer	Ipilimumab; nivolumab; radiotherapy	20	2019	n.a.
NCT04133948	Melanoma	Nivolumab; ipilimumab; domatinostat	45	2019	n.a.
NCT04136470	Non-small cell lung cancer; melanoma	ICIs	130	2019	n.a.
NCT04196465	Gastric cancer; esophageal cancer; liver cancer	IMC-001	48	2019	n.a.
NCT04291755	Non-small-cell lung cancer; colorectal cancer	Pembrolizumab	100	2019	n.a.
NCT03977571	Renal cell cancer	Ipilimumab; Nivolumab	400	2020	n.a.
NCT04063501	Lung cancer	Anti-PD-1 antibodies	80	2020	n.a.
NCT04090710	Renal cell cancer	Ipilimumab; nivolumab; radiotherapy	78	2020	n.a.
NCT04107168	Melanoma; renal cancer; lung cancer	Nivolumab; pembrolizumab; ipilimumab; durvalumab; tremelimumab; atezolizumab; bevacizumab	1800	2020	n.a.
NCT04189679	Non-small cell lung cancer	ICIs	60	2020	n.a.
NCT04207086	Melanoma	Pembrolizumab; lenvatinib	20	2020	n.a.

Table I. Continued.

Tim	Condition(s)	Anticancer therapy	Enrollment	Start date	(Refs.)
NCT04271384	Non-small cell lung cancer	Nivolumab; SOC	30	2020	n.a.
NCT04312308	Non-small cell lung cancer	Atezolizumab	100	2020	n.a.
NCT04333004	Non-small cell lung cancer (brain metastases)	Pembrolizumab; chemotherapy	40	2020	n.a.
NCT04392505	Non-small cell lung cancer	Durvalumab	100	2020	n.a.
NCT04435964	Melanoma; lung cancer; head and neck cancer; urogenital cancer; breast cancer	ICIs	400	2020	n.a.
NCT04566029	Urothelial cancer	SOC, ICIs	40	2020	n.a.
NCT04636775	Non-small cell lung cancer	ICIs	46	2020	n.a.
NCT04638751	Non-small cell lung cancer; colorectal cancer; triple negative breast cancer; pancreas cancer	ICIs, chemotherapy	4000	2020	n.a.
NCT04680377	Non-small cell lung; advanced lung cancer	Durvalumab	44	2020	n.a.
NCT04169074	Head and neck cancer	Nivolumab; abemaciclib	20	2021	n.a.
NCT04602078	Urothelial cancer	Atezolizumab; gemcitabine; cisplatin	66	2021	n.a.
NCT04698161	Non-Small cell lung cancer; melanoma	ICIs	50	2021	n.a.
NCT04711330	Non-small cell lung cancer	Durvalumab	126	2021	n.a.
NCT04743752	Non-small cell lung cancer	ICIs	200	2021	n.a.
NCT04804137	Non-small cell lung cancer; metastatic lung cancer	ICIs	80	2021	n.a.

ICIs, immune checkpoint inhibitors; SOC, standard of care; n.a., not available.

methods with which to identify the specific features of the individual immune system and direct it to better respond to ICIs represents the current challenge of ICI research (44). As it will be largely discussed below, the GM is a master regulator of the immune system; therefore, it directly affects both the efficacy and toxicity of ICIs (45,46) (Fig. 1).

Consequently, the GM can be used as a powerful source to identify novel diagnostic and prognostic microbial-derived biomarkers, as well as innovative therapeutic targets (47,48). In fact, recent milestone findings have highlighted the presence of specific gut bacterial species which are able to improve both the compliance to, as well as the effectiveness of anticancer therapies, particularly ICIs (49). Notably, in 2021, for the first time, to the best of our knowledge, two groundbreaking studies demonstrated that fecal microbiota transplantation (FMT) can efficiently boost the anticancer efficacy of ICIs in patients with AM and MM (50,51).

The present review summarizes the up-to-date studies on the role played by the GM in modulating the host immune system, thus influencing both the safety and the outcome to ICI-based anticancer therapy in cancer patients. Current findings indicate that each individual cancer patient has a specific GM footprint. Research efforts are presently focusing on developing effective strategies which can be used to manipulate the GM in a patient-tailored manner, with the aims of:

i) Improving the efficacy of ICIs; and ii) actively reducing the occurrence of irAEs linked with ICI administration.

Compared with the existing literature on this topic, the general aim of the present review was to provide a concise and complete overview of the milestone studies that have contributed to deciphering the complicated association between gut microbial health, the host immune system and ICI activity over the past decade. Overall, the hidden potential of treating cancer patients with a more holistic therapeutic approach is strongly emerging, through the administration of integrated therapies (e.g., ICIs combined with GM modulators) tailored around the features of each specific patient (including gut microbial composition and immune system reactivity).

## 2. Gut microbiota and the host immune system

A dynamic two-way association occurs between GM and the host immune system through the course of a lifetime (52). The GM plays a key role in both shaping and modulating the immune system. In turn, the immune system regulates the gut microbial balance and it helps to maintain a healthy gut homeostasis (53). Any imbalance in this association could contribute to the development of several pathological conditions, including immune-mediated disorders, as well as cancer (54).

Table II. Current ongoing clinical trials registered at *clinicaltrials.gov* using interventions to modulate intestinal microbiota in association with immune-checkpoint immunotherapy.

NCT Number	Condition(s)	Anticancer therapy	Gut microbial modulation	Enrollment	Start date
NCT03353402	Melanoma	ICIs	Fecal microbiota transplantation (from patients treated with ICIs in remission from 1 year)	40	2017
NCT03686202	Advanced solid tumors	ICIs	MET-4 (microbial ecosystem therapeutics)	65	2018
NCT04056026	Mesothelioma	Pembrolizumab	Fecal microbiota transplantation (From healthy family donors)	1	2018
NCT03341143	Melanoma	Pembrolizumab	Fecal microbiota transplantation	20	2018
NCT03595683	Melanoma	Pembrolizumab	EDP1503 (Bifidobacterium animalis)	70	2018
NCT03775850	Colorectal cancer; triple negative breast cancer; non-small cell lung cancer; bladder cancer; oeso-gastric cancer; renal cell cancer	Pembrolizumab	EDP1503 (Bifidobacterium animalis)	120	2018
NCT03817125	Melanoma	Nivolumab	Vancomycin pretreatment; SER-401 (adjunctive microbiome therapy)	14	2019
NCT03637803	Advanced solid tumors	Pembrolizumab	MRx0518 (Enterococcus gallinarum)	132	2019
NCT03772899	Melanoma	ICIs	Fecal microbial transplantation (From single healthy donor)	20	2019
NCT04116775	Prostate cancer	Pembrolizumab; enzalutamide	Fecal microbial transplantation	32	2019
NCT03829111	Renal cell cancer	Ipilimumab; nivolumab	CBM-588 (Clostridium butyricum)	30	2019
NCT04105270	Lung cancer	Durvalumab; cisplatin; carboplatin	Oral restorative microbiota therapy (microbial ecosystem therapeutics)	30	2020
NCT04114136	Advanced solid tumors	Nivolumab; pembrolizumab	Metformin; rosiglitazone (metabolism modulatory molecules)	108	2020
NCT04601402	Non-small cell lung cancer; head and neck cancer; urothelial cancer	Avelumab	GEN-001 (Single strain bacteria isolated from gut of healthy human volunteers)	93	2020
NCT04577729	Melanoma	ICIs	Fecal microbial transplantation (From patients treated with ICIs in remission from 1 year)	60	2020
NCT04130763	Gastrointestinal cancer	Anti-PD-1 antibodies	Fecal microbial transplantation (From healthy donors)	10	2020
NCT04163289	Renal cell cancer	ICIs	Fecal microbial transplantation (From healthy donors)	20	2020
NCT03819296	Melanoma	Infliximab; prednisone; vedolizumab	Fecal microbial transplantation	800	2020
NCT04038619	Genitourinary cancer	ICIs	Fecal microbial transplantation	40	2020
NCT04521075	Melanoma	Nivolumab	Fecal microbial transplantation	50	2020
NCT04758507	Renal cell cancer	ICIs	Fecal microbial transplantation	50	2021

ICIs, immune checkpoint inhibitors.

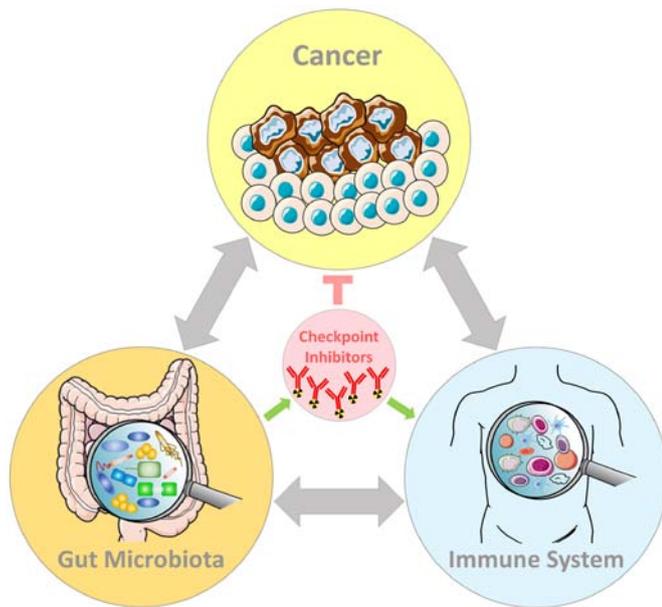


Figure 1. Complex interplay between gut microbiota, immune system and cancer during immune-checkpoint inhibition. Gut microbiota may positively or negatively modulate tumor growth; it may also regulate immune system response. Host immune system inhibits tumor growth and it can also be activated by gut microbiota. Cancer can alter host immune response by activating immunosuppressive pathways; also, cancer may modulate gut microbiota. Anticancer immunotherapy, based on the administration of immune checkpoint inhibitory antibodies, blocks tumor growth as it is able to inhibit immunosuppressive pathways. A favorable gut microbiota can increase immune checkpoint efficacy and moderate immune-adverse related reactions.

As regards innate immunity, since the very early years of life, the GM composition is actively shaped by the immune system and, in turn, the GM affects the development of the immune system (55). It has been demonstrated that since the maternal acquisition of the gut microbes during childbirth, critical interactions between the GM and the immune system may determine the establishment of both a eubiotic GM and a fully-functional immune activity (56). Any imbalance in the GM composition due, for example, to antibiotic-mediated depletion, may determine the instauration of immune-related diseases which can appear later in the adult life (such as asthma, or inflammatory bowel disease) (57,58).

The intestinal mucosal barrier represents the interface between the gut microbiota and the human body (59). Below the mucosal layer resides the gut lumen which is composed of the following: i) Intestinal epithelial cells; ii) enteroendocrine cells; and iii) intraepithelial lymphocytes and other immune cells (60). This conserved structure allows the interaction of the commensal GM with the host immune system, together forming the so-called gut-immune axis (54). Although the immune system in the intestine evolves to fight invading pathogens, there is a delicate balance which allows the development of tolerance to non-dangerous commensals, as well as food antigens, although it fights pathogenic microbes that may otherwise invade the gut lumen and trespass the gut barrier (61).

At the gut lumen, intestinal goblet cells secrete high levels of hyperglycosylated mucin able to compartmentalize gut microbes within the mucosal surface and distant from the

epithelial surface (62). Moreover, the glycans bound to mucin deliver tolerogenic signals, inducing intestinal local DCs to switch towards an anti-inflammatory state (62). Such DCs, once they internalize commensal microbes and express their antigens at the cell surface, selectively induce resident plasma cells to secrete IgA and protect the host from microbial invasion (63). In addition, Paneth cells of the small intestine secrete a range of antimicrobial peptides (AMPs), which restrain the expansion of potential microbial pathogens and hence help to maintain the GM homeostasis (64).

Pathogen-associated molecular patterns (PAMPs) comprise all the microbial-derived molecules, produced from both pathogens and commensals (65). PAMPs are actively recognized by pattern recognition receptors (PRRs), which are expressed by gut epithelial cells and local immune myeloid cells, and represent the main innate immune recognition pathway (65). PRRs are constantly exposed to PAMPs, also during gut homeostasis, in the absence of any infection (66).

PAMPs produced by gut commensals usually do not elicit a pro-inflammatory response. The specific context determines the outcome upon PRR activation (67). Only in the presence of epithelial damage, PAMPs enter the cytosolic cellular epithelial compartment (68). The elicited inflammatory response determines the activation of NF- $\kappa$ B signaling, further promoting the local secretion of pro-inflammatory cytokines, such as interferons (IFNs) (69). This pro-inflammatory response actively protects the intestine against microbial infections. On the contrary, in the absence of concurrent epithelial damage, PRR activation can be beneficial and may promote immune tolerance (70).

Similar to the innate response, the adaptive immune response is modulated in a two-way manner by GM, both locally and systemically (71). For instance, gut-associated B-cells secrete several grams of IgA per day within the gut lumen (72). This secretion can be either T-cell-independent or T-cell-dependent (72). Recently, it was also reported that gut mesenchymal cells can induce plasma cells to secrete IgA (73). In general, Foxp3<sup>+</sup> regulatory T-cells (Tregs) induce a diversified IgA repertoire, which in turn maintains a heterogeneous and eubiotic GM. In turn, the healthy GM sustains the homeostatic IgA responses in a positive feedback loop (74). IgA in the lumen coat pathogenic bacteria, preventing their potential invasion and a subsequent pro-inflammatory response (75).

T-cells play a pivotal role in regulating both local and systemic adaptive immunity related to GM homeostasis, as well as pathology. GM and GM-derived molecules can induce CD4<sup>+</sup> T-cell differentiation towards the main types: Th1, Th2, Tregs and Th17 (76). Th1 cells are essential against intracellular pathogens. Th2 cells are necessary during parasite-mediated infection. Tregs and Th17 are cellular phenotypes involved in the containment of the immune response. In particular, Tregs regulate the instauration of the immune tolerance (77). It has been observed that polysaccharide A (PSA) produced by *Bacteroides fragilis* induces the Th1 phenotype, while segmented filamentous bacteria (SFB) potentially trigger Th17 differentiation (78,79).

Th17 cells play a major role in mucosal immunity as they are able to prevent pathogen infection within the lamina propria, by secreting cytokines, including IL-17. IL-17 induces

intestinal epithelial cells to express tight junctions and to secrete AMPs (80). Moreover, IL-17 further stimulates the release of other pro-inflammatory cytokines by neutrophils, which can be recruited from the main bloodstream and directed towards the gut (81).

Additionally, GM-mediated immune-cell priming can shape the systemic immune response. When APCs, such as DCs, present their antigens within the mesenteric lymph nodes to Tregs and Th17 cells, these T-cells can travel through the bloodstream and promote distal immune responses against cross-reacting antigens located in other sites of the body (82,83). As a consequence, dysbiosis may affect systemic immune functions, thus increasing the susceptibility to certain infections and, for example, altering the response to vaccines (84).

Commensal gut microbes can actively secrete *de novo* produced molecules or transform the host's metabolites, which all may be sensed by nearby gut epithelial cells (85). Such bioproducts may have profound effects on the health of a host, including: i) Inducing immune-mediated protection against microbial pathogens; ii) maintaining gut barrier integrity; iii) metabolizing xenobiotics; iv) modulating the host's metabolism; and v) shaping and activating/inactivating the host immune system (86-89).

The GM is involved in the production of essential micronutrients, including vitamins K and B (90). In addition, a number of gut commensals can convert certain amino acids into signaling molecules, such as glutamate into gamma-aminobutyric acid (GABA) or histidine into histamine (90). Importantly, gut bacteria ferment dietary fibers to obtain a class of hormone-like bioproducts known as short-chain fatty acids (SCFAs), with multiple known functions in human health (91). For instance, SCFAs are transported to the liver representing an energy source. Additionally, SCFAs may control both glucose and lipid metabolism through the modulation of peptide hormone secretion by gut epithelial cells (92). Moreover, SCFAs (including butyrate) can enhance immunity, triggering the production of IgA by plasma cells (93). In turn, IgA inhibits bacterial adhesion to gut epithelial cells, hence blocking invasion (94,95). Moreover, SCFAs can interfere with the balance between anti-inflammatory and pro-inflammatory cytokines secreted by immune cells, both locally and systemically. The consequence is the modulation of homeostatic Treg vs. the Th17 cell ratio, resulting in an immune imbalance (96,97).

In summary, the preservation of a fine gut microbial equilibrium (in terms of the presence and relative abundance of commensal species) is imperative for sustaining and accomplish all the vital immune functions of the host. A healthy GM positively influences the immune system both locally and systemically. Conversely, once activated, the immune system may alter the GM balance. Gut eubiosis is thus of utmost importance for the maintenance of immune health (Fig. 2).

Notably, there is increasing evidence to indicate that gut dysbiosis may specifically affect local and systemic anti-tumor immunity. In fact, recurrent antibiotic exposure (which can impair intestinal eubiosis and favor the expansion of gut pathogens) is directly associated with an increased risk of cancer (98). In general, dysbiosis may influence tumor formation or ICI-based therapy failure (99). As largely depicted in the present review, a healthy (both enriched and diverse)

GM can activate the immune system to: i) Fight cancer; and ii) efficiently respond to anticancer immunotherapies, in particular ICIs (100).

### 3. Gut microbiota and cancer

Cancer is a multifactorial disease resulting from a combination of intrinsic factors (e.g., the stochastic accumulation of gene mutations and epigenetic alterations), environmental exposures (e.g., pollution, sunlight exposure and infections) and lifestyle habits (cigarette smoking, diet and sport) (101,102). The resulting overall risk of developing a given malignancy is mainly dependent on the dose, duration, as well as the combination of exposures, all coupled with the specific genetic and epigenetic background (103).

Presently, several biological agents are listed as carcinogens by the International Agency of Research on Cancer (IARC), including a number of viruses (i.e., Epstein-Barr virus, hepatitis B virus, hepatitis C virus, Kaposi's sarcoma-associated herpesvirus, human immunodeficiency virus-1, human papillomaviruses and human T-cell lymphotropic virus type 1), as well as the gastrointestinal bacterial pathogen, *Helicobacter pylori* (104). Individuals with *Helicobacter pylori* infection have a higher risk of developing stomach cancer as the infection directly causes chronic inflammation (105).

Generally, the influence of gastrointestinal microorganisms on cancer development is complex (106). In fact, the GM plays a dual role in cancer, as gut microbes can either positively or negatively affect tumorigenesis, depending on their nature (49). Bacteria *per se* or their products may directly or indirectly affect tumorigenesis. In general, beneficial bacteria which are normally part of an eubiotic GM exert an antitumor effect (107). On the contrary, pathogens prevailing in a dysbiotic GM are pro-tumorigenic, either directly or through the production of microbial-derived toxins. These effects have been documented in both local colorectal cancers (CRCs), as well as in distant tumors (108). The authors recently reviewed all the relevant findings regarding the dual role played by the GM in cancer, focusing on the specific gut microorganisms involved (49).

Several preclinical findings have demonstrated the pro-tumorigenic role of a number of gut microbes. For instance, a number of bacteria, mostly pathogens, can release toxins within the intestinal lumen which, once internalized by lumen epithelial cells, can directly promote genotoxic damage or, alternatively, they can activate pro-proliferative or anti-apoptotic pathways (49). For example, cytolethal distending toxins (CDTs), Shiga-like and Shiga toxins secreted respectively by *Escherichia coli*, *Helicobacter* spp., *Shigella dysenteriae*, as well as several others, may directly induce DNA damage (109). Moreover, the surface molecule FadA from *Fusobacterium nucleatum*, CagL from *Helicobacter pylori* and SopB from *Salmonella typhimurium* can trigger the WNT/B-catenin, MEK-ERK and STAT3 pathways, respectively, all inducing target cells to over-proliferate and/or not undergo apoptosis (110-112).

Additionally, the GM may alter the immune response activated by the host against the neoplasm, as observed in several tumor-bearing mice models (49,113,114). For example, enterotoxigenic *Bacteroides fragilis* infection in a mouse model of

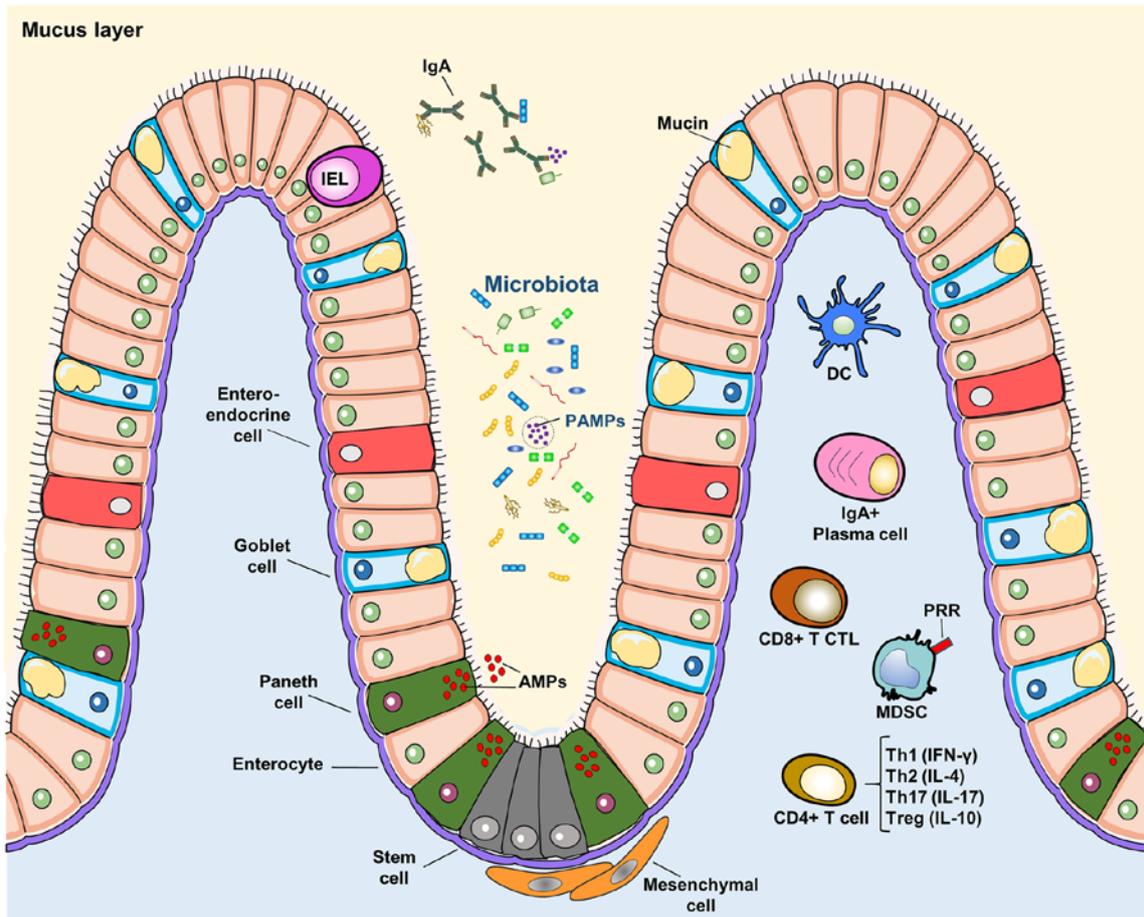


Figure 2. The gut-immune axis. The gastrointestinal lumen represents the interface between the GM and immune system. Intestinal cells constitute the villi structure and include: Enteroendocrine cells, goblet cells, Paneth cells, enterocytes and stem cells. Paneth cells secrete AMPs. IELs may reside within the epithelial structure. Goblet cells secrete mucin which enriches the intraluminal mucus layer. Gut microbiota and their derived molecules form PAMPs. PAMPs are recognized by PRRs expressed by immune cells and gut epithelial cells. IgA are secreted in the lumen and help to bind microbes and microbial-derived molecules. Immune cells are pivotal in the instauration of the immune-tolerance versus commensals and immune-reactivity against pathogens. Both innate and adaptive immunity are involved. Immune cells include: DCs, IgA-producing plasma cells, CD8+ CTLs, MDSCs and CD4+ T-cells. The latter can differentiate into different phenotypes involved in immune reactivity or tolerance (i.e., Th1, Th2, Th17 and Tregs). GM, gut microbiota; AMPs, anti-microbial peptides; IELs, intestinal intraepithelial lymphocytes; PAMPs, pathogen-associated molecular patterns; PRRs, pattern recognition receptors; DCs, dendritic cells; CTLs, cytotoxic T lymphocytes; MDSCs, myeloid-derived suppressor cells.

CRC attracts a colonic immune infiltrate. In particular, it has been demonstrated that *Bacteroides*-derived enterotoxin BFT promotes the differentiation of pro-tumoral myeloid-derived suppressor cells (MDSC), which in turn produce NO and suppress CD4<sup>+</sup> T-cell proliferation (115,116). In addition, the Treg response upon enterotoxigenic *Bacteroides fragilis* infection in mice with CRC triggers IL-17 and induces Th17 development which in turn, promotes tumorigenesis (117). Furthermore, *Fusobacterium nucleatum* has been shown to potentiate CRC tumorigenesis in a mouse model of CRC, driving MDSC infiltration within the intestinal tumors and inducing the activation of a general pro-tumoral immune-milieu (118).

By contrast, some bacteria have been shown to possess an anticancer function through the stimulation of the immune system of their host. For example, *Bifidobacterium* spp. promotes antitumor immunity in tumor-bearing mice through the activation of DCs, which enhance cytotoxic CD8<sup>+</sup> T-cell activity directed against tumor cells (119). *Akkermansia muciniphila* and *Enterococcus hirae* orally

administered to tumor-bearing mice have been shown to induce DCs to secrete IL-12, thus triggering the recruitment of CD8<sup>+</sup> cytotoxic T-cells and inhibiting tumor growth (120) (Fig. 3).

Given all the aforementioned preclinical examples, it is clear that the maintenance of a healthy GM through the life of an individual may represent a good strategy with which to prevent cancer. A number of groundbreaking studies (described in detail below) have further expanded this concept, demonstrating that GM contains diagnostic and prognostic cancer biomarkers, suitable to identify therapy-responder patients. More importantly, either the modulation of the GM towards a healthy phenotype or the selective enrichment of GM in specific beneficial bacterial types may represent an enforcement to anticancer therapy. In this context, different studies have demonstrated how the administration of specific probiotic strains may positively influence the GM with beneficial effects for cancer patients and human health (121,122). This concept particularly applies to immunomodulatory treatments and specifically to ICIs.

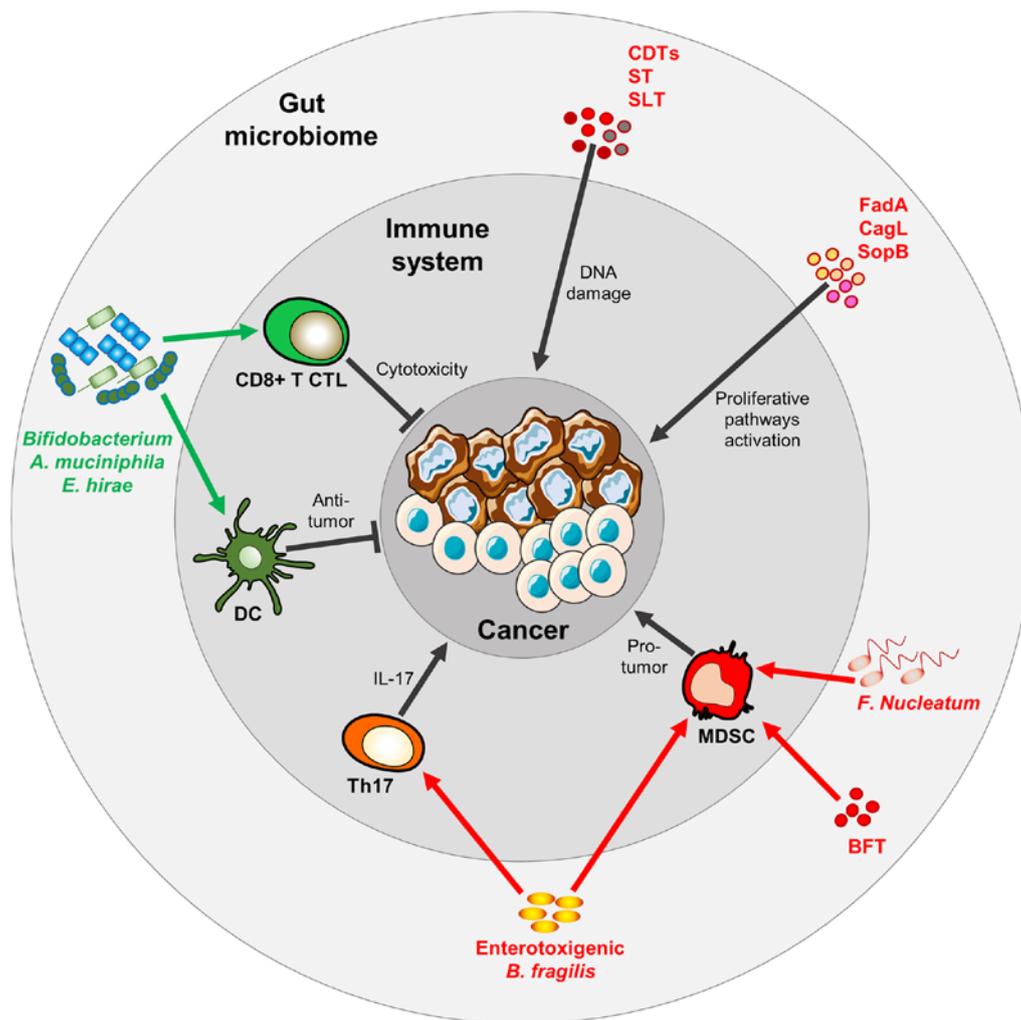


Figure 3. Gut microbiota play a dual role in cancer. Microbial-derived molecules, including CDTs, SLTs and STs may directly induce DNA damage and trigger cancer mutation. Other microbial surface molecules, such as FadA from *Fusobacterium nucleatum*, CagL from *Helicobacter pylori* and SopB from *Salmonella typhimurium* induce cancer cell proliferation. *Fusobacterium nucleatum*, *Bacteroides fragilis* or its derived Bacteroides fragilis toxin (BFT) can boost MDSCs which, in turn, favor a pro-tumoral milieu. *Bacteroides fragilis* may also trigger the Th17 T-cell phenotype, which is immunosuppressive and hence, pro-tumorigenic. *Bifidobacterium* spp., *Akkermansia muciniphila*, *Enterococcus hirae* promote DC activation and CD8<sup>+</sup> cytotoxic T-cell activation, both triggering an anti-tumor immune response. CDTs, cytolethal distending toxins; SLT, Shiga-like toxin; ST, Shiga toxin; MDSCs, myeloid-derived suppressor cells; DC, dendritic cell.

#### 4. Gut microbiota and immune checkpoint inhibition

A growing number of studies have shed further light on the association between the safety and efficacy of ICI-based immunotherapy and GM features in cancer patients. In this section, the authors aim to provide a temporal timeline demonstrating the progress made and milestones accomplished in this field.

In 2015, Vétizou *et al* (123) observed for the first time that tumors in antibiotic-treated or in germ-free mice did not respond to anti-CTLA4 immunotherapy. Pivotaly, they demonstrated that the antitumor effect of anti-CTLA4 was dependent on *Bacteroides* spp., and in particular on *Bacteroides fragilis*. In fact, a significant antitumor response was observed: i) With oral gavage of *Bacteroides fragilis*; or ii) with immunization with *Bacteroides fragilis*-derived polysaccharides; or iii) with the adoptive transfer of *Bacteroides fragilis*-specific *in vitro* activated T-cells (123). The authors of that study performed FMT in tumor-bearing mice treated with anti-CTLA4 antibody. Specifically, they employed stools from melanoma patient

donors with fecal abundance of *Bacteroides* spp. Following FMT, mice-derived feces were found to be selectively enriched in *Bacteroides fragilis*. This feature was negatively associated with tumor size following the CTLA-4 blockade in recipient mice. Hence, anti-CTLA4 antibody treatment could actively modify the abundance of immunogenic *Bacteroides* spp. in the gut, which in turn affected ICI-anticancer efficacy (123).

In the same year, Sivan *et al* (119) compared the growth of melanoma in mice grown in different breeding facilities, thus bearing different GM compositions. They demonstrated significant differences in melanoma growth, which reflected the different cancer-specific T-cell response. Pivotaly, anti-PD-L1-non-responder mice, when orally receiving either feces obtained from responder mice or *Bifidobacterium* alone, augmented their response to anti-PD-L1 therapy (119). In particular, a significant reduction in tumor outgrowth coupled with an augmented DC activity, leading to increased CD8<sup>+</sup> T-cell priming and T-cell accumulation in the tumor microenvironment was observed (119).

Both studies strongly suggested that manipulating the GM could enhance the antitumor efficacy of ICIs (119,123). These important observations paved the way for subsequent translational observations. In 2016, Dubin *et al* published a prospective study aimed at analyzing GM features in patients with MM treated with ICIs and developing colitis (124). The use of anti-CTLA4 antibody in cancer patients is often associated with dysbiosis and with inflammatory colitis as irAEs (124). The authors associated the fecal microbial composition at the baseline with the one following colitis manifestation. Notably, they found that an increased representation of bacteria belonging to the *Bacteroidetes* phylum was associated with augmented resistance to the development of ICI-induced colitis. On the contrary, microbiome analysis confirmed that patients lacking genetic pathways involved in polyamine transport and B vitamin biosynthesis had an increased risk of developing colitis (124).

In 2017, Frankel *et al* (125), through the use of metagenomic shotgun sequencing coupled with metabolomic profiling, identified the specific footprint of GM associated with the efficacy of ICIs in patients with MM. Of the 39 patients with MM, only a group exhibited a response to ICIs (corresponding to 67% of ipilimumab plus nivolumab-, and 23% of pembrolizumab-treated subjects). Despite the specific ICI used, feces from ICI-responders were enriched for *Bacteroides caccae* (125). In particular, responders treated with a combination of anti-CTLA4 and anti-PD-L1 had a GM enriched in *Faecalibacterium prausnitzii*, *Bacteroides thetaiotamicron* and *Holdemania filiformis*. However, responders treated with anti-PD-1 antibody had a GM enriched in *Dorea formicogenerans*. Among all the GM obtained from responders, the metabolite resulting consistently enriched was anacardic acid (125).

Also in 2017, Chaput *et al* (126) performed a prospective analysis on the fecal microbiota composition in 26 patients with MM. The analysis revealed that at the baseline, prior to any anti-CTLA4 antibody infusion, patients whose baseline microbiota was driven by *Bacteroides* exhibited a longer progression-free survival (PFS) than patients whose baseline microbiota was driven by *Faecalibacterium* genus plus other *Firmicutes*. Additionally, baseline colitis-associated phylotypes were selectively associated with the presence of *Firmicutes* (126). Upon ICI treatment, patients with MM belonging to the *Faecalibacterium*-driven cluster who developed anti-CTLA4-induced colitis exhibited a significant increase in the CD4<sup>+</sup> T-cell population and, in particular, in CD4<sup>+</sup> T-cells expressing the T-cell inducible T-cell COStimulator surface marker. This observation suggested that the baseline GM composition may represent an important determinant of the immune response in cancer patients, as well as of anti-CTLA-4 associated-colitis (126).

Overall, the aforementioned prospective studies have demonstrated the importance of combining metagenomics and metabolomics to study the GM in cancer patients. These studies clearly demonstrate that GM is affected by ICI treatments. Thus, a specific GM composition and/or metabolite enrichment may be predictive of a better prognosis.

In 2018, three landmark studies were published in *Science*, clearly demonstrating, for the first time, a direct association between GM composition and efficacy of ICI-based

therapy (120,127,128). In particular, Routy *et al* (120) examined the effects of FMT from NSCLC and renal cell carcinoma (RCC) ICI-responder and ICI-non-responder donors, in recipient epithelial (melanoma and sarcoma) tumor-bearing mice (either germ-free or antibiotic-treated). They found that FMT from responders significantly enhanced the efficacy of ICIs in reducing tumor growth in mice, whereas FMT from non-responder patients did not exert any effect (120). Metagenomics analyses of fecal samples from responders and non-responders clearly revealed that the GM composition affected the primary immune-resistance to ICIs. In particular, a positive association was observed between the relative abundance of *Akkermansia muciniphila* and the clinical response to ICIs. The oral gavage of *Akkermansia muciniphila* in mice receiving FMT from non-responders significantly restored the efficacy of anti-PD-1 and augmented the recruitment of CD4<sup>+</sup> T-cells at the tumor site, and increased the local secretion of IL-12 by DCs (120).

Additionally, Matson *et al* (127) analyzed the baseline GM composition of stool samples from 42 patients with MM prior to receiving ICI therapy. Metagenomics analysis revealed that *Bifidobacterium longum*, *Collinsella aerofaciens* and *Enterococcus faecium* species were significantly enriched in ICI-responder patients (127). FMT from responder donors in recipient tumor-bearing mice improved the T-cell response and the anti-PD-L1 anticancer efficacy. In addition, from fecal analyses, 10 bacterial species were found to be differentially enriched in responder vs. non-responder mice. In total, eight of these (i.e., *Enterococcus faecium*, *Collinsella aerofaciens*, *Bifidobacterium adolescentis*, *Klebsiella pneumoniae*, *Veillonella parvula*, *Parabacteroides merdae*, *Lactobacillus* spp., and *Bifidobacterium longum*) were more abundant in responders, whereas two (i.e., *Ruminococcus obeum* and *Roseburia intestinalis*) were more abundant in non-responders (127).

Finally, Gopalakrishnan *et al* (128) analyzed the gut microbiome of 112 patients with MM, demonstrating that responders had a significantly higher alpha diversity and a relative abundance of bacteria belonging to the Ruminococcaceae family. Moreover, from metabolomics analyses, the authors of that study observed a significant enrichment of anabolic pathways. Responder patients also exhibited an enhanced systemic and antitumor immunity, with increased cytotoxic CD8<sup>+</sup> T-cell tumor infiltrates (128). Accordingly, germ-free mice receiving FMT from responders exhibited favorable immune profiling and an improved response to anti-PD-L1 antibody treatment in terms of tumor growth, which was significantly reduced. Importantly, gut microbiome analyses revealed that feces of responders were enriched in Clostridiales, whereas the feces of non-responders were rich in Bacteroidales (128).

Since these milestone studies, several others conducted on cancer patients have further revealed the existence of a relevant bacterial gut footprint in ICI-responders vs. non-responders. In 2018, Derosa *et al* (129) demonstrated how antibiotics can alter GM health, triggering antibiotic-associated dysbiosis. In turn, dysbiosis may invalidate the response to ICIs. In the retrospective analysis, patients with advanced RCC and NSCLC (121 and 239 patients, respectively) treated with anti-PD-L1 antibody (either as monotherapy or in combination) were considered. Above all, 13% of patients with RCC

and 20% of patients with MM received antibiotics 30 days prior to the ICI administration. Of note, the antibiotics significantly reduced the benefits of ICIs in the cancer patients. In particular, PFS was significantly reduced in patients with RCC, whereas overall survival (OS) was decreased in patients with NSCLC (129).

In 2019, Jin *et al* (130) additionally confirmed that a favorable GM, as well as a healthy immune profile were associated with an improved response to anti-PD-1 immunotherapy. The authors considered 37 patients with advanced NSCLC. They performed the fecal microbiome analysis: i) At the moment of the anti-PD-1 therapy; ii) at the clinical evaluation; iii) following the progression of the disease. According to the Response Evaluation Criteria in Solid Tumor (RECIST) scale, patients were divided in responders and non-responders (130). Responders which exhibited a significantly prolonged PFS had a high microbiome diversity. Compared with the baseline, the feces of responders were enriched in *Alistipes putredinis*, *Bifidobacterium longum* and *Prevotella copri*. However, non-responders exhibited a prevalence in *Ruminococcus* spp. In addition, responders manifested an increase in CD8<sup>+</sup> T-cell and NK cell subsets in response to anti-PD-1 therapy (130).

Also in 2019, Zheng *et al* (131) performed gut microbiome profiling of a small cohort of patients with hepatocellular carcinoma (HCC), using metagenomic sequencing. Fecal samples from patients responding to anti-PD-1 immunotherapy exhibited a higher taxa richness and more gene counts than those of non-responders. Furthermore, dynamic sequencing analyses demonstrated that anti-PD-1 therapy increased the GM dissimilarity between responders and non-responders, with a prominence in such differences at 6 weeks post-treatment. A total of 20 responder-enriched species (including *Akkermansia muciniphila* and *Ruminococcaceae* spp.) were further identified as prominent. Subsequent metabolic pathway analysis demonstrated that carbohydrate metabolism and methanogenesis were selectively enriched in the responders (131).

Additionally, in 2020, Salgia *et al* (132) characterized the stool microbiome from 31 patients with metastatic RCC receiving ICIs (as single agents or combination) to assess treatment-related changes in GM composition over the course of treatment. They found that a higher microbial diversity was associated with better treatment outcomes. Temporal profiling of the microbiome indicated that the relative abundance of *Akkermansia muciniphila* significantly increased in patients obtaining clinical benefits from ICIs. Hence, dynamic changes in GM composition suggested the potential utility of modulating GM to reach more successful outcomes with ICIs (132).

Taken together, the clinical studies evidenced several microbial candidates, which can be suggested as predictive biomarkers of the patient population that may truly benefit from ICIs. Of equal importance is the elucidation of the molecular mechanisms responsible for the beneficial effect of the GM. For this purpose, several important preclinical studies have recently been published.

In 2019, Tanoue *et al* (114) isolated a consortium of 11 bacterial strains (comprised of seven *Bacteroidales* and four non-*Bacteroidales* species) from healthy human donor feces, capable of augmenting IFN- $\gamma$ -secreting CD8 T-cell levels

in the gut. The consortium, when inoculated into syngeneic CRC tumor-bearing germ-free mice, induced a robust MHC1 expression in DCs and enhanced the therapeutic efficacy of ICIs, with a concurrent decrease in tumor growth (114).

In 2020, Xu *et al* (133) evaluated the effects of GM in MSS-type CRC tumor-bearing mice treated with different antibiotics prior to anti-PD-1 anticancer treatment. The injection of antibiotics significantly counteracted the efficacy of anti-PD-1 antibody in inhibiting tumor growth. Furthermore, metabolomics analysis demonstrated the enrichment in the glycerophospholipid metabolic pathway, specifically within the antibiotic-treated group. Changes in GM composition may drive these metabolic changes. In fact, Xu *et al* (133) demonstrated that *Prevotella* spp. and *Akkermansia* spp. were able to support the efficacy of anti-PD-1 by affecting the metabolism of glycerolipids in MSS-type CRC tumor-bearing mice.

Of note, in 2020, Mager *et al* (134) explored the underlying mechanisms through which the GM enhanced ICI-mediated antitumor immunity, with particular focus on the T-cell adaptive response. In detail, *Bifidobacterium pseudolongum*, *Lactobacillus johnsonii* and *Olsenella* spp. significantly increased the efficacy of ICIs in four different mouse models of cancer. In particular, *Bifidobacterium pseudolongum* modulated the ICI response through the production of the metabolite, inosine (134). They further assessed that a decreased gut barrier functionality induced by ICI-immunotherapy increased the systemic translocation of inosine, which in turn activated antitumor CD8<sup>+</sup> T-cell activity. Importantly, the effect of bacterial-derived inosine was strictly dependent on the T-cell surface expression of the adenosine A2A receptor, whose stimulation was specifically required (134).

More recently, in 2021, Si *et al* (135) evaluated the therapeutic efficacy of administering a single bacterial strain, *Lactobacillus rhamnosus GG* (LGG) in combination with ICI immunotherapy. Pivotaly, the oral administration of LGG significantly improved the efficacy of ICIs and reduced tumor growth in CRC and melanoma mouse models. The authors further explored the molecular mechanism, evidencing that the augmented anti-tumor activity of anti-PD-1 was associated with increased tumor-infiltrating DCs and CD8<sup>+</sup> T-cells (135). Moreover, treatments with live LGG alone or in combination with anti-PD-1 triggered type I IFN production by DCs, enhancing the cross-priming of CD8<sup>+</sup> cytotoxic T-cells. In DCs, cyclic GMP-AMP synthase (cGAS)/stimulator of IFN genes (STING) was required for IFN- $\beta$  induction in response to LGG. In fact, LGG significantly boosted IFN- $\beta$  production via the cGAS/STING axis in DCs (135). The role of the STING pathway in potentiating the efficacy of immunotherapy, was also proven by Shi *et al* (136). In agreement with the findings of Si *et al* (135), Shi *et al* (136) observed that a specific gut microbe, *Bifidobacterium* spp., potentiated anti-CD47 immunotherapy via the stimulation of STING in DCs in tumor-bearing mice.

2021 was a breakthrough year in GM research associated with ICI therapeutic efficacy in human studies. In this year, two important publications in *Science* demonstrated, for the first time, that FMT significantly overcame the resistance to anti-PD-1 therapy in patients with MM (50,51). Firstly, Davar *et al* (50) demonstrated that in patients with MM, the GM composition was associated with the anti-PD-1 response.

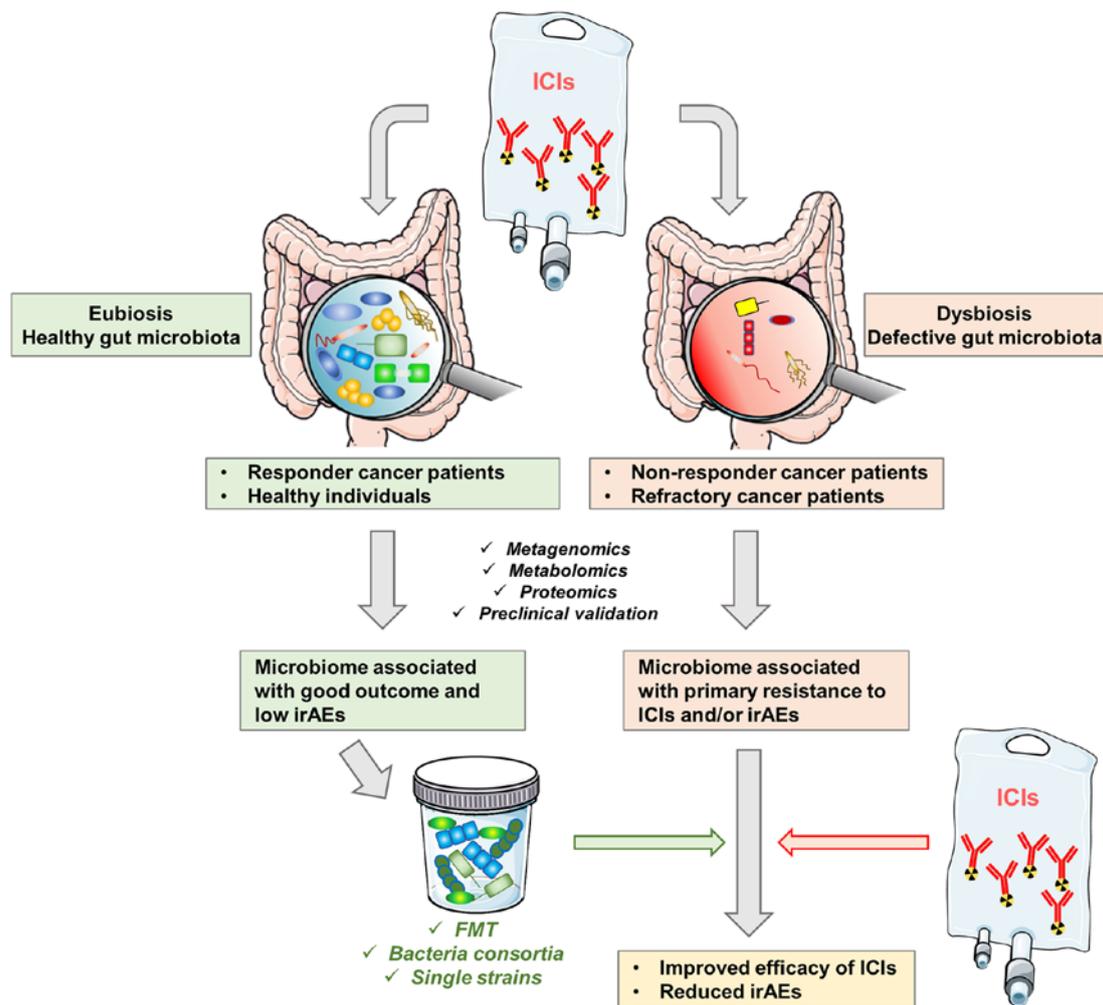


Figure 4. Gut microbiota as an adjuvant of immune checkpoint inhibition. ICIs affect the gut microbiota. In particular, responders have a eubiotic microbiota whereas non-responders have a dysbiotic and depauperated microbiota. Gut microbiota from responders or from healthy subjects is analyzed via metagenomics, metabolomics, proteomics and preclinical studies. This characterization allows to identify whole stools to perform a FMT or, alternatively, microbial consortia or single microbes that can be administered to non-responder cancer patients or cancer patients who (after an initial response) became refractory to ICIs. The therapeutic modulation of gut microbiota can be associated with ICIs to obtain an improved efficacy and/or a reduction in irAEs in refractory cancer patients. ICIs, immune checkpoint inhibitors; FMT, fecal microbiota transplantation; irAEs, immune-related adverse events.

Notably, the resistance to anti-PD-1 was overcome by directly modulating GM composition. The authors of that study evaluated both the safety and efficacy of responder-derived feces transplanted together with anti-PD-1 in recipient patients with PD-1-refractory melanoma (50). FMT with anti-PD-1 was well-tolerated and provided clinical benefit in 6 out of 15 patients. The combined treatment induced a rapid and robust gut microbiota perturbation. The six responders exhibited an increased abundance of taxa, such as Ruminococcaceae and Bifidobacteriaceae, that were previously shown to be associated with a response to anti-PD-1 associated with an increased CD8<sup>+</sup> T-cell activation, as well as with a decreased frequency of myeloid cells secreting IL-8 (119,128). In addition, the responders exhibited distinct proteomic and metabolomic signatures. The trans-kingdom network analysis confirmed that the GM directly regulated these changes (50).

In parallel, Baruch *et al* (51) performed a pilot phase I clinical study to assess both the safety and feasibility of FMT and anti-PD-1 immunotherapy re-induction 10 patients with anti-PD-1-refractory MM. Notably, they observed a positive

clinical response in 3 out of 10 patients (two partial responses and one complete response). Treatment with FMT was associated with favorable changes in immune cell infiltrates and immune-related gene expression profiles locally (at the level of the gut lamina propria), as well as distally (within the tumor microenvironment) (51).

Taken together, the reported findings provide strong evidence on the key role that the GM plays in modulating the ICI therapeutic response and potential-associated toxicity. Pivotaly, Davar *et al* (50) and Baruch *et al* (51) demonstrated that gut microbial modulation can reverse ICI resistance in patients with MM through the specific modulation of the individual immune system, both locally and systemically (Fig. 4).

Since 2015, a number of clinical trials have been performed with two main goals. One important aim is to characterize the gut microbiome signature, as well as the associated immune system changes upon a specific ICI immunotherapy. The outcomes of these studies may reveal the existence of specific diagnostic and prognostic gut microbial biomarkers (Table I).

Additionally, a growing number of clinical trials is currently evaluating both the safety and efficacy of actively modulating GM composition in association with ICI immunotherapy. Currently, a number of companies are investing in this direction and the number of registered trials is increasing exponentially (100). As presented in Table II, in order to 'turn bugs into drugs' a number of strategies are currently being tested, including the administration of: i) Single probiotics; ii) microbial communities; iii) synbiotics; iv) FMT; and v) metabolic modulators.

Of note, for certain malignancies, such as prostate cancer, researchers have shown the limited efficacy of ICIs (137). This may be partially explained by the fact that prostate cancer is immunologically 'cold' compared to both melanoma and lung cancer, thus with a lower tumor mutational burden (TMB) (138,139). Previous clinical studies have tested ICIs administered in combination with miscellaneous agents that may improve the overall efficacy of ICIs (140,141). For instance, the concurrent administration of androgen inhibitors has been shown to significantly improve the OS of patients with metastatic castration-sensitive prostate cancer (142). Coherently, one ongoing trial reported (presented in Table II) is evaluating both the safety and efficacy of combining anti-PD-1 antibody with the anti-androgen enzalutamide and the FMT in prostate cancer patients not responding to initial ICI and androgen deprivation therapy. Prostate cancer patients with a significant initial response to anti-PD-1 and anti-androgens, will be used as fecal donors (NCT04116775).

## 5. Conclusions

Since birth, each individual inherits a specific GM footprint. Moreover, the GM is constantly shaped with age, diet and lifetime exposures. Current research has demonstrated the dual role played by the GM in cancer (49). In particular, the GM deeply affects the host immune system and vice versa. During dysbiosis, several microbial species can overpopulate the gut. Consequently, they may enhance inflammation and promote the formation of a pro-carcinogenic environment (143).

On the contrary, the re-establishment of a healthy GM may be beneficial for cancer patients, inducing a healthy immune system. In line with this observation, it has been further demonstrated that a specific ICI-responder gut microbial footprint is associated with a reduction in irAEs and an improvement of ICI-efficacy (120,127,128). Furthermore, specific gut microbial modulatory therapy (based on FMT from healthy/responders donors) may revert ICI-resistance in patients with advanced cancer (50,51). This latter groundbreaking observation is paving the way towards a growing number of ongoing clinical trials in cancer patients to test the administration of 'good' gut microbes as adjuvants in association with ICI-based therapy (Table II).

In spite of the tremendous progress made, some questions still remain unanswered. In fact, since cancer patients are often immunocompromised, special care needs to be taken to select the correct therapeutic strategy to modulate the GM, in order to maximize the positive outcomes of microbial-modulators, and reduce the potential harmful side-effects due to the possibly fragile immune system. For instance, a number of

factors may affect the overall clinical outcome, including the use of concurrent medications such as antibiotics, which have been associated with a reduced response to ICIs (144). In addition, specific dietary associations are crucial and they can be efficiently used as predictors of FMT success, as demonstrated for patients with a higher intake of dietary fiber (145). The consumption of specific nutrients or bioactive food-derivatives may favor the FMT engraftment, particularly in cancer patients, who are often affected by nutritional and metabolic issues (e.g., vomiting, swallowing difficulties, reduced food adsorption and inadequate food consumption).

In conclusion, anticancer therapy is increasingly becoming holistic. In the near future, anticancer treatments will be tailored to the specific cancer patient, based on the GM of the individual, as well as the immune signature. Henceforward, larger clinical longitudinal studies will help to increase the current knowledge on the long-term safety and robustness of FMT as adjuvants of ICIs, in order to expand and standardize their use in a number of types of cancer.

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## Availability of data and materials

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## Authors' contributions

All authors (SV, LF, GCL, MS and ML) participated in the writing of the manuscript, and preparing the figures and tables, as well as in the revisions. SV and LF confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

ML is the principal investigator of a research grant founded by Dicofarm Spa to his University Department. In light of the fact that this manuscript is a Review of the existing literature, as well as of the ongoing clinical studies, the Dicofarm Spa will not benefit specifically from any of the findings described. The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential competing interest.

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