Host microbiomes in tumor precision medicine: how far are we?

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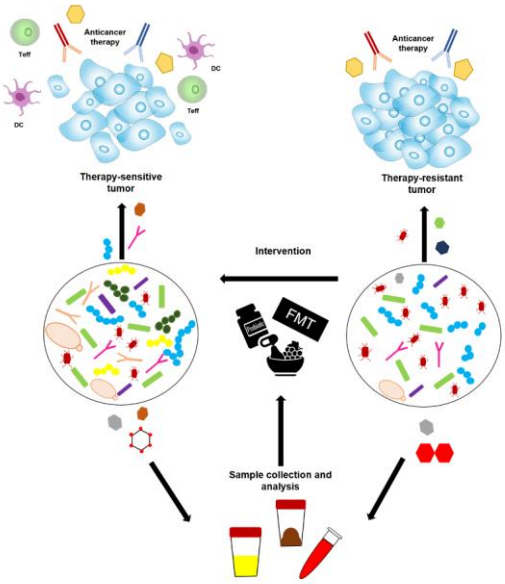
Abstract

The human gut microbiome has received *a crescendo* of attention in recent years, due to the countless influences on human pathophysiology, including cancer. Research on cancer and anticancer therapy is constantly looking for new hints to improve the response to therapy while reducing the risk of relapse. In this scenario, the gut microbiome and the plethora of microbial-derived metabolites are considered a new opening in the development of innovative anticancer treatments for a better prognosis. This narrative review summarizes the current knowledge on the role of the gut microbiome in ~~cancer~~ the onset and progression of cancer, as well as in response to chemo-immunotherapy. Recent findings regarding the tumor microbiome and its implications for clinical practice are also commented on. ~~The c~~Current microbiome-based intervention strategies (*i.e.*, prebiotics, probiotics, live biotherapeutics and fecal microbiota transplantation) are then discussed, along with key shortcomings, including ~~the~~ a lack of long-term safety information in patients who are already severely compromised by standard treatments. ~~In this scenario~~Thus, ~~t~~he implementation of bioinformatic tools applied to microbiomics and other omics data, such as machine learning, has an enormous potential to push research in the field, ~~allowing~~ enabling the prediction of health risk and therapeutic outcomes, for a truly personalized precision medicine.

Graphical Abstract



Personalized microbiome-based interventions are critical ~~to~~ for ~~ensure~~ ensuring antitumor immune responses, circumventing resistance to chemo-immunotherapy.

Keywords: Gut microbiome, Microbial metabolites, Tumor microbiome, Anticancer therapy, Probiotics, Fecal microbiota transplantation, Next-generation probiotics, Machine learning

1. Introduction

In ~~the last few~~recent years, the microbial community inhabiting the gastrointestinal tract (*i.e.*, the gut microbiome – GM) has received special attention, not only for the well-known supporting functions of host homeostasis [1,2], but also for its involvement in ~~cancer~~ the onset and progression of cancer, as well as in the outcomes of anticancer therapy [3]. Tumor development and patient failure ~~of to patient~~ respon~~se~~d to anticancer approaches (*i.e.*, chemotherapy, radiotherapy, and immunotherapy) are among the leading causes of death worldwide [4]. For this reason, research is moving towards new fields that could help overcome these obstacles; host-associated microbes, as well as their products, have recently been identified as unexpected key orchestrators in these branches.

Herein, we discuss the role of GM composition and functionality in promoting the development and progression of local and distant tumors, as well as recent evidence on the intratumor microbiome. Particular attention is given to immunological tumors (e.g., leukemia and lymphoma) and to complications related to hematopoietic stem cell transplantation (HSCT), for which an abundant and consistent microbiome-centered literature is available.

In ~~an~~ attempt to fully explore the possibility of using microorganisms/microbiomes as a therapeutic target/tool in the anticancer field, we comment on ~~the~~ microbiome-tailored intervention strategies currently in use, as well as ~~on~~ the most recent clinical trials involving the use of prebiotics, (traditional and next-generation) probiotics and fecal microbiota transplantation (FMT). Finally, we discuss the translational potential of bioinformatics, particularly machine learning, ~~for~~ to stratify~~ing~~ patients, predict~~ing~~ outcomes, and design~~ing~~ personalized precision intervention strategies for a better patient quality of life. See **Figure 1** for a summary of the role of host microbiomes in tumor onset and progression, as well as in response to therapy, and the available microbiome manipulation tools that could help improve patient prognosis.

2. Exploring the human gut microbiome and its functions from eubiosis to dysbiosis

The GM is a key contributor to ~~the~~ maint~~enance of~~aining ~~the~~ host physiological homeostasis [1,2]. While this sentence may seem foregone in 2021, countless studies still attempt to advance our understanding of the close and complex relationship between the GM and the human host. Going back in time, the first signs of the presence of microbes inside the human intestinal tract took place in the late 1800s in Europe, when the German pediatrician Theodor Escherich consolidated the study of ~~the~~ "human gut flora" [5,6]. Indeed, Prof. Escherich discovered what he called "*Bacterium coli commune*" (*i.e.*, currently *Escherichia coli*) in human feces and afterwards "*Bacillus bifidus communis*" (*i.e.*, *Bifidobacterium animalis*) in the gut of newborns and breast-fed infants. For the first time, Escherich and colleagues

spoke of "good bacteria" ~~in a period in which~~at a time when the only link between microorganisms and their host was in the development of ~~pathologies~~ diseases (e.g., cholera, anthrax and tuberculosis) [5]. From then to the present day, GM research has always played an important role in science.

GM is a deeply complex ecosystem that includes not only bacteria, which are the most represented, but also archaebacteria and fungi, along with viruses [7]. Until recently, a frequently repeated slogan was that the human body contained 10 times more microorganisms than human cells. However, this was a rough calculation made more than 40 years ago [8], while today we can state that the ratio of human to microbial cells is likely to be 1:1 with the balance slightly in favor of microbes [9]. As just mentioned, the most examined fraction of GM is the bacterial one, 90% of which belongs to Firmicutes and Bacteroidetes phyla, while the remaining 10% is distributed among the subdominants Actinobacteria, Proteobacteria, Fusobacteria and Verrucomicrobia [2]. So far, it is estimated that the collective genome of GM, known as the microbiome, harbors 150 to 500 times more genes than the host, which are implicated in providing functional traits that complement the human repertoire and ~~that~~ are relevant to our metabolic, immunological and neurological homeostasis [10-12].

In the individual's lifespan, the first microbial stimuli derive from the early moments of the infant's life and are closely linked to the birth mode, the maternal microbiota, antibiotic exposure and early-life feeding practices [13,14]. At this time, when ~~the~~ infant-GM symbiosis is ~~going to~~ being established, GM is featured by low bacterial diversity and functional complexity, as well as a higher degree of interpersonal variation ~~compared to~~than the adult-type GM profile [15,16]. Both structural components of microbes and products of their metabolism have been found to be involved in the development, maturation, and education of the child's immune system [17-19], as well as in the regulation of the endocrine and central nervous systems [20]. It is therefore not surprising that GM disruption (*i.e.*, dysbiosis) ~~during~~ in ~~the~~ early- life ~~window can~~ may be associated with several disorders [21,22], such as type 1 diabetes, atopic disease, asthma and childhood obesity [23-25]. At weaning, with the cessation of breastfeeding, there is a rapid increase in the ~~the~~ structural and functional diversity of the infant GM, which progressively evolves towards ~~the~~ a mature adult-like state [26]. The development of the adult GM is regulated by a complex interplay between the host and several environmental factors, such as diet, lifestyle, or the so-called geographical effect [27,28] or, more generally, the exposome (*i.e.*, the totality of internal and external exposures that an individual faces throughout his life) [29]. ~~In~~ Under healthy conditions, all of these factors contribute to shaping the microbial community, selecting a eubiotic GM configuration (*i.e.*, a stable, resistant, and resilient GM, with high diversity and functional redundancy) [30], which provides the functional traits necessary for host homeostasis, such as the barrier effect against infectious threats and the production of several bioactive small molecules that support the GM-host metabolic, immunological and neurological connections (e.g., vitamins, fatty acids, protein metabolites, bile acids, polyamines, etc.) [31-33].

As a matter of fact, GM is dominated by species (mainly from *Ruminococcaceae* and *Lachnospiraceae* families) capable of degrading complex carbohydrates, otherwise indigestible for humans, such as glycans and mucins (called Microbiota-Accessible Carbohydrates) [34]. The end products of this fermentation are short-chain fatty acids (SCFAs) [13], mainly acetate, propionate and butyrate, which are indisputably beneficial to health, acting as local (butyrate) and peripheral (acetate and propionate) energy sources, inflammation modulators, vasodilators and regulators of gut motility, wound healing, metabolism and epigenetics [35]. SCFAs also influence the proliferation and differentiation of colonic epithelial cells, including through the modulation of gene expression, and contribute to the protection against pathogens, promoting the integrity of the epithelial barrier, acidifying the intestinal milieu and stimulating the production of bacteriophages [36-39]. ~~At the systemic level~~Systemically, SCFAs act as signaling molecules that drive the expansion and function of hematopoietic and non-hematopoietic cell lineages [35]. For example, SCFA-mediated inhibition of histone deacetylase promotes tolerogenic and anti-inflammatory functions that are crucial for the maintenance of immune homeostasis [19,33]. Many other immunoregulatory properties of SCFAs are associated with the activation of G protein-coupled receptors expressed by nearly all types of immune cells, including epithelial cells, neutrophils, monocytes and macrophages [40,41].

Another worthy example of GM-produced metabolites that act as key immunoregulators are polyamines (e.g., putrescine, spermine, and spermidine), which are usually produced by amino acid decarboxylases [42]. These molecules are essential for host cell function, barrier integrity and ~~pathogen~~ defense against pathogens, as well as for local and systemic adaptive immunity [43-46]. Alterations in polyamine metabolism with higher levels of these compounds have been ~~shown to be~~ associated with cell growth bugs, and acute and chronic inflammation up to carcinogenesis [47]. Indeed, highly proliferative cells, such as tumor cells, require polyamines, among others, to support rapid growth. For example, increased circulating and urinary levels of polyamines have been observed in patients with colorectal cancer (CRC), as well as ~~with~~ skin and hormone-related (*i.e.*, breast and prostate) ~~tumors~~ cancers [49-50]. As recently demonstrated, a "polyamine blocking therapy", based on the reduction of intratumoral polyamine availability, could therefore have an antiproliferative effect but also reverse immunosuppression in the tumor microenvironment and heighten antitumor immune responses [51,52]. On the other hand, it should be mentioned that in autophagy-competent tumors, treatment with spermidine (as an autophagy-inducing caloric restriction mimetic) improved the efficacy of anticancer chemotherapy and enhanced immunosurveillance [53].

Not only the metabolites produced or contributed by GM but also the structural components of the microbes are able to influence the host immunological landscape. Since birth, the human immune system coexists with a plethora of microorganisms and develops pattern recognition receptors (PRRs), capable of detecting microbe-associated molecular patterns (MAMPs) (e.g., lipopolysaccharide (LPS), peptidoglycan, flagellin and unmethylated bacterial DNA CpG

motifs) [54]. This intimate GM-immune system crosstalk is strategic for maintaining the delicate balance between tolerance towards commensal microbes and recognition and attack towards pathogens or pathobionts [32,55]. Upon PRR-mediated response activation, a complex signaling cascade is initiated ~~that~~ lead~~s~~ing to the release of host immune system effectors, such as cytokines, chemokines, and acute phase proteins [56,57]. Furthermore, MAMPs are involved in the modulation of immune cell function, such as neutrophil migration and function [58] and differentiation of T cell populations into helper cells (T_H) (*i.e.*, T_H1, T_H2 or T_H17) or T regulatory cells (T_{reg}) [54]. For example, in preclinical models, *Bacteroides fragilis*-specific structural polysaccharide (PSA) has been shown to restore a T_H1/T_H2 imbalance through stimulation of Toll-like receptor (TLR) 2 signaling and interleukin (IL)-12 production by dendritic cells, and suppress inflammation by driving IL-10 production [59,60].

As mentioned above, during human lifespan, GM is able to respond and adapt to changes in endogenous and exogenous variables, such as diet, lifestyle and geography. This is made possible by the great GM plasticity, *i.e.*, the ability to oscillate between different healthy states, without losing diversity, stability, as well as ~~and~~ microbe-microbe and microbe-host interactions [30]. However, it is also well known that ~~in~~ under certain conditions, such as intake of antibiotics or other drugs, pathogen infection and inflammation, just to name a few, stability is compromised and unbalanced (dysbiotic) states are established, which can be resilient and explain the onset and progression of diseases, as well as resistance to the ~~efficacy~~ effectiveness of treatments [12]. Although the exact boundaries of a healthy GM are still ~~lacking~~ missing [61], disease-associated GM profiles are typically featured by less biodiversity and distinct compositional alterations, which fall~~ing~~ into the following categories: selective suppression of certain health-associated members (generally SCFA-producing, oxidative stress-sensitive *Lachnospiraceae* and *Ruminococcaceae* taxa) and/or burst of subdominant taxa, including overt pathogens or pathobionts [62]. These alterations are generally reflected in an inappropriate pattern of metabolites, which can improperly regulate human biology, with even deleterious consequences for health [63,33].

3. The gut microbiome in cancer development and progression

3.1 The role of the gut microbiome ~~role~~ in ~~the~~ tumor onset: CRC and beyond

~~Until now,~~So far unhealthy states of GM have been found in the context of multiple intestinal but also metabolic, immunological, hepatic, respiratory, cardiovascular, neurological, psychiatric, and oncological disorders [3]. In most cases, it is still impossible to define whether or not GM has a causative role ~~or not~~, even if some hypotheses supporting ~~causality~~ causation have been advanced, especially in obesity and related complications [64]. Notwithstanding this, it must be said that for some disorders, the related literature is already quite substantial (e.g., for inflammatory bowel disease (IBD) and CRC) [65-67], while for others, such as different tumor types, there is still a long way to go to understand the

GM-disease relationship. As expected, the loss of intestinal homeostasis has been linked to both local (*i.e.*, CRC and gastric cancer) [68,69] and distant tumors, such as pancreatic [70], laryngeal [71] and gallbladder [72] carcinomas.

To date, microbial pathogens are known to drive 20% of carcinogenesis [73,74]. Carcinogenesis is a multistep process, ~~whose~~ the progression of which is characterized by the gradual accumulation of slow and random genetic and epigenetic mutations that can take more than 10 years depending on the frequency [75]. In particular, as far as the GM field is concerned, the most frequent (and obvious) connection has been made with CRC. CRC is sporadic ~~for~~ in approximately 90% of cases and develops gradually from normal epithelium to adenomatous polyps until the settlement of an invasive carcinoma [76]. In addition to genetic predispositions that can increase the risk of developing CRC (e.g., adenomatous polyposis coli gene mutation), leading to the development of hundreds to thousands of polyps, several environmental factors have been shown to be involved in CRC onset, including diet, chronic inflammation (e.g., IBD) and GM [77]. As for the latter, several studies have ~~shown~~ highlighted a CRC-associated GM profile enriched with opportunistic pathogens, such as *Fusobacterium*, several members of the *Enterococcaceae* family and *Campylobacter*, as well as other pro-inflammatory taxa, *i.e.*, *Erysipelotrichaceae* and *Collinsella* (recently proposed as a potential marker of metabolic disorders) [78-81]. In parallel, a reduction ~~of~~ in health-associated microbial partners, including the butyrate producers *Faecalibacterium* and *Roseburia*, is frequently observed [82]. However, all of these studies, although milestones in the CRC-associated GM literature, are purely observational and ~~have~~ therefore have not explored the mechanisms by which GM members can influence CRC or, more importantly, the triggers that shift the GM profile towards a tumor-associated one. Most of these questions were answered by coupling next-generation sequencing-based approaches with animal models, which helped to better outline the role of GM microbes in tumor onset. Research conducted in recent years has highlighted the fairness of the bacterial driver-passenger model developed in 2012 ~~from~~ by Tjalsma et al. [83] Briefly, several environmental (e.g., diet, pathogen infection) and genetic (e.g., chronic inflammation, mutations) factors can push the GM homeostatic profile towards a dysbiotic, pro-inflammatory one that settles in the gastrointestinal tract. CRC can therefore be promoted by commensal bacteria with pro-carcinogenic features (known as bacterial drivers) that drive the DNA damage of the colon epithelium, leading to CRC development. Afterwards, the local microenvironment is altered as a result of ongoing inflammation and carcinogenesis, which paves the way for bacterial passengers, *i.e.*, microorganisms that show a competitive advantage in the tumor microenvironment and allow for cancer progression [83]. Therefore, inflammation is a trigger for initiating the GM-dependent pro-inflammatory cycle, which is detrimental to the host health [69]. The bacterial drivers identified so far are mostly subdominant components of GM, capable of inducing a harmful inflammatory loop, synthesizing genotoxins and other toxic molecules that can directly damage host cells, and activating dietary heterocyclic amines to pro-carcinogens [69]. ~~Some e~~Examples ~~are~~ include superoxide-producing *Enterococcus faecalis* strains [84,85], toxigenic strains belonging to the *B. fragilis* species [86,87], and genotoxin-

producing *Salmonella enterica* and *E. coli* strains [88-91]. As for bacterial passengers, again ~~they~~ these are usually subdominant GM commensals, which may however show either tumor-promoting or tumor-suppressive properties, depending on the microorganism type. Indeed, ~~the~~ tumor tissue is selectively colonized by opportunistic pathogens, such as *Clostridium septicum* [92], *Fusobacterium nucleatum* [93], *Streptococcus gallolyticus* [94], and several *Enterobacteriaceae* members [83], but sometimes also ~~sometimes~~ enriched in health-associated bacteria, such as *Roseburia* and *Faecalibacterium*, for which a possible protective role as CRC quenchers has been advanced [95].

It is clearly very simple to explain the relationship ~~of~~ between GM ~~to~~ and CRC, but the pro-carcinogenic role of commensal microbes extends far beyond the gastrointestinal tract [74,96]. Due to its physical proximity and close physiological links, the liver is one of the organs most affected by GM. The development of hepatocellular carcinoma (HCC) may be related to various GM-derived functions and metabolites, including LPS, ~~whose~~ the presence of which potentiates HCC tumorigenesis through the activation of innate immune system effectors, such as TLR4 [97]. Moreover, some GM taxa play the role of oncogenic drivers by producing secondary bile acids (*i.e.*, deoxycholic acid, DCA), deriving from ~~the~~ GM-mediated deconjugation and metabolism of primary bile acids [98]. Once absorbed from the gastrointestinal tract, DCA can reach the liver through the portal circulation, where it can exert tumorigenic functions by inducing DNA damage and senescence on hepatocytes, with the establishment of a pro-inflammatory liver environment [99,100]. Consistently, in murine models fed high-fat diets, ~~it has been observed that the~~ enrichment of GM species belonging to the *Clostridium* genus, including *C. scindens*, *C. hiranonis*, *C. hylemonae* and *C. sordelli*, capable of producing DCA [101], led to progression from non-alcoholic steatohepatitis to HCC [99]. The same tumor-driving actions by GM members have recently also been reported in esophageal tumors [100]. Finally, it is worth mentioning that GM dysbiosis and the consequent dysregulation of metabolite production have been shown to be involved in the development of breast cancer [102,103]. In particular, gut microbes are able to metabolize liver-derived estrogens through beta-glucuronidase and beta-glucosidase activities in the gastrointestinal tract (the so-called estrobolome, *i.e.*, "the aggregate of enteric bacterial genes whose products are capable of metabolizing estrogens") [104]. This GM role in modulating the systemic ~~estrogen~~ pool of estrogens could affect their enterohepatic circulation and reabsorption, thus contributing to an increased risk of hormonal cancers, such as breast cancer [105-107].

3.2 Gut microbiome middles with anticancer therapies

A new frontier of research is the understanding of the bidirectional relationship between GM and drugs (*i.e.*, pharmacomicrobiomics) [108]. GM can in fact modulate the host response to therapies through several mechanisms, including immune system interactions and drug metabolism [109], and, in turn, drugs can affect the GM structure and thereby its mutualistic relationship with the host [110]. Identifying the pivots of this relationship can therefore be crucial

for improving patients' clinical outcomes, as it can inform the development of novel, evidence-based intervention strategies, aimed at manipulating GM to enhance therapeutic efficacy, reduce toxicity and possibly, also the risk of relapse [111,112].

The first research on GM in anticancer therapies dates back to 1890, when two heat-inactivated microbes belonging to the *Streptococcus* genus were injected intratumorally as an attempt to cure cancer in humans. In those years, Dr. Coley thought that a local bacterial infection could boost the patient immune response against inoperable tumors. For more than 40 years, more than 1000 patients were injected intratumorally with microbes or microbial products, with excellent results mostly in inoperable bone and soft-tissue sarcomas. From this moment on, albeit rudimental, this approach was called Coley's Toxins and defined as the "father of immunotherapy" [113,114]. Several years later, the same approach was used to treat patients with bladder cancer, in whom *Mycobacterium bovis* was intratumorally injected right after tumor resection, resulting in reduced tumor recurrence through activation of local immune responses [115]. Furthermore, oral administration of the well-known probiotic species *Lactobacillus casei* has been associated with the decrease in recurrence of superficial bladder cancer [116]. Later on, all these studies confirmed the intuition of those researchers: the antitumor responses were stimulated by the microbial activation of two important effectors of the immune system: natural killer cells and macrophages [117]. Although these are rudimentary and "seasoned" studies, they have paved the way for many clinical trials, some still ongoing, using attenuated GM members to aid cancer treatments [118]. Recently, ~~it has been observed that~~ *Mycobacterium obuense* [119,120] and genetically modified *Salmonella* Typhimurium [121,122] have been shown to promote anticancer responses in several refractory solid tumors (e.g., pancreatic, melanoma), by activating the host immune system and exerting a cytotoxic effect on tumor cells. While very promising, many studies are still needed to refine microbial therapies before they can be used in clinical routine. Still today, the action and toxicity of microbes inside the tumor are very hazy and mainly correlated to the long microbial half-life with the possibility of antibiotic resistance accumulation, as well as the onset of mutations reverting the attenuated bacterial phenotype [123].

In addition to the intratumoral effect of individual microbes, the GM has recently been associated with the therapeutic outcome of anticancer treatments [124]. Since the discovery of the cytotoxic effects of mustard gas during the Second World War, cytotoxic chemotherapeutic ~~cytotoxic~~ agents (*i.e.*, alkylating agents, platinum-based drugs and cytotoxic antibiotics) have been developed and are still the major staple of anticancer approaches [125]. However, some ~~tumors~~ cancers fail to respond to treatment and/or ~~tumor~~ the cancer ~~relapse oc~~recurs. To overcome these hurdles, novel anticancer approaches are constantly in progress [126]. The first advancement in this field was the development of targeted immunotherapy [127,128] and, of course, research focusing on the relationship between GM~~-~~ and anticancer therapy has ~~relationship~~ followed the same trend. Gut microbes have been shown to influence drug pharmacokinetics, anticancer activity and toxicity of chemo-immunotherapy treatments to varying degrees [110,129]. A striking example of a GM-

drug interaction is represented by irinotecan, a chemotherapy drug administered parenterally in an inactive form to patients with CRC, which is activated by host enzymes, detoxified in the liver and subsequently excreted in the intestinal lumen via the bile [130]. Here, GM members can reverse the detoxification process through bacterial beta-glucuronidases, which catalyze drug deconjugation and reactivation, resulting in intestinal toxicity [131]. In this regard, the use of specific enzyme inhibitors has been shown to prevent irinotecan-induced diarrhea while maintaining its efficacy in animal models [132]. As for the GM influence on anticancer activity, one of the first milestone studies in the field showed that the antitumor effect of oxaliplatin or cisplatin treatment on subcutaneous transplantable tumors was ~~dramatically~~ drastically reduced in germ-free or GM-depleted mice by broad-spectrum antibiotics [133]. The so-called platinum resistance of these models has recently been linked to the role of GM members in promoting oxidative stress and, subsequently, apoptosis of ~~tumor~~ cancer cell ~~apoptosis~~. Consistently, mice with lung tumors treated with antibiotic-coupled cisplatin therapy have been shown to have reduced long-term survival and developed even larger tumors [134]. On the other hand, when cisplatin was combined with the administration of probiotics, ~~like~~ such as *Lactobacillus acidophilus*, the same animal models showed improved response to chemotherapy, through the activation of pro-apoptotic genes and effectors within the tumor aggregate and the promotion of a proper tumor-specific immune response. Similar to platinum-derived compounds, chemotherapy treatments based on the alkylating agent cyclophosphamide coupled with ~~the~~ oral administration of microbes (*i.e.*, *Lactobacillus johnsonii* and *Enterococcus hirae*) have been shown to promote the conversion of T cells from naïve to pro-inflammatory T_H17, with the final outcome of improved therapeutic efficacy in tumor-bearing mice [135-137]. These findings were also confirmed in advanced lung or ovarian cancer studies, in which patients with GM enrichment of *E. hirae* and *Barnesiella intestinihominis* showed a more favorable prognosis after chemo-immunotherapy [138].

With specific regard to immunotherapy, different studies have highlighted that the administration of CpG oligodeoxynucleotides (*i.e.*, synthetic molecules mimicking microbial DNA) strongly stimulated the host immune system, pushing endogenous anticancer activity in several types of cancer [139]. After *in vivo* intratumoral injection of CpG oligodeoxynucleotides coupled with anti-IL-10 receptor antibody, ~~the~~ host immune cells were activated near the tumor site to produce pro-inflammatory tumor necrosis factor (TNF), leading to reduced tumor growth due to hemorrhagic necrosis. ~~With~~ By a similar mechanism, the administration of *Alistipes shahii* and *Ruminococcus* in antibiotic-treated mice stimulated the production of TNF with a notable improvement of the anticancer therapeutic outcomes [133]. As the literature currently stands, GM members are involved in the intrinsic efficacy of another class of immunotherapy drugs known as immune checkpoint inhibitors, which are commonly used to treat different types of solid tumors. These molecules are capable of blocking immune-inhibitory pathways, thus modulating the activation of T cells against the targeted tumor cells [140-142]. Currently, the checkpoint inhibitors put in place are monoclonal antibodies that target

cytotoxic T lymphocyte-associated antigen 4 (CTLA4) and programmed cell death 1 (PD1) located on T cell surfaces, as well as its ligand (*i.e.*, programmed cell death ligand 1, PD-L1) [143]. The mechanisms of action are both T cell-specific but while anti-CTLA4 therapy is able to regulate T cell proliferation early during the immune response within the lymph nodes, anti-PD1 suppresses T cell activation later in the body periphery [144]. In this scenario, a landmark study by Vétizou and colleagues [145] showed that antibiotic-treated or germ-free mice with subcutaneous tumors treated with anti-CTLA4 responded poorly to therapy, but the response was significantly increased when GM was enriched in *Bacteroides fragilis* and *Burkholderia cepacia* [145]. Furthermore, oral feeding of GM-depleted mice with different *Bacteroides* species (*i.e.*, *B. thetaiotaomicron* or *B. fragilis*) restored the therapeutic response to anti-CTLA4 by inducing immune cell response in tumor-draining lymph nodes. When *B. fragilis* and *B. cepacia* were administered together in the same murine models~~, *B. fragilis* and *B. cepacia* were administered together~~, ~~the~~ restoration of the anti-CTLA4 response was confirmed but, unlike ~~the~~ administration of single *Bacteroides* taxa, therapy-related side effects, such as intestinal damages and colitis, were also significantly reduced. These findings were ~~confirmed~~ upholded in melanoma patients treated with anti-CTLA4, where the abundance of the Bacteroidetes phylum was positively correlated with the reduction ~~in~~ of therapy-associated colitis. In particular, the GM profiling of these patients revealed three different configurations: one was dominated by *Prevotella* spp., whereas the other two were mostly characterized by the presence of various *Bacteroides* spp. Subsequently, these different GM configurations were used to perform FMT ~~to~~ on germ-free mice. Only the GM profile enriched in *B. thetaiotaomicron* or *B. fragilis* resulted in high responsiveness to anti-CTLA4 treatment in non-responder mice. Taken together, these observations are extremely relevant as they suggest not only that some GM members may affect immunotherapy responses but also that GM manipulation may favor antitumor activity in non-responders. On the same line ~~of~~ as anti-CTLA-4 therapy, Sivan et al. found that in melanoma-bearing mice, the efficacy of PD-L1-targeted antibodies was enhanced in the presence of a GM ecosystem enriched with different *Bifidobacterium* spp., including *B. breve*, *B longum* and *B. adolescentis* [146]. Oral administration with a commercially available probiotic cocktail (*i.e.*, with *B. breve* and *B. longum*) during anti-PD-L1 therapy, was able to activate the immune T cell response and hold tumor growth, while the combined treatment (bifidobacteria and anti-PD-L1) nearly abolished tumor outgrowth. From this moment onwards, multiple translational works have been carried out. Among the most relevant, it is certainly noteworthy that of Routy et al.~~,~~ [140] who found that patients with melanoma treated with antibiotics during anti-PD-L1 immunotherapy showed a lower survival rate [140]. By comparing the GM of responders vs non-responders, the authors were able to identify the GM compositional signatures of response to therapy, which consisted of ~~an~~ enrichment in *Akkermansia* and *Alistipes*. Again, they performed FMT from patients to germ-free mice and found that *Akkermansia muciniphila*, alone or in combination with *E. hirae*, increased intratumoral cytotoxic T cell infiltrates, favoring the PD-1 blockade response. In parallel, similar compositional differences between responders and non-responders to anti-PD-L1

therapy were found out by Gopalakrishnan et al. [141]. Notably, responders to melanoma-targeted therapy were characterized by higher microbial diversity, as well as increased relative abundance of *Ruminococcaceae* and *Faecalibacterium*, both associated with improved effector T cell function in the peripheral and intratumoral environment. On the other hand, patients showing poor immunotherapeutic response possessed lower microbial diversity and higher relative abundance of Bacteroidales, which ~~was~~ correlated with reduced systemic and antitumor immune responses. Another GM metagenomic characterization in patients with melanoma treated with immune checkpoint inhibitors further corroborated the above findings (*i.e.*, that responders have a distinct GM profile from non-responders), although in this case the efficacy of anti-PD-L1 therapy was associated with *B. longum*, *E. faecium* and *Collinsella aerofaciens* [142].

It should be noted that, despite the huge number of microbial species inhabiting the gastrointestinal tract, to date only a few of them have been suggested to play a role in anticancer responses and only a handful of strains have shown the potential to manipulate ~~the~~ host physiological functions *in vivo* [147,148]. For example, Tanoue and colleagues [149] isolated a consortium of 11 microbial strains (mainly belonging to *Bacteroides* spp.) from feces of healthy human donors, which are able to robustly induce T cell activation within the intestine. *In vivo* colonization with the 11-strain mixture enhanced the therapeutic efficacy of immune checkpoint inhibitors in syngeneic tumor models, confirming the great potential of these microbes as widely effective biotherapeutics in anticancer approaches. Furthermore, three strains belonging to *B. pseudolongum*, *L. johnsonii*, and *Olsenella* have recently been tested in tumor-bearing mice, where they significantly increased the efficacy of immune checkpoint inhibitors [150]. In particular, *B. pseudolongum* enhanced the immunotherapy response through the production of the metabolite inosine, which was able to systemically translocate due to immunotherapy-induced decrease in gut barrier function, and thus activate antitumor T cells. Several questions about the safety of administering live microbes to very often immunocompromised patients during anticancer therapy have been raised over the years and are still largely unanswered. In this context, the usage of prebiotics could be a valid alternative, as discussed below (see the paragraph "Modulation hypothesis: prebiotics, probiotics, live biotherapeutics and FMT, as adjuvant cancer therapy"). As an example, prebiotics (*i.e.*, mucin and inulin) have been shown ~~in syngeneic mouse models~~ to induce antitumor immunity and concomitantly control tumor growth in syngeneic mouse models [151].

3.3 The gut microbiome and hematological malignancies: a focus on hematopoietic stem cell transplantation and related complications

As discussed above, most studies on the role of GM in influencing therapies have focused on solid tumors, particularly melanoma. In parallel, another popular line of research has dealt with profiling the GM of patients suffering from hematological neoplasms (e.g., acute leukemia, lymphoma, and myeloma), with particular regard to the patients' clinical outcomes during and after ~~the~~ chemo-immunotherapeutic treatment. For patients with various blood tumors, first-line and

life-saving therapy is considered HSCT, a combination of stem cell therapy, conventional treatments (*i.e.*, chemotherapy and radiation) and immune therapy [152]. Unfortunately, HSCT can lead to life-threating complications, such as graft-versus-host disease (GvHD, *i.e.*, when alloreactive donor T cells attack host organs, such as skin, liver and gut), and local and systemic infections, and ~~tumor~~ cancer may relapse~~s may occur~~ [153]. In this context, recent studies in mice and humans suggest important links between GM and clinical outcomes, as well as a role of GM in immunological reconstruction in HSCT recipients [154-156]. Indeed, HSCT practices significantly affect GM balance with a reduction in diversity and sometimes monodominance by Proteobacteria members, *Enterococcus* or *Streptococcus* [157]. In a retrospective study, the reduction of GM diversity in patients undergoing HSCT was associated with a significant increase in mortality (*i.e.*, 52%) compared to patients with a high-diversity GM profile (*i.e.*, 8%) [158]. In addition, during the post-transplant period, antimicrobial treatments are commonly used to treat febrile neutropenia with the ultimate consequence of affecting the GM structure, which can result in increased susceptibility to bacterial infections [159]. For example, an increase in the level of *Enterococcus* spp. after antibiotic exposure correlated with an increased risk of developing bacteremia [157]. Similarly, enrichment of *Enterobacteriaceae* members and other Gram-negative microbes was associated with increased mortality [158]. Recent studies in animal models have shown that the development of GvHD is also associated with a peculiar GM dysbiosis, featured by increased levels of *Enterobacteriaceae* and a reduced amount of obligate anaerobes, mostly belonging to the Clostridiales order [160,161]. These findings were then confirmed in several clinical studies on both adult and pediatric patients [159, 162-165]. By reconstructing the GM dynamics across HSCT, some of these studies have suggested that the so-called "anti-inflammatory Clostridia", *i.e.*, members of the families *Clostridiaceae*, *Lachnospiraceae*, *Ruminococcaceae* and *Eubacteriaceae* (the main producers of SCFAs in the intestine), might exert a counteracting effect on GvHD onset and progression [166,167]. As further confirmation, a study on a large cohort of adults showed that the relative abundance of *Blautia*, a well-known health-associated SCFA-producing microorganism belonging to the *Lachnospiraceae* family [168], was correlated with reduced mortality from GvHD [169]. Increased proportions of *Blautia*, along with increased SCFA production, have recently been demonstrated in pediatric patients receiving post-transplant enteral rather than parenteral nutrition [170]. Interestingly, none of the enterally fed patients showed evidence of bloodstream infections, stressing the importance of maintaining a eubiotic GM configuration, capable of producing health-promoting metabolites, to reduce the risk of HSCT-related complications [170,171]. Bacterial-derived SCFAs, especially butyrate, have also been shown in a mouse model to improve the junctional integrity of intestinal epithelial cells, reduce apoptosis, and mitigate GvHD severity [166].

4. The big issue of the tumor microbiome

Since the work by Geller et al. [172] on pancreatic ductal adenocarcinoma, accumulating and robust evidence has confirmed the existence of intratumoral microbes, which can act as intrinsic and essential components of the tumor microenvironment, thus influencing cancer and cancer therapy. These microbes do not necessarily include only "oncomicrobes", *i.e.*, microbes that are known to initiate cancer through genotoxin-mediated mutagenesis (such as fragilisin- and reactive oxygen species-producing *B. fragilis* or colibactin-producing *E. coli*) [173,174] or by interfering with important pathways involved in differentiation and morphogenesis (such as *F. nucleatum* expressing FadA that binds to E-cadherin and activates Wnt-beta-catenin signaling) [175]. Indeed, it has recently emerged that many other microorganisms may not be causative but rather complicit, acting mainly ~~acting~~ through ~~the~~ modulation of the host immune system (immunosuppression or immunogenicity) or molecular mimicry [176].

To date, traces of bacterial DNA have been identified using next-generation sequencing approaches in at least 30 types of cancer, including pancreatic, bile duct, lung, breast, ovarian, cervical, uterine, testicular, prostate, bladder, melanoma, thyroid, kidney, leukemic, bone and brain cancers [177,178]. Notably, most major cancer types appeared to be featured by unique microbial signatures, not only at the tissue level but also in the blood, thus paving the way for the use of plasma-derived cell-free microbial DNA in novel microbiome-based diagnostic tools [178]. In particular, the ratio between taxa belonging to the phylum Proteobacteria and those of Firmicutes varied in the different types of cancer, with the breast cancer microbiome being the richest and most diverse of those analyzed to date [177]. Alongside the compositional traits, it is worth noting that a tissue-specific enrichment of some bacterial functions has been hypothesized, which could be related to the clinical features of the different tumor types [177]. For example, bacterial degradation of hydroxyproline, deriving from bone collagen and particularly high in bone diseases, including cancer, was overrepresented in bone tumors. Likewise, the pathways involved in the degradation of chemicals in cigarette smoke were enriched in lung cancer. While these findings are expected, as they may be the result of host-driven or top-down selection, it should be pointed out that they were generated through inferred metagenomics, with obvious interpretative limitations. The presence of microbial components within some tumor tissues was also investigated by immunohistochemistry, which allowed to confirm that not only nucleic acids but also structural components can be found, such as LPS from Gram-negative bacteria and lipoteichoic acid from Gram-positive bacteria~~, can be found~~ [177]. However, while LPS was frequently detected, lipoteichoic acid was ~~mainly~~ found mainly in melanoma. These data were apparently in contrast to ~~with those of sequencing~~sequencing-derived ones, according to which many Gram-positive bacteria were represented in any tumor type, but they may reflect an altered cell morphology (with lack of cell wall), as also hypothesized based on transmission electron microscopy imaging and previous literature [179]. Based on the staining patterns, intratumor bacteria were mostly localized in the cytoplasm and nucleus of cancer cells, as well as in immune cells, *i.e.*, in leukocytes and especially in macrophages [177]. As for their number, in pancreatic ductal adenocarcinomas, an average of one bacterium per about

150 human cells has been estimated [172]. According to tumor mapping by Nejman et al. [177], the percentage of tumors positive for bacterial DNA ranged ~~between~~ from 14.3% in melanoma to >60% in breast, pancreatic and bone tumors. Based on these estimates, Sepich-Poore et al. [176] ~~came to the conclusion of~~concluded about 10^5 to 10^6 bacteria per palpable 1-cm^3 tumor, or about 34 bacteria per mm^2. The source of these microorganisms/microbial components is not yet clear, but they could likely be part of other host-associated ecosystems, such as GM, translocate ~~through~~ across compromised mucosal barriers (e.g., leaky gut), and then reach tumor masses, facilitated by their disorganized and leaky vasculature. For example, this has been strongly suggested for CRC liver metastases, where >99.9% nucleotide identity was found for *Fusobacterium* isolates from the primary tumor and metastatic site, although tissue collection occurred months if not years later [81]. Once in the bloodstream, *F. nucleatum* has been hypothesized to translocate also ~~to~~ in breast cancers, colonization of which is made possible by the binding of its lectin Fap2 to galactose and N-acetyl-d-galactosamine residues, abundantly expressed on cancer cells [180]. Obviously, work ~~is~~ still ~~needed~~ needs to be done to confirm all of these findings, including the dead/alive issue. Similarly, ~~it will have to be determined~~ whether these bacteria are actually involved in tumor pathogenesis or are mere opportunistic residents/passengers, which take advantage of a nutrient-rich and immunosuppressed environment, should be determined. Regardless, they may play a critical role in promoting tumor growth and/or mediating chemoresistance, thus affecting patient response and survival. This has been seen for example for Gammaproteobacteria that can inactivate the chemotherapeutic drug gemcitabine, ~~by~~ through the expression of a long isoform of the bacterial enzyme cytidine deaminase [172], and for *F. nucleatum*, which activates the autophagy pathway (by targeting TLR4 and MYD88 innate immune signaling and specific microRNAs), thus preventing chemotherapy-induced apoptosis [181]. *F. nucleatum* may also accelerate tumor growth by inducing apoptosis in lymphocytes, as suggested by the reduced levels of CD4+ and CD8+ T cells in breast cancers [180]. Notably, the administration of antibiotics (*i.e.*, metronidazole) inhibited *F. nucleatum*-induced tumor enlargement, stressing once again how microbial manipulation can have significant repercussions on clinical outcomes. Finally, it is worth mentioning that greater intratumoral diversity has been correlated with long-term survival in patients with pancreatic adenocarcinoma [182]. Long-term survivors also showed potentially favorable intratumoral microbial signatures (*i.e.*, *Saccharopolyspora*, *Pseudoxanthomonas*, *Streptomyces* and *Bacillus clausii*), which could promote the recruitment and activation of CD8+ T cells, with overproduction of interferon (IFN)-gamma, thus contributing to the antitumor immune response and influencing the natural history of the disease. Although the data are still preliminary, using FMT from long-term survivors with no evidence of disease in tumor-bearing mice, the authors observed immune system activation and antitumor response, thus opening the way to immense microbiome-based therapeutic opportunities. What is certain is, therefore, that future therapeutic strategies ~~can~~ will no longer be able to ignore the presence of intratumoral microbes, rather they

could be improved through integration with precision microbiome manipulation tools, targeting microbes and/or the mechanisms in which they are involved.

5. Modulation hypothesis: prebiotics, probiotics, live biotherapeutics and FMT, as adjuvant cancer therapy

In recent times, enormous strides have been made in improving anticancer therapies, expanding the plethora of treatments available and significantly reducing side effects, while paying attention to patient compliance [183]. As discussed above, multiple lines of evidence have placed an increasing emphasis on how microbiome modulation may ~~represent~~ be a crucial adjunct to current anticancer therapies [141,184-186]. Microbiome-tailored precision medicine is based on the use of prebiotics, (traditional and next-generation) probiotics and FMT, to be personalized for the best efficacy and safety according to the patient's microbiome configuration and other host metadata~~, for the best efficacy and safety~~. Herein, we will discuss the most recent and relevant clinical trials that have been planned (some still ongoing) to shed light on the therapeutic potential of GM manipulation in cancer patients (using the tools mentioned above), in terms of improved response and mitigation of adverse events. See **Table 1** for a summary of the clinical trials that have been registered in the last two years.

5.1 Prebiotics

Prebiotics are typically referred to as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" [187]. In particular, they exert their beneficial effects by promoting the expansion and/or metabolic activity of specific groups of commensals, including keystone taxa. Other induced effects at the microbial ecosystem level include growth promotion through cross-feeding interactions and inhibitory effects against pathogens or pathobionts, through displacement, production of antimicrobial metabolites or other changes in microbial fitness [185,188,189]. Evidence~~s~~ ~~gathered~~ from ~~in~~ *in vitro* and *in vivo* studies suggest~~s~~s that administering prebiotics is a promising and safe therapeutic strategy in different clinical settings [151,185]. A recent study has also demonstrated that discrete dietary fiber structures (*i.e.*, chemically modified resistant starches with small structural differences) are able to induce divergent and highly specific effects on GM, which direct~~ly~~ change~~s~~s in SCFA production, thus paving the way for precision manipulation of ~~the~~ GM through *ad hoc* designed carbohydrates [190]. With specific regard to cancer, however, only a limited number of interventional studies in humans are available to date, with sometimes conflicting results. For example, oral consumption of amylase-resistant starch as a prebiotic formulation, administered in combination with chemotherapy, was not conclusive in the prevention of acute radiation proctitis in patients with cervical cancer [191]. Conversely, a prebiotic regimen based on fructooligosaccharides, xylooligosaccharides, polydextrose, and resistant dextrin, administered 7 days prior to ~~surgery for~~ CRC surgery, improved serum immunological markers, reversing the surgical stress-induced surge of

opportunistic pathogens in GM [192]. The interventional clinical trial conducted by Garcia-Peris and colleagues ([193]; NCT01549782) on 40 women undergoing radiotherapy for endometrial neoplasms confirmed the hypothesis that a mixture of fructooligosaccharides and inulin modulates the representation of *Lactobacillus* and *Bifidobacterium* within the GM community, while reducing tissue damage at the enterocyte level. As briefly mentioned above, in a mouse melanoma model, inulin and mucin also stimulated *Bifidobacterium* spp. and *A. muciniphila* [151], both previously identified as beneficial GM components, capable of eliciting effective antitumor immunity [140,146]. Regarding clinical trials in cancer patients still ongoing and started in the last two years (**Table 1**), one study foresees the enrolment of 120 participants with gastrointestinal cancer and chemotherapy-related diarrhea (NCT04447443) and focuses on a 2-week supplementation with prebiotic fiber ~~supplement~~ along with loperamide hydrochloride ~~administration~~. Longitudinal monitoring of the GM configuration and subsequent comparison with the results obtained from the administration of placebo (*i.e.*, maltodextrin) and loperamide will allow researchers to dissect the effects specifically induced by prebiotics. A second study (NCT04624568) aims to compare the regression rate of cervical intraepithelial lesions in 150 women after 6-month administration of a vaginal gel composed of hyaluronic acid and prebiotic extract of *Coriolus versicolor*, which improves the re-epithelialization of the uterine cervix [194]. Creating a protective biofilm on the cervix would help restore a niche environment conducive to regression of intraepithelial lesions and human papillomavirus clearance. Although the aforementioned studies are still in their infancy, they have the potential to provide valuable insights into how prebiotic administration modulates the microbiome of cancer patients, while influencing disease markers and clinical outcomes.

5.2 Probiotics and live biotherapeutics

Probiotics, defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit to the host" [195], are a GM manipulation tool with a long history of use. Those currently most used and studied are certainly bifidobacteria and lactobacilli, but also, to a lesser extent, strains of *Lactococcus* spp., *Streptococcus thermophilus*, *Saccharomyces boulardii* and *E. coli* Nissle 1917. Clinical efficacy, mechanisms of action and caveats in the field are admirably discussed elsewhere (see for example Suez et al.~~,~~ [196]). In the context of cancer patients, strains of *Lactobacillus* and *Bifidobacterium* have stood out for their ability to delay tumor formation, inhibit tumor cell proliferation and prevent life-threatening side effects associated with chemotherapy treatments, in addition to ~~the~~ binding and degradation of carcinogenic compounds, ~~the~~ inhibition of carcinogen-producing enzymes, and immunomodulating and anti-inflammatory properties [197-201]. It is also worth noting that probiotic strains of *Bifidobacterium* and *Clostridium*, when administered intravenously, have been shown to colonize hypoxic tumors, preferably thriving in solid malignancies [202]. However, despite the encouraging results, most probiotic therapies in oncology are still in the preclinical stage and very few studies have ~~have~~ reported the effects of probiotics in humans [201,203] (see also **Table**

1). Among them, a clinical trial in CRC patients showed that probiotic (*Bifidobacterium lactis* Bl-04 and *L. acidophilus* NCFM) administration promoted the expansion of beneficial butyrate-producing microbes in both mucosa and feces, and tended to reduce *Fusobacterium* proportions (NCT03072641; Hibberd et al., [204]). Furthermore, preoperative probiotic (*S. boulardii*) therapy resulted in reduced levels of IL-1beta, IL-10, and IL-23 mRNA within the colonic mucosa of CRC patients following resection, when compared to controls who received the anticancer treatment alone (NCT01895530) [205]. As for other cancer types, improved relapse-free survival was observed after administering an oral preparation of *L. casei* for one year to patients with superficial bladder cancer, after completion of transurethral resection therapy followed by intravesical administration of epirubicin, although no difference in overall survival was observed compared to the control group [206]. Beneficial effects on GM are also expected in an ongoing clinical trial, which involves a short course of probiotic therapy (with the following 13 species: *S. boulardii*, *Lactobacillus plantarum*, *Bacillus subtilis*, *B. lactis*, *Bifidobacterium bifidum*, *Lactobacillus rhamnosus*, *B. breve*, *L. casei*, *Lactobacillus salivarius*, *L. acidophilus*, *Lactobacillus brevis*, *B. longum* and *Lactobacillus paracasei*) in patients with operable breast cancer before surgery (NCT03358511). Interestingly, variations within the tumor microenvironment are also expected, with in particular an increase in the resident CD8+ T cell subpopulation. In another clinical trial, ~~the~~ researchers aim to evaluate the efficacy of the probiotics administered (no information is available on the strains or species used) prior to surgery in 40 patients with breast and lung cancer, while assessing systemic and intratumoral immunomodulatory effects (NCT04857697). The enrollment of 40 patients with potential/resectable non-small cell lung cancer is instead planned to evaluate the safety and effect of neoadjuvant chemotherapy and immunotherapy combined with probiotics (again, no compositional information is available) (NCT04699721). Within the Thoracic POISE project [207], the efficacy of a probiotic blend of *Lactobacillus* and *Bifidobacterium* spp. (*i.e.*, Pro 12) will be assessed in reducing surgical adverse events, prolonging overall survival and pioneering integrative care delivery in 40 patients with esophageal cancer (NCT04871412).

As next-generation probiotics, live biotherapeutics, defined as “live organisms designed and developed to treat, cure, or prevent a disease or condition in humans” [208], have the potential to ~~represent~~ be a decisive tool for the improvement of current anticancer therapies [209]. This category encompasses GM members that have emerged thanks to advances in massive sequencing technologies but also engineered microbes, *i.e.*, GRAS (generally recognized as safe) organisms or commensals, which are used as a delivery vehicle for a bioactive molecule or to express certain functionalities [210]. The former includes, for example, *A. muciniphila* (identified as a predictor of response in melanoma) [140], as well as *Bacteroides ovatus* and *Bacteroides xylanisolvens*, both of which have been associated with enhanced cancer immune surveillance [211,212]. As for recombinant bacterial therapeutics, although they are currently being tested in clinical trials, none of them have so far been approved for use in humans. Among these, *B. longum* expressing the pro-inflammatory IL-12 transgene (bacTRL-IL-12) was selected to evaluate the beneficial effects on solid tumors, in terms

of stimulation of the local and systemic anticancer immune response (phase I, Symvivo). The clinical trial focusing on the safety, tolerability and preliminary evaluation of the anticancer efficacy of bacTRL-IL-12 following intravenous infusion was recently conducted on 5 participants (NCT04025307). *E. coli* Nissle 1917, engineered to produce cyclic adenosine diphosphate, a stimulator of the STING (STimulator of INterferon Genes) pathway (SYNB1891; phase I Synlogic), has also been identified as a promising live biotherapeutic agent for the adjuvant treatment of solid tumors but, to date, clinical trials are still ongoing. In particular, the study NCT04167137 involves intratumoral injection of SYNB1891 in patients with diagnosed advanced/metastatic solid tumors and lymphomas, undergoing imaging to assess tumor response and safety monitoring. After determination of dose-limiting toxicity, SYNB1891 will be administered in combination with immunotherapy treatment (*i.e.*, atezolizumab). It is worth noting that *E. coli* Nissle 1917 has also been engineered to bind to the surface of CRC cells and secrete myrosinase, an enzyme capable of converting glucosinolates in cruciferous plants into isothiocyanates such as sulforaphane, a small molecule with known anticancer activities [213]. In murine models of CRC, ~~,~~ fed with engineered microbes and a cruciferous vegetable diet, the authors observed significant tumor regression and reduced tumor recurrence. Finally, a double-blind, randomized interventional study on 100 women with breast cancer has been planned to evaluate the effect of an investigational product (a probiotic from BIOHM Health LLC, engineered to address the key role of fungi in digestive health) administered in combination with standard anticancer therapy, ~~,~~ on the breast cancer microbiome and GM (NCT04362826).

On the other side of the coin, some studies have shown deleterious effects for probiotics in cancer patients, even using the same strains, such as an increase in tumor penetrance and multiplicity [214]. Discordant and heterogeneous evidence of efficacy strongly underscores the need for a precision tailored approach, which ~~takes into account~~considers the individual microbiome configuration (in terms of composition and functionality), host metadata (e.g., genetics, anthropometrics and immune profiling) and varying environmental exposures [196,215]. However, it remains undeniable that extreme caution should be taken when administering live microbes to individuals who are very often immunocompromised, due to primary disease and/or therapeutic treatments [196,216].

5.3 FMT

As already defined, FMT is the therapeutic procedure that involves the transfer of microbes from healthy individuals to recipients hosting a dysbiotic GM layout, with the aim of normalizing its structure and functionality towards a~~n~~ eubiotic state [186, 217-219]. Since 2018, increasing attention has been paid to the manipulation of GM through FMT in ~~the field of~~ oncology, with particular regard to immune checkpoint blockade [141,220]. From the perspective of microbiome-based medicine, FMT could be administered as a drastic tool for cancer patients who are unresponsive to therapies, to improve systemic and antitumor immune responses. As discussed above, in a pioneering study in an animal model ~~study~~, FMT

from non-responding cancer patients to tumor-bearing mice conferred the resistance phenotype to the recipient, while infusions from responding patients restored reactivity to PD-1 blockade [140]. Subsequently, clinical benefits were obtained as a result of FMT in patients with immunotherapy-resistant metastatic melanoma. In particular, ~~the~~ administration of anti-PD1 in combination with FMT, performed every 14 days for up to 90 days, induced objective or complete responses in three out of five patients, crossing the 6-month progression-free survival landmark [221]. The shifts induced in the GM composition after treatment included the expansion of bacterial species potentially favorable to immunotherapy, *i.e.*, *Ruminococcus* spp. (*R. gnavus* and *R. callidus*) and *B. adolescentis*. In parallel, the tumor microenviroment also underwent a reprogramming, consisting in an upregulation of the IFN-gamma-mediated signaling pathway, together with effector T functions. In a second recent study on 16 patients with advanced melanoma, a single FMT from seven different donors was administered in combination with PD-1 blockade [222]. PD-1 refractory patients exhibited a shift towards donor GM composition, along with significant metabolic changes and reprogramming of the tumor microenvironment, thereby overcoming primary resistance to immunotherapy. Taken together, these pivotal studies led to the first proof of concept that FMT transfers clinical benefits to patients with immunotherapy-resistant metastatic melanoma, by shifting their GM towards a donor-like profile associated with immune activation, mitigation of anti-inflammatory tone and modification of host metabolism.

Among the clinical trials on FMT in cancer patients, registered in the past two years and currently underway (**Table 1**), only two studies are in the active recruitment phase. The first trial (NCT04721041) involves the enrollment of 40 participants and focuses on the treatment of oncotherapy-related intestinal complications by evaluating the efficacy of washed microbiota transplantation (WMT), a new stage of FMT. Consisting of sequential microfiltration and centrifugation steps, WMT has been shown to reduce the rate of adverse events potentially associated with classic microbiome-based treatment (e.g., fever, diarrhea, abdominal pain, nausea and vomiting), without compromising the effectiveness of ~~the~~ procedure [223,224]. The second randomized controlled clinical trial (NCT04758507) aims to evaluate the efficacy of FMT in improving response rates to immune checkpoint inhibitors in 50 patients with advanced renal cell carcinoma, by selecting donors who respond to therapy. Recipient patients will receive the first infusion ~~by~~ via colonoscopy, while frozen fecal capsules ~~at~~ three and six months after the first treatment. Four of the remaining five clinical trials listed in **Table 1** concern the evaluation of the safety and efficacy of FMT, as well as the enhancement of immunotherapy treatment in 20 patients with advanced lung cancer (NCT04924374), 15 participants with metastatic CRC (NCT04729322), 50 patients with metastatic or inoperable melanoma or non-small cell lung cancer (NCT04521075), and 60 patients with malignant melanoma prior refractory to immune checkpoint inhibitors (NCT04577729). Finally, the efficacy of FMT will be assessed in the prevention of allogenic HSCT complications, particularly GvHD, in a prospective multi-center randomized phase II clinical trial on 150 participants (NCT04935684).

While it is proving to be a valid and promising tool for modulating GM, as expected, the safety of FMT is still debated, especially as large cohort studies on long-term safety are currently lacking [225]. The risk of adverse events potentially caused by FMT treatment suggests that great caution should be taken in choosing the most suitable microbiome-tailored treatment, especially in cancer patients already severely compromised by standard chemo-immunotherapy treatments.

6. Application of novel microbiome-based approaches in cancer medicine: machine learning as thea key for to patient stratification and outcome prediction

6.1 The promise of machine learning

As discussed in the previous paragraphs, research in recent decades has highlighted the dramatic impact of GM on multiple aspects of human pathophysiology, including the development and progression of cancer. This was possible thanks to 'omics' techniques (*i.e.*, 16S rRNA gene sequencing, shotgun metagenomics and metatranscriptomics, metaproteomics and metabolomics), which led to a paradigm shift in the field of microbiology, moving from the study of single microbial colonies to a high-resolution taxonomic and functional profiling of microbial communities. However, it is undeniable that we are still far from a full understanding of the terms of the GM-host interaction, a *sine qua non* for the development of truly effective preventive and intervention strategies. In parallel, enormous progresses has haves been made in the field of analytical approaches to data, whose collection, organization and mining are fundamental steps for the analysis of complex interaction networks. Overall, technological advances in molecular biology and computer science are driving medical science towards big data. This large amount of data can potentially be explored via artificial intelligence methods, such as machine learning (ML) approaches that can handle large-scale datasets. ML is a data-driven approach capable of mining complex data, discovering informative patterns. In a nutshell, ML identifies algorithms capable of learning patterns from data in a self-manner, which enables the machine to solve a specific task, and deal with invisible data without explicit programming. In principle, the more heterogeneous data are used to train the model, the better the algorithm can generalize the problem when dealing with new data. Within ML, deep learning algorithms can better handle complex, multi-modal data. The premise of the emergence of ML techniques in various scientific fields, including healthcare, is the possibility of automating certain repetitive tasks, with the aim of achieving greater accuracy than that achievable by human experts, with also the possibility of estimating and predicting parameters, such as health risk factors. ML methods can adopt and combine different sources of health-related data, leveraging the tasks of diagnosing, prognosis, disease risk and potential treatments, with the aim of progressing towards a treatment tailored to the patient profile. ML algorithms can be supervised, *i.e.*, we know *a- priori*, based on manual curation, how the samples are tagged. Supervised techniques are adopted to answer specific problems, by training the algorithm to recognize distinct features of the dataset. Halfway between supervised and unsupervised learning, semi-supervised learning can be applied

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when we have incompletely labelled datasets, as it can be a real-word scenario [226]. With unsupervised algorithms, common features from input data are extracted, for instance, by grouping the samples based on the metagenomic profile. Unsupervised tasks are implemented through clustering (e.g., k-means, hierarchical) and dimensionality reduction algorithms, helping to explore and visualize similarities between samples. Overall, non-linear dimensionality reduction approaches (PCoA, UMAP), and autoencoders are adopted for microbial data, as these techniques are suitable for handling sparse data. Different types of algorithms can be used both for ~~both~~ classifications, identifying a sample as healthy or diseased based on the metagenomics profile, and for regression, for instance determining what the expression value would be for a given bacterial species upon treatment. Furthermore, ensemble strategies combine multiple models~~, to obtain~~ for more robust and accurate results. When building a new model, the crucial steps are training and testing the model. To validate its performance, the dataset on which the model is trained is divided into training and testing subsets. The more data we can start with, the higher ~~the are~~ the chances that the algorithm can be better trained. The training set will be used to train the model, while the testing set must be a dataset not previously seen by the model during the training phase, in order to evaluate retrospectively and in an unbiased manner its performance. Shortcomings related to the model performance include overfitting, when the model is well trained on the dataset used for training, leading to high accuracy when applied to test data from the same dataset, but poor results when dealing with new datasets (*i.e.*, the model has little power to generalize the problem). On the other hand, the model may be underfitted to the data and not be able to generate predictions with sufficient accuracy even on the testing dataset. Very important components of model training in supervised tasks are the dataset annotation and the degree of curation of the data. In this regard, a certain level of expertise on the data based on the application task and data type, would be another important component of the ML workflow. The performance of a~~n~~ ML model is also subject to the computational power at disposal, which plays an important role in model training, especially when dealing with deep learning models. Furthermore, reproducibility, pipeline standardization and data accessibility are other major challenges. All this is even more true in the field of GM, whose complexity and high inter-individual and temporal variability stress the need for standards and cross-study validation of models. In this regard, the COST Action CA18131 "ML4Microbiome" project aims to tackle the issues related to the advent of ML in the microbiome field [227].

6.2 Machine learning in clinical oncology practice

Following the trend of increasing data collection, ML algorithms have been successfully applied to various problems, predicting human faces, targeting consumer behaviors, and also in relation to protein structure and function [228], drug discovery [229], and cancer detection [230]. ML models and algorithms are highly flexible between different scientific fields. However, data filtering and preparation require some knowledge. In oncology, ML approaches have already some

applications. Just to name a few based on imaging data, ML models have been used for breast density assessment [231], and for the detection of malignant lung nodules [232]. Furthermore, CURATE.AI is an artificial intelligence platform that has been trained on prostate cancer patient's health records and used by doctors to choose the optimal dose of drugs [233]. While for model training on imaging data~~,~~ the detection accuracy is comparable to that of radiologists, or in some cases even better performing, ML models based on health records still need further evaluation. In any case, the model results require a review by the physician, as in some cases they may go against clinical guidelines. One major limitation is the inability to benchmark these tools towards a larger real-world dataset. In order to deploy ML tools in real clinical practice, several aspects must be taken into consideration and continuous and extensive collaboration between clinicians and informaticians is required, as well as data curation and longitudinal studies to monitor clinical outcomes [234].

6.3 Machine learning application in oncology with omics data

Nowadays, the boost of microbiome studies ~~in the microbiome field~~ and the availability of large datasets in public repositories are enabling the application of ML to metagenomics, which could lead to the identification of microbial species or other biomarkers, such as genes/enzymes and metabolites, for cancer diagnosis and prognosis [235]. Microbiome data combined with patient genetic information, but also with other types of omics data (transcriptomics, proteomics, metabolomics), could therefore return a comprehensive picture of the biological complexity of the disease and play a leading role in defining a personalized medicine approach. For example, predictions over microbiome-drug interactions could be the key to guiding precision therapeutic solutions. Recently, metagenomic data from CRC patients and healthy subjects ~~have been~~were used to train a random forest classifier [236]. The model identified six key microbial species, *i.e.*, *Porphyromonas asaccharolytica*, *Peptostreptococcus stomatis*, *Fusobacterium* spp., *Parvimonas* spp., *Streptococcus vestibularis* and *Flavonifractor plautii*, which discriminated between controls and patients. Another work based on a random forest classifier and CRC~~,~~ was trained on the metagenomic and metabolomics data from an Indian cohort of 30 patients and 30 healthy subjects. This work identified *F. plautii* as a cancer biomarker, and also found discriminating microbial genes for CRC. Interestingly, the authors hypothesized that flavonoid degradation by *Flavonifractor* is a key component for cancer progression [237]. More recently, Jang et al. [238] applied a Bayesian network model to find out species signatures in patients responding to chemotherapy treatment, while Kharrat et al. [239] adopted an ensemble method, including a Bayesian network model, to identify CRC-related microbial species. We would also like to mention the recently established Gut OncoMicrobiome Signatures project, which aims to identify microbial signatures of cancer progression and response to therapy [235]. These applications have defined or aim to define marker microbial species that could help better stratify patients, as well as guide GM remodeling via microbiome-based strategies, as outlined above. In this regard, ML could be used to screen large datasets in order to find potential new probiotics,

which will then have to be experimentally validated, or novel compounds to be evaluated for their therapeutic potential, as well as to refine/personalize drug therapies. A rRecent work [240] based on unsupervised ML techniques, has identified structural similarities between drugs that can be metabolized by bacteria, with important implications in pharmacological research. For example, it has been shown that chemical groups such as the amide and ester groups can be hydrolyzed by Bacteroidetes members. A similar application led to the implementation of the Drugbug database, a resource that collects data on the bacterial metabolism of drugs. These data were used to train a random forest model that allowed for the classification of compounds based on microbial metabolism [241]. By exploiting different types of omics data (from metagenomics to metabolomics), ML approaches could therefore help to delineate microbiome-drug interactions, with the possibility of predicting drug response and related toxicity. On the other hand, it should be remembered that certain drugs, other than antibiotics, can shape the GM structure, eventually leading to a dysbiotic state. New studies are investigating this aspect [240,242,243].

In summary, ML can set the direction of personalized precision medicine, helping to overcome the barrier of huge volume of data analysis, with the ability to perform classification and prediction tasks. ML can be adopted to stratify patients based on individual characteristics, including microbiome profile, and predict clinical outcomes (including response to therapy), as well as identify novel health- or disease-promoting taxa/compounds, determine microbiome-drug interactions and therefore guide the design of microbiome-targeted strategies to prevent/fight cancer, ensuring a long-term positive response. ML methods applied to the microbiome/cancer fields offer a valuable bench-free way to sift through possible solutions, which will then need to be validated by experimenters and clinicians. To speed up research in this field, collaborations between among the clinical, biotechnological and bioinformatic parties for data model evaluation and results interpretation will be mandatory.

7. Conclusions and perspectives

The gut microbiome has recently taken a leading role in research focused on maintaining the physiological wellbeing of the host. On the other hand, evidence of a direct relationship between certain microbes and cancer development, as well as the recent involvement of microbiomes in the outcomes of anticancer therapy, have left the door open to a new frontier in microbiome-based research in these fields. In this scenario, the introduction of multi-omics approaches and novel bioinformatic tools are helping to understand the role of microbial ecosystems in these unimaginableed lapels of the relationship with the host. However, there are still few studies in large cohorts and many knowledge gaps to be filled, especially in terms of underlying mechanisms and the development of safe and effective intervention strategies. Only through transdisciplinary collaborations, it will be possible to move forward with the development of personalized microbiome-based interventions, to overcome resistance to anticancer treatments and reduce the risk of relapse.

Consent for Publication

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Conflict of Interest

The authors declare no conflict of interest, financial or otherwise.

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Figures/Illustrations

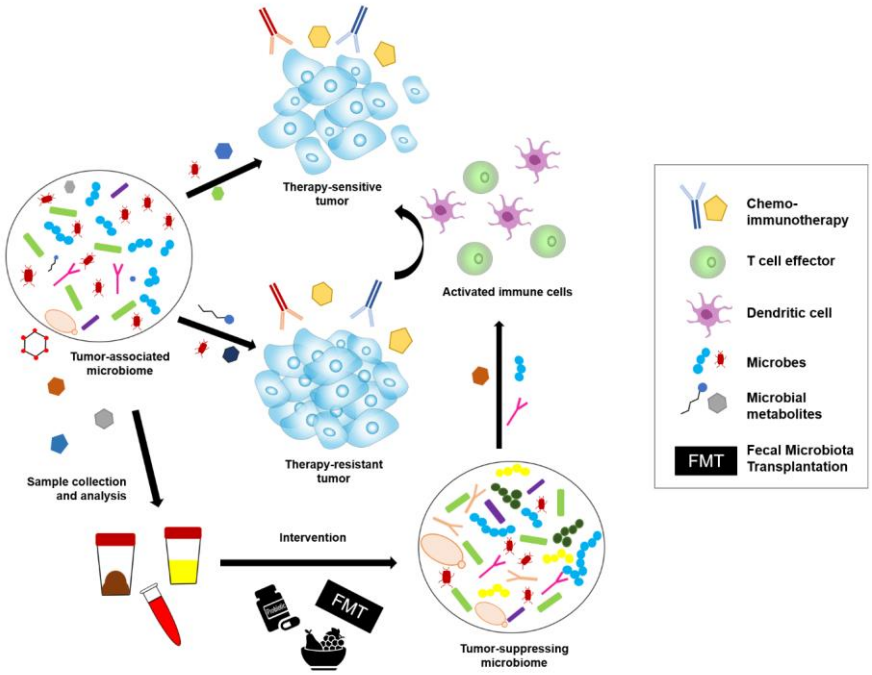

Figure 1. Characterization of the gut microbiome in cancer onset and response to anticancer therapies in order to develop novel personalized microbiome-based intervention strategies. An unbalanced gut microbial ecosystem and its metabolites may be involved in the development of cancer, as well as in a poor response to anticancer therapy. The collection and analysis of biological samples (e.g., feces, blood and urine) through novel sequencing-based and metabolomic approaches, as well as bioinformatic tools, are needed to gain knowledge about the microbiome, cancer and anticancer therapies~~y~~. In particular, machine learning approaches have great potential in enabling the development of personalized microbiome-based interventions (*i.e.*, prebiotics, probiotics and FMT), which, through the activation of the host immune system, could favor the response to therapy and tumor clearance.

Tables:

Table 1. Clinical trials registered in the last two years on ClinicalTrials.gov (as accessed on July 2021) concerning the application of prebiotics, probiotics, and fecal microbiota transplantation (FMT) as adjuvant therapy in cancer patients. Search terms included "cancer", in combination with "prebiotics", "probiotics" or "FMT".

	Title	Status	Results	Condition	Intervention	Location	URL
Prebiotics	Impact of Dietary Fiber as Prebiotics on Chemotherapy-related Diarrhea in Patients With Gastrointestinal Tumors	Recruiting	Not available	Chemotherapy-related Diarrhea	Dietary supplement with prebiotic fiber + loperamide hydrochloride vs. maltodextrin + loperamide hydrochloride	China	https://ClinicalTrials.gov/show/NCT04447443
	Papilocare®: Effects on Regression of Histologically Confirmed Cervical Intraepithelial Lesions 1 and Tolerance	Recruiting	Not available	Squamous Intraepithelial Lesions of the Cervix, Human Papilloma Virus Infection, Cervix Lesion	PAPILOCARE® device	France	https://ClinicalTrials.gov/show/NCT04624568
Probiotics	Study to Investigate Efficacy of a Novel Probiotic on the Bacteriome and Mycobiome of Breast Cancer	Not yet recruiting	Not available	Breast Cancer	Novel probiotic vs. placebo	United States	https://ClinicalTrials.gov/show/NCT04362826
	Effects of Probiotics on the Gut Microbiome and Immune System in Operable Stage I-III Breast or Lung Cancer	Not yet recruiting	Not available	Anatomic Stage I, IA, IB, II, IIA, IIB, III, IIIA, IIIB, IIIC Breast Cancer AJCC v8	Dietary supplement with probiotic	United States	https://ClinicalTrials.gov/show/NCT04857697
	Clinical Study of Neoadjuvant Chemotherapy and Immunotherapy Combined With Probiotics in Patients With Potential/Resectable NSCLC	Recruiting	Not available	Non-small Cell Lung Cancer Stage III	dietary supplementation with probiotics, nivolumab +paclitaxel (albumin-bound type) + carboplatin AUC5	China	https://ClinicalTrials.gov/show/NCT04699721
	The Thoracic Peri-Operative Integrative Surgical Care Evaluation Trial - Stage II	Not yet recruiting	Not available	Lung Cancer, Gastric Cancer, Esophageal Cancer	Dietary supplement with probiotic (Pro12)	Canada	https://ClinicalTrials.gov/show/NCT04871412
FMT	Fecal Microbiota Transplant and Re-introduction of Anti-PD-1 Therapy (Pembrolizumab or Nivolumab) for the Treatment of Metastatic Colorectal Cancer in Anti-PD-1 Non-responders	Not yet recruiting	Not available	Metastatic Colorectal Adenocarcinoma, Metastatic Small Intestinal Adenocarcinoma, Stage IV, IVA, IVB, IVC Colorectal Cancer AJCC v8, Stage IV Small Intestinal Adenocarcinoma AJCC v8	FMT + nivolumab, FMT + pembrolizumab	United States	https://ClinicalTrials.gov/show/NCT04729322

	Microbiota Transplant in Advanced Lung Cancer Treated With Immunotherapy	Active, not recruiting	Not available	Lung Cancer	FMT + anti-PD1 therapy vs. anti-PD-1 therapy	Spain	https://ClinicalTrials.gov/show/NCT04924374
	Washed Microbiota Transplantation for The Treatment of Oncotherapy-Related Intestinal Complications	Recruiting	Not available	Intestinal Complications, Cancer	Washed Microbiota Transplantation (WMT)	China	https://ClinicalTrials.gov/show/NCT04721041
	A Phase Ib Trial to Evaluate the Safety and Efficacy of FMT and Nivolumab in Subjects With Metastatic Melanoma or NSCLC	Not yet recruiting	Available	Melanoma Stage IV, Unresectable Melanoma, NSCLC Stage IV	FMT	Israel	https://ClinicalTrials.gov/show/NCT04521075
	Faecal Microbiota Transplantation After Allogeneic Stem Cell Transplantation	Not yet recruiting	Not available	Acute Leukemia in Remission, Myelodysplastic Syndromes, Myeloproliferative Syndrome, Hodgkin Lymphoma, Lymphoma, Non-Hodgkin, Myeloma, Chronic Lymphocytic Leukemia	FMT	France	https://ClinicalTrials.gov/show/NCT04935684
	The IRMI-FMT Trial	Not yet recruiting	Not available	Malignant Melanoma Stage III, Malignant Melanoma Stage IV	Allogenic FMT vs. autologous FMT	Austria	https://ClinicalTrials.gov/show/NCT04577729
	Fecal Microbiota Transplantation to Improve Efficacy of Immune Checkpoint Inhibitors in Renal Cell Carcinoma	Recruiting	Not available	Renal Cell Carcinoma	FMT vs. placebo	Italy	https://ClinicalTrials.gov/show/NCT04758507

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