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Metabolite and microbiome interplay in cancer immunotherapy

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Abstract

The role of the host microbiome has come to the forefront as a potential modulator of cancer metabolism, and could be a future target for precision medicine. A recent study revealed that in colon cancer, bacteria form polysaccharide matrices called biofilms at a high frequency in the proximal colon. Comprehensive untargeted and stable isotope assisted metabolomic analysis revealed that the bacteria utilize polyamine metabolites produced from colon adenomas/carcinomas to build these protective biofilms, and may contribute to the inflammation and proliferation of colon cancer. This study highlighted the importance of finding the biological origin of a metabolite and assessing its metabolism and mechanism of action. This led to a better understanding of host and microbial interactions, thereby aiding therapeutic design for cancer. In this review we will discuss methodologies for identifying the biological origin and roles of metabolites in cancer progression, and discuss the interactions of the microbiome and metabolites in immunity and cancer treatment, focusing on the flourishing field of cancer immunotherapy.

Keywords

Metabolomics; microbiome; cancer; immunotherapy

Introduction

Cancer is a multifaceted, heterogeneous disease and its treatment remains complex with variable responses between patients. Over the past decade there has been resurgence in studies that are focused on cancer metabolism as it is now understood that metabolites play a

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Conflict of interest

The authors declare no conflict of interest.

major role in tumor cell proliferation and can be used as targets to probe dysregulated metabolic pathways and networks [1]. One source of metabolites is the microbiota, which typically exists in a symbiotic relationship with the host, providing essential nutrients and metabolites, regulating immune function and protecting the host from pathogens. Microbial imbalance known as dysbiosis can perturb this intricate relationship causing direct effects on the immune response and disease pathogenesis.

Microbiota exist on all surfaces that interface with the external environment (nose, mouth, stomach, intestine, lung, skin and vagina) and have a close relationship with the immune system, which work to maintain a balance of beneficial versus pathogenic factors. Changes to the microbiome have been associated with various inflammatory diseases, such as atopic dermatitis [2] and psoriasis [3] as well as chronic lung diseases (chronic obstructive pulmonary disease (COPD) and cystic fibrosis), which are associated with the state of the lung microbiome [4]. Interestingly patients with COPD have an increased incidence of inflammatory bowel disease (IBD) [5], another disease that is associated with dysbiosis. The microbiota has also been implicated in various cancers, including pancreatic [6], gastric [7], liver [8] and colorectal [9], a recent review highlights the increased risk of developing pancreatic cancer associated with dysbiosis of mouth and gut microbiota [10]. The authors put forth a hypothesis that the microbiota generates low levels of immune system activation that promote inflammation in the tumor microenvironment. In general, there is increasing evidence that the dynamic changes within the microbiome due to environmental factors such as diet and xenobiotics can affect both immune and cancer cells, whereby certain microbiota compositions and functions may be beneficial for cancer prevention, while other components may promote cancer. High-protein diets for example can reduce beneficial microbial species and metabolites, downregulating genes involved in immunoprotection [11]. Whereas high-fiber diets have the opposite effect on the microbial communities and metabolites, indirectly increasing the differentiation of regulatory T cells (Tregs) and regulating host immunity [12]. In addition high-fat diets can reduce microbial species that protect the intestinal barrier causing microbial antigens to cross and activate immune cells causing inflammation [13]. In addition, these findings are expanding beyond organs that are proximal to the gut microbiota (e.g. colon), suggesting that systemic processes may be modulated by aspects of the microbiome, such as the example of COPD and IBD incidence [5].

Assessing the role of microbiota in cancer is confounded by the fact that much of it is undefined and cannot be studied in culture, which complicates the identification of specific bacteria driving a biological process. Advances in acquiring ‘omic’ data, including 16S ribosomal RNA (rRNA) sequencing and metabolomics are providing a path forward to understand this complex system. To fully benefit from the data being collected there will need to be additional progress on data integration and analysis techniques. With that said, insights from on-going research are elucidating the interplay between microbiota, genetics, environmental toxins, diet, and drugs, and show that the common denominator is metabolites. Thus understanding the origin, production and actions of metabolites will lead to a better understanding of host and bacterial processes aiding in therapeutic design for cancers.

Bacteria use host metabolites to build biofilm and propagate cancer

Recent studies investigating the role of the microbiome in disease have shown the importance of metabolism in understanding how bacteria interact as a community as well as determining the functional roles of individual species [14]. One such growing area of research is in the role of polymicrobial biofilms in disease pathogenesis. Bacterial biofilms are matrix-enclosed microbial accretions which adhere to surfaces and have been observed in the colon, inner ear, teeth as well as many other locations [15]. On surfaces such as the colon they line the mucosa and indicate disruption to the normal colonic mucous barrier [16]. Thus, they have been implicated in the pathogenesis and maintenance of inflammatory bowel conditions such as ulcerative colitis [15]. More recently biofilms were observed on colorectal cancers [17, 18]. In the patient cohort examined, almost all proximal colon cancer exhibited biofilms, whereas only 12 % of distal cancers had them, furthermore 16S rRNA sequencing and fluorescence in situ hybridization (FISH) analysis revealed no distinct differences between the species of microbiota on tissues with biofilms compared to those without [17]. Particularly intriguing is that patients with proximal colon cancers have a higher risk of mortality from the disease than those with distal cancers [19]. There are some differences in genetic characteristics between these two areas of the colon which may play a role, but it is still not fully understood why bacteria preferably form biofilms on proximal colon cancers. In the case of healthy individuals approximately 15 % will have thin biofilms but these are not specific to the proximal colon [17]. Thus, it is possible that other environmental influences are involved in biofilm development such as diet and smoking [20, 21].

Clearly, the microbiome plays a major role in metabolite production and metabolism which is self-regulated so that individual species or the community can adapt to changing environmental conditions, indeed metabolites have been shown to have a role in biofilm formation and persistence [22]. We assessed the role of metabolites in colon cancer biofilm metabolism, revealing further interplay between the microbiota, metabolites and cancer pathogenesis [23]. Our study used a combination of four mass spectrometry-based metabolomic platforms and orthogonal biological approaches to identify key metabolites and metabolic pathways. We also determined the biological origins (mammalian or bacterial) of dysregulated metabolites and their effects in patients with colon cancer [17, 23]. It was observed that surgically-resected cancer tissues from colon cancer patients produced polyamine metabolites (spermine, spermidine, N^1-acetylspermine, N^1-acetylspermidine and N^1, N^{12}-diacetylspermine (DAS)) at a significantly higher concentration compared to their paired normal tissues [23].

Polyamines and their metabolites have been previously associated with increased cellular proliferation and cancer, and is reviewed extensively elsewhere, but it is not known whether they are a cause or consequence of the disease [24]. Interestingly the metabolic differences between colon tissues with and without biofilms were small but highly significant; DAS was seen in much higher concentrations in tissues with biofilms. DAS was assessed to be an end-product of metabolism using stable isotope-assisted metabolomics, therefore it was hypothesized that the microbiota use host-derived polyamines to form biofilms, and DAS was observed in our study as a metabolic product in this process. It is also possible that DAS

can be derived from dietary metabolism and downstream host cellular production resulting from increased ornithine decarboxylase activity [24].To elucidate the origin of increased DAS in biofilm-containing tissues a number of techniques were used. Antibiotic treatment in a new cohort of patients disrupted bacterial biofilms and lowered DAS tissue concentrations to those seen in biofilm negative colon cancer samples, suggesting that microbes induce the elevation of host generated DAS. Considering that the number of individuals in this part of the study was relatively small (~n=10/group) such a positive statistical correlation provided strong evidence that biofilms increase the production of this metabolite. Furthermore immunohistochemistry analysis showed no change in host protein levels for enzymes involved in DAS production. Using a combination of histology and a powerful metabolite imaging technique called nanostructure imaging mass spectrometry (NIMS), it was possible to observe higher concentrations of DAS localized to the colonic mucosal layer *in situ*, showing its direct association and accumulation in bacterial biofilms. Orthogonal biological analysis of the biofilm-associated colon cancer samples by Dejea et al., revealed increased inflammation and cellular proliferation, even in normal-adjacent tissues that exhibited biofilms [17]. Furthermore it is known that polyamine production enhances the activity of solute carrier family member SLC3A2 for their export from the cell. This protein also imports arginine which produces pro-inflammatory nitric oxide, therefore providing a direct link between polyamines and inflammation [25]. As nitric oxide is an effective antimicrobial agent that induces biofilm formation [26], further studies are needed to assess the relationship between arginine metabolism, biofilm production and inflammation. Thus these two studies were able to reveal metabolites that were produced by colon cancer cells and subsequently utilized by microbiota to contribute to increased inflammation and cellular proliferation in proximal colon tissues [17, 23]. Moreover this study importantly highlighted the need to develop technologies for identifying the biological origin of the metabolite, a question that presents a complex yet important challenge.

Metabolomics shows a clear role for metabolites in biofilms in proximal colon cancer yet it remains unclear why these biofilms are penetrant. Biofilm formation is of high importance since direct epithelial contact with the gut microbiota induces inflammatory responses and increases the likelihood of bacterial invasion of the epithelial layer; moreover this finding also suggests an alteration to mucin catabolism. Mucosal tissues express a series of mucin polypeptides that become heavily glycosylated, providing a protective mucin layer that separates the single layer epithelium of the gut from the densely populated microbiota. The loose outer layer of mucin provides a continuous energy source for commensal microbes specialized in liberating and metabolizing mucin glycans. Alterations in mucin glycosylation are frequently observed in pathologies such as cystic fibrosis, Crohn’s disease and cancer [27, 28], thus it is possible that mucins are altered in colon cancers that have biofilms. Therefore understanding the role of mucin glycan composition (N- vs. O-glycans) in the formation of biofilms in proximal versus distal colon is of importance. Further studies are now underway to examine the role of the gut microbiota in glycan catabolism and the formation of penetrant biofilms in subjects with colon cancer.

Host versus bacterial metabolite origin

The studies mentioned here demonstrate that microbiota influence host metabolism and contribute to the production of a complex pool of both primary and secondary metabolites. However in order to understand the interplay between microbiota and host metabolism, to guide effective therapeutic interventions it is important to elucidate the metabolites that are co-metabolized by the microbiota. As the metabolome is a culmination of both host and microbial activities, various models and strategies can be used to elucidate the origin of metabolites and to interpret their actions. The most effective methods to ascertain the direct effects of microbial metabolism on the host are antibiotic treatment which decreases the bacterial load in the gut, or germ-free models that are free of microbial colonization. Germ-free models are particularly useful as it is possible to study the effect of recolonization with bacterial species from controlled sources, thus revealing the link between metabolite production, specific species and community interactions [29]. In addition microbial species have distinct genomes and thus different enzymatic capabilities; it is therefore possible to identify the role of the individual bacterial species in these models before assessing their community effects of metabolism.

Germ-free models have helped to reveal a significant interplay between bacterial and mammalian metabolism. In particular a seminal mass spectrometry-based metabolomics study on plasma from germ-free and conventional mice revealed bacterial-mediated production of indoxyl sulfate, indole-3-propionate, hippurate and p-cresol sulfate [29]. These metabolites are signature metabolites for mammalian-bacterial co-metabolism of amino acid and phenolic compounds, and have paved the way for future metabolomic and microbiome-based studies. A study by Zheng et al., also revealed hundreds of urinary and fecal metabolites that were altered in rats exposed to a broad spectrum antibiotic in a specific pathogen free (SPF) environment, thus both studies showed widespread metabolic changes as a result of microbial dysbiosis [30].

The cecal contents are highly enriched for microbial metabolites and analysis can provide an effective model for drawing hypotheses. A recent study on germ-free mice revealed how cecal metabolite concentrations are altered by individual bacterial species and complex communities; metabolites were modeled in relationship to colonization with individual strains and by additive metabolite-bacterial community interactions, where more than one strain was responsible for metabolite production [31]. This type of cooperative metabolism is carried out by the consortia of bacteria abound in the gut microbiota. Quinic acid for example was at its highest concentrations when *Odoribacter splanchnicus* were present and *Escherichia coli* absent, whereas lysophosphatidylethanolamine was increased by the presence of *Bacteroides ovatus* or *Bacteroides vulgatus*, but at its highest concentrations when both were present [31].

An additional strategy used to assess human host microbiota interactions involves the transplantation of human gut communities (fecal microbiome transplantation) into germ-free mice in order to establish metabolite/phenotype relationships. Ridaura et al. showed that gut bacteria transplanted from obese and lean humans induced weight gain versus no weight gain respectively in recipient gnotobiotic mice [32]. Similarly, bacterial changes can be

induced by the diet; a two week food exchange in which rural Africans and African Americans switched diets resulted in large changes to bacterial species and metabolites, and their risk of developing cancer. Higher risk was associated with higher levels of secondary bile acids such as deoxycholic and lithocholic acid which causes DNA damage, and lower levels of short-chain fatty acids such as butyrate in the colon [33].

Metabolomic technologies can also be used to help elucidate the biological origin of a metabolite. For instance our study of bacterial biofilms in colon cancer demonstrated how stable isotope-assisted metabolomics may be used to trace a stable-isotope labeled precursor and determine its metabolic fate in an untargeted manner. Qualitative and quantitative differences in metabolite flux and products can also reveal how the host, and/or bacterial species are utilizing the precursors. Mass spectrometry imaging (IMS) techniques that are optimized for small molecular analysis, such as NIMS can show the *in situ* localization of metabolites. When overlaid with bacterial sequencing information or histological analysis direct co-localization of metabolite and microbiota or biological information can be made. A recent study used mass spectrometry-based metabolomics, 16S rRNA sequencing and computational tools to reveal the co-localization of metabolites and microbiota through extraction and culture of skin swabs producing a molecular map of the skin surface [34]. This study demonstrated the effect of the skin microbiome on metabolite production for the identification of chemical drivers of disease, in particular skin cancer. Another novel computational platform was recently developed, that uses model simulations to predict metabolic interactions within the microbiome; Community And Systems-level Interactive Optimization (CASINO)[35]. The authors were able to show altered fecal and serum amino acid concentrations in response to dietary changes in individuals, and how individual bacterial species contribute to host metabolism. MetaboNetworks was also recently created that examines the interconnectivity of metabolic reaction networks between different organisms, thereby making it possible to view the overlap in metabolite production between mammals and bacteria [36].

From Data to Knowledge

Studies of the microbiome's role in cancer have the potential to generate vast amounts of data. Yet, to impact human disease, data on its own is not sufficient. The data must be transformed into knowledge that strengthens our biological understanding, which can in turn lead to actionable interventions to prevent or treat disease. In general, data storage is no longer a major issue. Databases already exist for the microbiome (Human microbiome project; QIIME; MG-RAST [37–39]) and metabolites (METLIN; human metabolome database [40, 41]), however databases that specifically annotate microbial metabolites are rare and are needed to advance this field. Another issue is that there is an overlap between metabolites that are externally derived, produced by host metabolism, and produced through co-microbial metabolism, which adds to the difficulty in setting up these types of databases, N^1, N^{12}-diacetylspermine is one such metabolite. Data post-processing and analysis can be time consuming and difficult to properly implement [42]. Data post-processing is a critical step and as diverse yet complementary experimental data (e.g. multi-omics) are incorporated into a single analysis, data processing techniques (e.g. normalizations) specific for each data type should be utilized [43].

Following data processing, various analysis methods can be employed to look for group differences or study the biological system in an integrated manner. For example, traditional approaches assessing statistical differences and clustering analyses can be applied. However, as datasets grow in size, multiple comparisons inherent within the analysis may limit some statistical approaches due to the random chance of identifying a significant difference defined simply by the sheer number of comparisons being made. A recent study focused on colorectal cancer, integrated microbiome (16S rRNA) and metabolomics data to demonstrate that the microbial composition was similar between healthy and colorectal patients, but that short chain fatty acids and other metabolites differed between the two groups [44].

Additional data analysis methods focus on the use of systems biology and metabolic networks to integrate the data into a framework defined by a specific biological network or structure. Recent reviews highlight various types of mathematical models applied to cancer metabolism [45] and host-microbial metabolomics [46]. Markert and Vazquez highlight several different types of models, including flux balance models [47], which have been successfully applied to study cellular metabolism under steady state conditions and are being expanded to include kinetic data. While Heinken and Thiele compare three major systems modeling approaches for metabolomics data: top-down metabolomics, topological network modeling and constraint-based models. In addition, methods that allow for integration of high-throughput microbiome and metabolomic data have been published providing groundwork for these types of studies [48–50]. Each modeling approach has various strengths and weaknesses and it is expected that the modeling approaches will continue to evolve to manage the complexities inherent in the multi-omic data generated when studying the microbiota's role in health and disease.

Microbial metabolic influences on immunotherapeutic efficacy

Fully understanding the interactions between microbes, metabolites and the immune system in cancer is essential for leading precision medicine. This is especially the case for treatment with cancer immunotherapeutic drugs which have variable efficacy in patients. These drugs target the patient's immune system, allowing it to adapt and attack cancer cells, and recent studies have shown that the microbiome could be involved in their efficacy [51].

Indirectly, microbial co-metabolism can produce butyrate, amino acids and fatty acids that affect T cell signaling pathways and production, in particular pro-inflammatory T_H17 and anti-inflammatory Tregs [52–54]. Therefore changes to the local environment elicited by microbial co-metabolism could determine the efficacy of immunotherapeutics and cancer cell survival [55–57]. Recent studies have also shown direct roles for microbiota in modulating the host immune system. *Bacteroides fragilis* can increase the secretion of the immunodulator polysaccharide A [58], and monocolonization of germ free mice with *Bifidobacterium infantis* and *Bacteroides fragilis* can increase the conversion of CD4+ T cells into Tregs [59]. Viaud et al., used this concept of bacteria immunomodulation to study the anti-tumor efficacy of cyclophosphamide. This drug altered the composition of the microbiome in the small intestine of mice, causing the selective translocation of distinct Gram positive bacteria to mesenteric lymph nodes and spleens. The production of helper T cells T_H1 and T_H17 was stimulated facilitating immune-mediated cancer cell death [60]. In

tumor-bearing germ-free mice and antibiotic-treated SPF mice, there was a blunted T_H17 response and resistance to cyclophosphamide, the tumors in these mice continued to grow steadily in size. Iida et al., also investigated immune modulation of the microbiome for a different class of drugs, namely the toll-like receptor (TLR) agonist CpG oligonucleotides (CpG-ON) [61]. The immune response triggered by TLR agonists is complex, and includes increased cancer cell death due to both increased cellular and humoral immunity. Germ-free and antibiotic-treated SPF mouse models of lymphoma, colon carcinoma and melanoma were treated with CpG-ON. Decreased tumor growth was only observed in the non-antibiotic treated SPF mice. They identified tumor necrosis factor (TNF) as an important component of the efficacy of CpG-ON, with higher levels of TNF production noted in non-antibiotic treated SPF mice when compared to germ-free mice. These results indicate the strong association of microorganism-metabolic immunomodulation effects.

It has been shown that microbiota play a role in modulating the tumor response to checkpoint inhibitors. In 2011, the U.S. Food and Drug Administration approved the use of a checkpoint inhibitor anti-cytotoxic T-lymphocyte associated protein 4 (CTLA-4)-antibody, ipilimumab, for the treatment of melanoma, and in 2014 approved the use of two more checkpoint inhibitors for the same disease; anti-programmed cell-death protein 1 (PD-1)-antibodies pembrolizumab and nivolumab [62]. Sivan et al, studied the effects of anti-PD-1/PD-L1 monoclonal antibodies in two mice strains with melanoma [55]. The mice were from two different facilities, the Jackson Laboratory (JAX) and Taconic Farms (TAC). The authors reported that the JAX mice had a significantly reduced rate of tumor growth compared to TAC mice after treatment with anti-PD-1/PD-L1 antibodies. They then transferred fecal suspensions from the JAX mice into the TAC mice which decreased tumor growth after treatment with anti-PD-1/PD-L1. They observed that the JAX mice and the TAC mice that received JAX fecal transfers both had increased tumor-specific T cell responses and increased intratumoral $CD8^+$ T cells. They identified the genus *Bifidobacterium* as the key promoter of this response after administering a *Bifidobacterium* species cocktail to TAC mice with melanoma. Treatment with the checkpoint inhibitor then resulted in reduced tumor growth compared to non-*Bifidobacterium* cocktail treated TAC mice. Ipilimumab was also shown to have a decreased tumor response in germ-free mouse models of sarcomas, melanoma and colon cancer compared to untreated SPF mice [56]. *Bacteroides* was the predominant commensal species responsible for this effect, and was revealed to be causal after *Bacteroides* inoculation in antibiotic-treated SPF mice and germ-free mice. When treated with anti-CTLA-4 antibodies, tumors in both groups of mice displayed decreased growth. Another beneficial effect of the transfer of *Bacteroides* in these mice was a decrease in the severity of colitis, one of the toxic side effects of the anti-CTLA-4 antibody treatment. As an added clinically-relevant analysis, the authors transferred fecal isolates from melanoma patients that had been treated with ipilimumab into the germ-free mice with melanoma; the feces from these patients had increased colonization with the immunogenic bacteria *Bacteroides thetaiotamicron* and *Bacteroides fragilis*. They found that the abundance of *Bacteroides fragilis* in these mice negatively correlated with tumor size.

These studies therefore show clear evidence for the role of the microbiome in modulating the host immune response, which increases the efficacy of immunotherapeutic anti-cancer agents in a murine model. It is important to determine the involvement of metabolites in

these mechanisms aiding translation into the clinic. In addition to modulating the efficacy of immunotherapeutics, the microbiome may play a role in the adverse events (AEs) associated with these treatments. Common AEs include rash, mucosal irritation, diarrhea and colitis, which are primarily treated with dose modification and immunosuppressive therapy, such as corticosteroids. In a prospective study of immune-mediated colitis, patients with greater bacteria from the Bacteroidetes phylum, particularly, Bacteroidaceae, Rikenellaceae and Barnesiallaceae, were less likely to develop colitis [63]. In addition, microbial modules for polyamine transport and the biosynthesis of B vitamins were more abundant in patients that did not develop colitis. A model was constructed from this information to predict the risk of developing colitis. The model resulted in a sensitivity of 70% and a specificity of 83%. It will be interesting to see if these results can predict future occurrence of colitis as more patients are treated with immunotherapeutics. A similar approach has utilized the state of the microbiome as a potential biomarker for pancreatic diseases [6]. While this effect may explain the variable response to immunotherapies in humans, it remains to be seen whether these exciting results will be reproduced in the clinical setting.

Future directions

This review serves to highlight the active role of the microbiome and metabolome in immune modulation, both of which have consequences on cancer progression. As highlighted by previous studies, the interplay between bacteria-metabolite-cancer is dependent on the bacterial species present and how the bacterial community is organized, where the effects of individual bacteria versus bacterial communities are quite different. This was highlighted in the pronounced role of bacteria's hierarchal structure (biofilms) on colon cancer. The interrelationship between bacteria, cancer and their metabolic ability to modulate the immune system is one of the most exciting areas of cancer research, and now our role as scientists is to learn how to disrupt this synergy.

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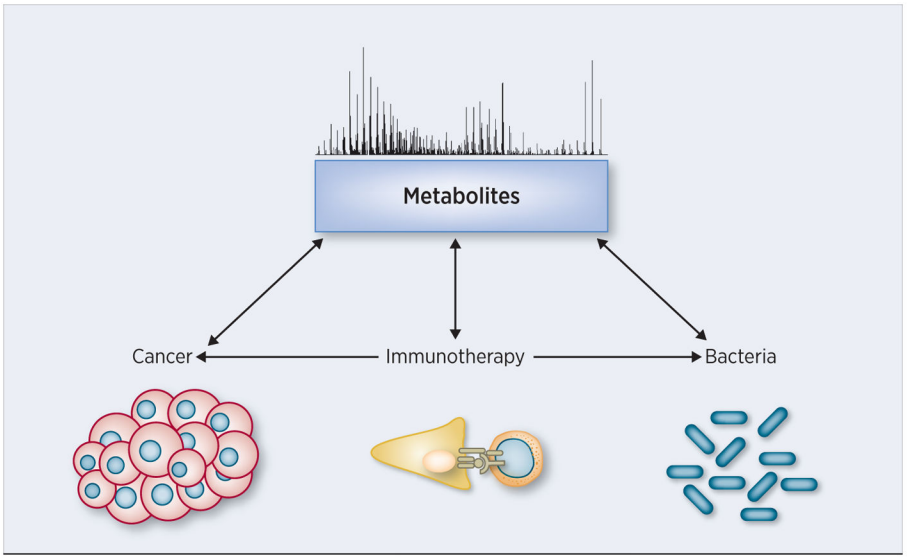

Figure 1.
The interplay between host and bacterial metabolism in cancer and immunotherapeutic treatment.