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## Melanoma and microbiota: Current understanding and future directions

**Bertrand Routy<sup>1,2,41</sup>, Tanisha Jackson<sup>3,41</sup>, Laura Mählmann<sup>4,41</sup>, Christina K. Baumgartner<sup>5</sup>, Martin Blaser<sup>6</sup>, Allyson Byrd<sup>7</sup>, Nathalie Corvaia<sup>8</sup>, Kasey Coutts<sup>9</sup>, Diwakar Davar<sup>10</sup>, Lisa Derosa<sup>11,12,13</sup>, Howard C. Hang<sup>14</sup>, Geke Hospers<sup>15</sup>, Morten Isaksen<sup>16</sup>, Guido Kroemer<sup>17,18,19</sup>, Florent Malard<sup>20</sup>, Kathy D. McCoy<sup>21</sup>, Marlies Meisel<sup>22,23</sup>, Sumanta Pal<sup>24</sup>, Ze'ev Ronai<sup>25</sup>, Eran Segal<sup>26</sup>, Gregory D. Sepich-Poore<sup>27,28</sup>, Fyza Shaikh<sup>29</sup>, Randy F. Sweis<sup>30</sup>, Giorgio Trinchieri<sup>31</sup>, Marcel van den Brink<sup>32,33,34</sup>, Rinse K. Weersma<sup>35</sup>, Katrine Whiteson<sup>36</sup>, Liping Zhao<sup>37</sup>, Jennifer McQuade<sup>38,\*</sup>, Hassane Zarour<sup>39,\*</sup>, Laurence Zitvogel<sup>11,12,13,40,\*</sup>**

<sup>1</sup>University of Montreal Research Center (CRCHUM), Montreal, QC H2X 0A9, Canada

<sup>2</sup>Hematology-Oncology Division, Department of Medicine, Centre Hospitalier de l'Université de Montréal (CHUM), Montreal, QC H2X 3E4, Canada

<sup>3</sup>Melanoma Research Alliance, 730 15th Street NW, Washington, DC 20005, USA

<sup>4</sup>Seerave Foundation, The Seerave Foundation, 35–37 New Street, St Helier, JE2 3RA Jersey, UK

<sup>5</sup>Oncology Discovery Research, AbbVie Inc, North Chicago, IL 60064, USA

<sup>6</sup>Center for Advanced Biotechnology and Medicine, Rutgers University, Piscataway, NJ 08854, USA

<sup>7</sup>Department of Cancer Immunology, Genentech, Inc., South San Francisco, CA 94080, USA

<sup>8</sup>MaaT Pharma, 69007 Lyon, France

<sup>9</sup>Department of Medicine, Division of Medical Oncology, University of Colorado School of Medicine, Aurora, CO 80045, USA

<sup>10</sup>Department of Medicine and UPMC Hillman Cancer Center, University of Pittsburgh, Pittsburgh, PA 15213, USA

<sup>11</sup>Gustave Roussy Cancer Center, ClinicoBiome, 94805 Villejuif, France

<sup>12</sup>Université Paris Saclay, Faculty of Medicine, 94270 Kremlin Bicêtre, France

<sup>13</sup>Inserm U1015, Equipe Labellisée par la Ligue Contre le Cancer, 94800 Villejuif, France

\*Correspondence: jmcquade@mdanderson.org (J.M.), zarourhm@upmc.edu (H.Z.), laurence.zitvogel@orange.fr (L.Z.).

### AUTHOR CONTRIBUTIONS

Review and editing and first author: B.R. Workshop director and co-first authors: T.J. and L.M. Curating of relevant literature and review: C.K.B. Review and editing: M.B., A.B., N.C., K.C., G.H., F.M., K.D.M., M.M., S.P., Z.R., E.S., F.S., R.K.W., K.W., and L. Zhao. Workshop notes, conceptualization, and writing – review and editing: D.D. and L.D. Writing – review and editing: H.C.H. and M.I. Wrote the chapter “Inflammation and ‘Omics’” with input from L. Zhao: G.K. Conceptualization and writing – review and editing: G.D.S.-P. and R.F.S. Discussion group leader and review and editing: G.T. and M.v.d.B. Co-chair and co-last author and co-corresponding author: J.M. and H.Z. Conceptualization, review, editing, co-chair, and co-last and co-corresponding author: L. Zitvogel.

<sup>14</sup>Departments of Immunology & Microbiology and Chemistry, Scripps Research, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA

<sup>15</sup>Department of Medical Oncology, University of Groningen, University Medical Center Groningen, 9713 GZ, Groningen, The Netherlands

<sup>16</sup>Bio-Me, 0349 Oslo, Norway

<sup>17</sup>Centre de Recherche des Cordeliers, Equipe labellisée par la Ligue contre le cancer, Université Paris Cité, Sorbonne Université, Inserm U1138, Institut Universitaire de France, 75006 Paris, France

<sup>18</sup>Metabolomics and Cell Biology Platforms, Institut Gustave Roussy, 94905 Villejuif, France

<sup>19</sup>Institut du Cancer Paris CARPEM, Department of Biology, Hôpital Européen Georges Pompidou, AP-HP, 75015 Paris, France

<sup>20</sup>Sorbonne Université, Centre de Recherche Saint-Antoine INSERM UMRs938, Service d'Hématologie Clinique et de Thérapie Cellulaire, Hôpital Saint Antoine, AP-HP, Paris, France

<sup>21</sup>Department of Physiology & Pharmacology, Snyder Institute, Cumming School of Medicine, University of Calgary, Calgary, AB T2N 4N1, Canada

<sup>22</sup>Department of Immunology, University of Pittsburgh School of Medicine, Pittsburgh, PA USA

<sup>23</sup>Cancer Immunology and Immunotherapy Program, UPMC Hillman Cancer Center, Pittsburgh, PA USA

<sup>24</sup>Department of Medical Oncology and Therapeutics Research, City of Hope Comprehensive Cancer Center, Duarte, CA 91010, USA

<sup>25</sup>Sanford Burnham Prebys Discovery Medical Research Institute, La Jolla, CA 92037, USA

<sup>26</sup>Weizmann Institute of Science, Computer Science and Applied Mathematics Department, 234th Herzl st., Rehovot 7610001, Israel

<sup>27</sup>Feinberg School of Medicine, Northwestern University, Chicago, IL 60611, USA

<sup>28</sup>Micronoma Inc., San Diego, CA 92121, USA

<sup>29</sup>Johns Hopkins School of Medicine, Department of Oncology, Baltimore, MD 21287, USA

<sup>30</sup>Section of Hematology/Oncology, Department of Medicine, University of Chicago, Chicago, IL 60637, USA

<sup>31</sup>Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

<sup>32</sup>Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA

<sup>33</sup>Department of Immunology, Sloan Kettering Institute, New York, NY 10065, USA

<sup>34</sup>Weill Cornell Medical College, New York, NY 10065, USA

<sup>35</sup>Department of Gastroenterology and Hepatology, University of Groningen, University Medical Center Groningen, 9713 GZ Groningen, The Netherlands

<sup>36</sup>Department of Molecular Biology and Biochemistry, University of California, Irvine, Irvine, CA 92697, USA

<sup>37</sup>Department of Biochemistry and Microbiology, New Jersey Institute of Food, Nutrition and Health, Rutgers University, New Brunswick, NY 08901, USA

<sup>38</sup>Department of Melanoma Medical Oncology, University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA

<sup>39</sup>UPMC Hillman Cancer Center, University of Pittsburgh, Pittsburgh, PA 15232, USA

<sup>40</sup>Center of Clinical Investigations in Biotherapies of Cancer (CICBT), Gustave Roussy, 94805 Villejuif, France

<sup>41</sup>These authors contributed equally

## SUMMARY

Over the last decade, the composition of the gut microbiota has been found to correlate with the outcomes of cancer patients treated with immunotherapy. Accumulating evidence points to the various mechanisms by which intestinal bacteria act on distal tumors and how to harness this complex ecosystem to circumvent primary resistance to immune checkpoint inhibitors. Here, we review the state of the microbiota field in the context of melanoma, the recent breakthroughs in defining microbial modes of action, and how to modulate the microbiota to enhance response to cancer immunotherapy. The host-microbe interaction may be deciphered by the use of “omics” technologies, and will guide patient stratification and the development of microbiota-centered interventions. Efforts needed to advance the field and current gaps of knowledge are also discussed.

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## INTRODUCTION

Immune checkpoint inhibitors (ICIs) have revolutionized the treatment of melanoma and other cancers, in early stages and advanced settings, by inducing durable and sometimes curative clinical responses in subsets of patients. However, most cancer patients do not benefit from ICIs, and strategies to enhance responses remain an unmet clinical need.<sup>1</sup> Host microbiota plays an important role in immunotherapy response and resistance.

Several lines of evidence support the notion that the gut microbiota regulates immune and clinical responses to cancer. In mouse models, certain gut commensals mediate efficacy of anticancer therapies, including chemotherapy, radiotherapy, and immunotherapies such as adoptive T cell transfer, intratumoral CpG-oligonucleotides, and ICIs.<sup>2–7</sup> In addition, broad-spectrum antibiotics (mainly beta-lactams) taken prior to immunotherapy markedly reduce the clinical benefit of ICIs, chimeric antigen receptor (CAR)-T cells, stem cell transplantation in patients, and immunogenic chemotherapy in preclinical tumor models.<sup>3,4,8–10</sup> The microbiota influences the cancer-immune set point, which is the threshold beyond which an immune response can be elicited in a given individual.<sup>11</sup> This statement relies on several lines of epidemiological and experimental evidence. First, correlations have been drawn across cancer indications and geographical sites between the taxonomic composition of the gut (and to some degree tumor) microbiota, and

ICI responses in patients with melanoma and other cancers.<sup>10,12–19</sup> Second, exposures including medications, especially antibiotics and proton pump inhibitors, and diet that shape the microbiota are associated with ICI responses in patient cohorts and preclinical models.<sup>5,10,13,18,20</sup> Third, cancer patients can suffer from stress ileopathies that perturb the commensal community toward profiles found in chronic inflammatory processes.<sup>21–23</sup> Fourth, cause-effect relationships between the prevalence or relative abundance of particular taxa, and clinical regression or progression, have been shown in animal models.<sup>21,24–27</sup> Finally, pioneering clinical studies in patients with melanoma showed that fecal microbiota transplant (FMT) could circumvent primary and secondary ICI resistance,<sup>28,29</sup> or ameliorate ICI response rates.<sup>30</sup> Furthermore, sufficient dietary fiber intake was associated with improved progression-free survival (PFS) with ICIs, while untargeted probiotic exposure was associated with worse outcomes, and preclinical melanoma models supported microbiota as mediators between these exposures and ICI outcomes.<sup>13,20</sup> However, many questions remain regarding mechanisms of host-microbiota-immune interactions, as well as optimal strategies of microbiota modulation to improve patient outcomes. This article is focused on melanoma, but given that the data in this field are still emerging, we have also included data from other populations/models and recognize that observations made in other cancers need to be validated in melanoma.

In March 2023, the Melanoma Research Alliance in partnership with the Seerave Foundation convened a workshop entitled “Melanoma and the Microbiome”. Experts in microbiology, immunology, computational biology, and oncology met and discussed the current state of the field, unanswered questions, and how to move the field forward. Based on these discussions, we provide a conceptual framework underpinning the interplay between microbiota and the host’s immune system in melanoma (and other cancers) alongside multi-omic characterization approaches and rational strategies to exploit or manipulate these mechanisms for patient benefit.

## **EFFECTS OF MICROBIOTA ON INNATE AND ADAPTIVE IMMUNITY: MECHANISMS OF ACTION**

Various mechanisms for the bidirectional interactions between the microbiota and human immunity have been elucidated, involving innate and adaptive immune components (Figure 1).<sup>20,27,31</sup> Regarding innate immunity, gut commensals provide ligands for pattern recognition receptors—including toll-like receptors (TLR)2-TLR4, TLR5, nucleotide oligomerization domain (NOD)2, and cGAS-stimulator of interferon genes (STING)—that modulate the functional status of professional antigen-presenting cells, including dendritic cells (DCs) and macrophages. Under the control of intestinal microbiota, type I interferons (IFN-I) are expressed by plasmacytoid DCs to prime the host against pathogens.<sup>31</sup> Microbiota-derived STING agonists such as c-di-AMP (derived from *Akkermansia* spp. or fiber-enriched diets) induce IFN-I release by monocytes present within tumors that, in turn, contribute to intratumoral natural killer (NK) cell activation.<sup>20</sup> STING also mediates the immunostimulatory capacity of macrophages in pancreatic tumors following exposure to trimethylamine-N-oxide (TMAO), dietary choline, or indirectly by CutC-containing fecal bacteria, which generate TMAO precursors.<sup>32</sup> Bacterial products,

partly through TLR4 activation, condition tumor-associated myeloid cells to respond to CpG-oligodeoxynucleotide TLR9 agonists in melanoma and colorectal cancer preclinical models.<sup>4</sup> In parallel, preclinical experiments demonstrated a direct impact of the gut microbiota on colon cancer. For example, *Escherichia coli* strains may express polyketide synthase, a microbial genotoxin that causes DNA damage in epithelial cells.<sup>33</sup> In addition, *Fusobacterium nucleatum* directly activates beta-catenin signaling via TLR4,<sup>34</sup> and *Peptostreptococcus anaerobius* activates integrin  $\alpha_2/\beta_1$  signaling, resulting in trophic, pro-inflammatory and immunosuppressive signals.<sup>35</sup> Whether the gut microbiota may affect oncogenic pathways in non-gastrointestinal cancers through similar direct interactions remains to be studied. During CTLA-4 blockade in various cancers, *Bacteroides fragilis* triggers TLR2–4-dependent interleukin (IL)-12 release that is associated with anticancer effects.<sup>5</sup> In contrast, the natural immunosurveillance of cancers can be negatively impacted by gut commensals through TLR5- and IL-6-dependent inflammation.<sup>36</sup> Thus, microbial-derived ligands directly modulate innate antitumor immunity.

With regard to adaptive immunity, distinct gut microbial genera and species are associated with CD8<sup>+</sup> T cell infiltration and survival in patients with melanoma.<sup>16</sup> The gut biomass encodes microbial antigens that are molecular mimics of tumor antigens. For example, upon exposure to cyclophosphamide, *Enterococcus hirae* translocation leads to the activation of CD8<sup>+</sup> T cells that recognize a latent bacteriophage encoded by the *E. hirae* genome and cross-react with tumor antigens, hence attenuating tumor progression.<sup>37</sup> Moreover, the detection of HLA-bound bacterial peptides has been associated with an inflamed tumor microenvironment. Intracellular bacteria contained in metastatic melanoma cells enable the binding of bacterial peptides to major histocompatibility complex class I and II molecules, thereby facilitating tumor recognition and killing by tumor-infiltrating lymphocytes.<sup>38,39</sup>

In other malignancies, distinct bacteria can induce follicular T helper cell and B cell priming, as well as tertiary lymphoid structure formation in tumor beds,<sup>40</sup> as shown in mice.<sup>41</sup> Conversely, mucosal commensalism can compromise cancer immunosurveillance. Cancer immunotherapy focuses on only one of the PD-1 ligands, namely PD-L1 (CD274). However, a second PD-1 ligand, PD-L2 (CD273), has not yet been targeted in successful clinical trials. In mice, dysbiosis causes the upregulation of PD-L2 on DCs and increased expression of the PD-L2 receptor repulsive guidance molecule b (RGMb) on CD8<sup>+</sup> T cells, hence explaining the resistance of cancers to PD-L1 blockade. In this context, simultaneous blockade of either PD-L1 or RGMb could restore the anticancer effects of PD-L1 blockade.<sup>42</sup> Altogether, these results suggest that modulation of the PD-L2/RGMb pathway may represent one mechanism used by gut microbiota to influence the clinical response to PD-1/PD-L1 blockade. Of note, *Coprobacillus cateniformis* has the capacity to downregulate the PD-L2/RGMb system in preclinical experiments, suggesting the possibility to develop immunostimulatory probiotics acting on this pathway.<sup>42</sup>

Certain microbes may release metabolites that directly activate T lymphocytes, act on epithelial or endothelial intestinal barriers, or act on the tumor cells themselves.<sup>17,43,44</sup> The microbial metabolite inosine promotes the anticancer effect of CpG. Inosine promotes the production of IL-12 by conventional DCs, thereby triggering IL-12R $\beta$  signaling in CD4<sup>+</sup> T cells, as well as CD8<sup>+</sup> Tc1 differentiation and effector functions.<sup>17</sup> High levels of

microbiota-derived arginine are associated with improved PFS in patients receiving ICIs. In murine tumor models, boosting L-arginine levels enhances the antitumor effects of ICIs in a T cell-dependent manner.<sup>45,46</sup> Microbial-derived TMAO produced by gut microbiota in patients with melanoma promoted antitumor CD8<sup>+</sup> T cell activity in mouse tumor models.<sup>32,47</sup> In addition, the microbiota-derived tryptophan metabolites, indole-3-acetic acid and indole-3-aldehyde (I3A), increased the efficacy of chemotherapy in mouse models of pancreatic cancer and are enriched in patients who respond to chemotherapy.<sup>48,49</sup> Notably, *Lactobacilli reuteri* translocates from the intestines to tumor beds and produces I3A *in situ*, inducing aryl hydrocarbon receptor (AhR)-dependent CREB activity, which drives Tc1 effector and tumoricidal functions. *L. reuteri*-derived I3A improved ICI efficacy in mice, and melanoma patients responding to ICIs harbored higher serum I3A levels compared with nonresponders.<sup>49</sup>

However, another study showed that dietary tryptophan metabolism to indoles by gut-derived *Lactobacillus* spp. reduced anti-tumor CD8<sup>+</sup> T cell quantity and increased pancreatic tumor cell growth.<sup>50</sup> These conflicting results may indicate that the dose and scheduling of a tryptophan-enriched diet, or tryptophan-derived metabolites, can influence clinical outcomes. Microbes can interfere in the composition of fecal and plasma biliary acids. Biliary acids can regulate colonic T regulatory (Treg) cell homeostasis<sup>51</sup> and the translocation of enterotropic IL-17-producing Treg cells to distal tumors,<sup>52</sup> promoting immunoresistance, or alternatively participate in the recruitment of effector cells, keeping liver malignancies in check.<sup>53</sup> The influence of short-chain fatty acids (SCFAs) produced by specific species on systemic immunity remains controversial. High serum levels of butyrate and propionate have been associated with resistance to anti-CTLA-4 antibodies and radiotherapy and increased Treg cell numbers in patients with melanoma.<sup>7,54</sup> However, other studies have shown that butyrate can activate CD8<sup>+</sup> T memory cells.<sup>55</sup> In mice, butyrate and pentanoate induce mammalian target of rapamycin activation and histone deacetylase inhibition in adoptively transferred T cells, resulting in enhanced CAR-T antitumor activity.<sup>56</sup> In cancer patients treated with anti-PD-1 antibodies, stool SCFAs are associated with favorable clinical outcomes.<sup>57</sup> In addition, a diet rich in fiber results in enrichment of fiber-fermenting, SCFA-producing bacteria, such as *Faecalibacterium prausnitzii*, which have been associated with ICI response. Habitual consumption of a high-fiber diet has also been associated with improved PFS in ICI-treated patients with melanoma.<sup>13</sup> In mice, dietary fiber *deprivation* was further shown to abrogate response to ICIs and decrease stool propionate.<sup>13</sup> However, the functional links among dietary fibers, serum- and fecal-derived SCFAs, and antitumor immunity remain to be determined. Pending further confirmation, these results suggest that SCFAs display context-dependent immune effects that range from the differentiation of Treg cells to the enhancement of effector T cell functions.<sup>56,58,59</sup>

Data increasingly suggest that intestinal commensals can emigrate from the gut to distal tumor lesions and reprogram the tumor microenvironment (TME). Cancer progression is associated with  $\beta$ 2-adrenergic receptor signaling-dependent stress ileopathy, causing transient intestinal permeability.<sup>21</sup> High-salt diet-induced gut permeability also enables translocation of *Bifidobacterium* to tumors, improving the function of NK cells and decreasing tumor size.<sup>60,61</sup> The combination of two ICIs promotes mucosal inflammation

and induces the migration of commensal-harboring DCs to mesenteric lymph nodes (LNs), thereby enabling microbes to reach distal lymphoid organs and tumor beds.<sup>62,63</sup> Likewise, immunosuppressive T cells can migrate from the gut to tumor beds. This T cell trafficking is in part controlled by a gut immune checkpoint called mucosal addressing cell adhesion molecule-1 (MAdCAM-1). The interaction between MAdCAM-1 expressed by high endothelial venules in Peyer patches and its ligand integrin  $\alpha 4\beta 7$  expressed on T cells plays a key role in the homing of T cells to the gut-associated lymphoid tissue (GALT). Antibiotics and more specifically, a relative over-dominance of gram-positive bacteria of the *Enter-ocloster* genus (such as *E. clostridioformis* or *E. boltea*) can cause MAdCAM-1 loss. Upon MAdCAM-1 downregulation, immunosuppressive  $\alpha 4\beta 7$ -expressing FoxP3<sup>+</sup>, ROR $\gamma$ T<sup>+</sup> regulatory (Tr17) cells are no longer retained in the GALT and hence migrate to tumor-draining LNs and into tumor beds, where they compromise the outcome of PD-1 blockade.<sup>52</sup> Cohort studies unveiled that low serum levels of soluble MAdCAM-1 identified patients with poor survival following anti-PD-1 treatment across several malignancies.<sup>52</sup>

Of note, recent reports suggest that specific pathogenic bacteria such as *Helicobacter pylori*, which causes gastric cancer, can be associated with ICI resistance. Cytotoxin-associated gene A encoded by *H. pylori* triggers activation of the ERK/MAPK pathway in gastric epithelial cells, thereby stimulating the development of mucosa-associated lymphoid tissue lymphoma.<sup>64</sup> In the context of ICIs, three retrospective studies demonstrated that the presence of circulating anti-*H. pylori* antibodies is associated with resistance to immunotherapy in patients with non-small cell lung cancer (NSCLC), melanoma, and gastric cancer.<sup>65–67</sup> In mice, *H. pylori* infection decreases the activation of CD8<sup>+</sup> T cells by reducing the cross-presentation activity of DCs. These results indicate that colonization by certain pathogens can lead to ICI resistance.

The following topics remain open conundrums. First, the regulatory events controlling the seesaw-like balance between beneficial versus harmful bacteria and/or keeping bacterial translocation in check are still unknown. This question needs to be addressed not only in the context of natural or therapy-induced immunosurveillance but also for drug-associated side effects that are dependent on the microbiota. Doing so may further improve understanding of enterofecal compatibility during FMT and its links with systemic inflammation and immune activation. Second, whether and how individual components of the extended repertoire of commensals—bacteria, fungi, viruses, archaea, and candida phyla—target innate and/or cognate immune responses remains largely elusive. Current studies focus mostly on the bacterial gut flora, and there is little knowledge on how other phyla including the virome and mycobiome influence immunotherapy responses. Internal transcribed spacer sequencing revealed that the interplay between fungi and bacteria profoundly influences the development of various diseases such as inflammatory bowel disease, as well as the efficacy of FMT against *Clostridium difficile* infection.<sup>68–70</sup> In the realm of oncology, it appears that an intestinal expansion of *Candida albicans* triggers macrophage activation, which in turn, promotes the development of colon cancer.<sup>71</sup> With respect to archaea, *Methanobrevibacter smithii* was found enriched in patients with melanoma (Wang, Abstract e21512, presented at ASCO 2023), and patients with NSCLC who responded to ICIs harbored *Akkermansia muciniphila*.<sup>15</sup> This observation was validated in two other studies on patients with NSCLC.<sup>72,73</sup> However, the mechanistic underpinning of this correlation



remains unexplored. Third, adequate experimental models are urgently required beyond specific pathogen-free or gnotobiotic mice orally gavaged with monoclonal bacteria, or consortia, to better translate findings to humans. Indeed, wild mice harbor an immune system closer to humans than vendor-derived specific pathogen-free mice.<sup>74,75</sup>

Lastly, the skin possesses a distinctive microbiome dominated by gram-positive bacteria including *Staphylococcus* and *Veillonella*, which play an important role in wound healing, and various potentially pathogenic microorganisms such as *Cutibacterium acnes* in acne vulgaris and *Staphylococcus aureus* in psoriasis, diabetic foot ulcers, and lupus erythematosus. For non-melanoma skin cancer, one study compared the local microbiota of premalignant actinic keratosis lesions to cutaneous squamous cell carcinoma (SCC). Several *S. aureus* species were more abundant in SCC than in keratosis.<sup>76</sup> This observation has been validated, and an SCC cell line cocultured with *S. aureus* exhibited upregulation of the potentially oncogenic beta defensin-2.<sup>77</sup> Regarding melanoma, a culture-based analysis of skin swabs of 27 acral melanoma patients revealed a stage-associated increase in *Corynebacterium*. Patients that harbored *Corynebacterium* had a higher frequency of Th17 cells, which produce IL-17 that can promote melanoma growth.<sup>78,79</sup> This latter observation was validated in mice, in which topical application of *Corynebacterium accolens* resulted in the dermal recruitment of IL-17A-producing  $\gamma\delta$  T cells.<sup>80</sup> These findings suggest that *Corynebacterium* species may stimulate melanoma progression through an IL-17-dependent pathway. These findings underscore the potential impact of the skin microbiota on local cancers.

## INFLAMMATION AND “OMICS”

Spatial and temporal control of inflammation is central for maintaining human health<sup>81</sup> and fails as aging advances.<sup>82</sup> Thus, failure to limit inflammatory processes to transient and localized phenomena underlies pathology. This phenomenon also applies to cancer control, as unrestrained inflammation contributes to (often age-related) oncogenesis as well as tumor progression, in part due to impaired immunosurveillance.<sup>83</sup> Leukocytes, especially myeloid cells, can contribute bidirectionally to procarcinogenic inflammation and tumor-suppressive immune responses, depending on their precise phenotype, functional state, and interactions with other immune cells (such as B, NK, and T lymphocytes), adding complexity.<sup>84</sup> Thus, the relationship between neoplastic cells and inflammation-modulating immune cells should be conceptualized and characterized at local and systemic levels.

This systemic or “ecological” dimension<sup>85</sup> offers the possibility to investigate the composition of cancer-related, body-wide, and ideally, easily accessible analytes related to inflammation, such as circulating immune cells and the fecal microbiota, rather than the tumor itself (Figure 2). Systemic pro-inflammatory states with enhanced gut permeability are characterized by a relative reduction of anti-inflammatory bacteria (including those producing SCFAs) and an expansion of pro-inflammatory species such as *Streptococcaceae* family and *Veillonella*.<sup>22</sup> These species are often associated with poor prognosis and lack of responses to cancer immunotherapy.<sup>15,19</sup>

Recent advances in “omics” technologies provide the opportunity to identify clinically actionable, inflammation-related, circulating and fecal-derived biomarkers for preemptive diagnosis, primary prevention, or interception of early melanoma lesions, as well as prognosis, ranging from patient survival to predicted, personalized therapeutic responses (Figure 2). Biomarker identification that considers and exploits host-microbial interactions, such as the relationship between serum MAdCAM-1 loss (due to dysbiotic gut microbiota) and poor ICI response, can provide insights otherwise missed when focusing on host or microbial constituents alone.<sup>52</sup> Elucidating specific metabolites, immune cell subsets, and/or microbial strains associated with a low risk of cancer or progression during treatment can suggest future preventive or therapeutic interventions.<sup>86–88</sup> Although correlative diagnostic markers are important, identification of “mechanistic” biomarkers is essential for prognostic and therapeutic design and could change the medical oncology landscape.<sup>89</sup>

Besides metagenomics and intestinal barrier-related markers (such as sCD14, sST2, LPS-binding protein, and calprotectin), multiple other “omics” technologies provide proxies of systemic inflammation and range in cost from relatively affordable technologies, such as plasma mass spectrometric metabolomics to more expensive methods including plasma proteomics, extensive immunophenotyping of circulating leukocytes (“immunomics”), deep bulk exome sequencing, and single-cell versions of these (such as scRNA-seq) (Figure 2). Microbiota are broadly characterized through genomic sequencing, either amplicon or shotgun-based methods; the decision is often a trade-off among biomass levels (e.g., sparse in tumors, abundant in feces), desired taxonomic resolution, and budget. Notably, gastrointestinal microbiota are incompletely sampled using feces, overrepresenting colorectal-resident species; whereas ileal microbiota may yield more functional insights.<sup>90</sup> Certain methods can simultaneously characterize host and microbial molecules (e.g., untargeted sequencing, metabolomics), increasing efficiency; additionally, when low biomass and/or contamination must be accounted for, taxonomic characterization can rationally guide subsequent multi-omic analyses (e.g., proteomic spectra matching).<sup>38</sup> Prospective population studies usually prioritize affordability over breadth, and methods that simultaneously interrogate inflammation-relevant human molecules plus microbes may be particularly advantageous for characterizing ICI response mechanisms.

The absence of universal standardization among sampling, aliquoting, cryopreservation, extraction, and bioinformatic approaches in host-microbe biomarker discovery complicates comparisons and reproducibility.<sup>91</sup> Although computational batch correction methods and/or uniform re-analyses can reconcile disparate results,<sup>12</sup> they are not a panacea.<sup>92</sup> This is further aggravated by the fact that bioinformatics-driven data interpretation suffers from the absence of universal guidelines for data quality control, normalization strategies, and the utilization of artificial intelligence tools. Because many methodological choices are still being optimized, especially for microbiota sampling, a realistic strategy for improving inter-laboratory comparisons of datasets may involve providing external standards (such as spike-in controls<sup>93</sup>)—benefitting both host and microbial data—rather than enforcing non-optimized standard operating procedures. The comparison of intra-dataset correlations appears to be more robust than attempts to normalize raw data collected by distinct operators. External standards would not remove variation caused by controls (such as

healthy volunteers or untreated patients), which are often truly distinct in different geographic locations.<sup>12,19,94</sup>

“Omic” data annotation comprises another challenge. Careful annotation of bacterial data is a prerequisite for successful identification of the species responsible for a given phenotype. One reason that different bacterial species identified in different parts of the US or Europe are linked to given phenotypes may be the accuracy of annotation, a point that deserves careful attention. Moreover, fecal DNA analyses typically exclude host-derived DNA and include DNA only from microbial taxa that are assignable to distinct genera, species, subspecies, or strains before the results are used to compare intra- and inter-individual microbiota differences. Similarly, metabolomics datasets often contain few annotatable mass spectrometric peaks (i.e., defined metabolites), with many more peaks that lack chemical assignments, and usually rely on known molecules for downstream analyses. Both approaches discard data on the basis of incomplete databases and/or inadequate annotation methods. However, alternative strategies exist. For instance, one can agnostically assign object identifier codes to each microbial sequence and compute intra- and inter-individual differences without taxonomic assignment; this procedure has been used to identify microbial “guilds.” These guilds are functional clusters of distinct microorganisms exhibiting cooperative relationships and co-abundance patterns; they interconnect to form co-occurrence networks, highlighting the interplay of unique microorganisms within the larger ecosystem. By correlating variations at the guild level with specific phenotypes or mechanistic markers, one can infer their health-related functions, including when lower resolution taxa-based analyses would miss them.<sup>95</sup> Analogously, unannotated metabolomics peaks can still be used to construct co-occurrence networks in the search for inflammation-relevant biomarkers indicative of pathogenic processes.<sup>96</sup> As future research improves underlying databases, currently unannotated metabolites, or microbes, may become interpretable, but it is unnecessary to wait to associate their data with pathophysiology. Furthermore, identification of microbial guilds, metabolites, or other molecular entities (such as plasma proteins, although the accuracy of currently available proteome platforms remains a challenge)<sup>97</sup> may generate new composite biomarkers that are multi-omic and/or multi-species in nature.

A spectrum of sampling strategies exists for “omics”-based biomarker discovery. On one end, samples may be drawn and banked from a large, still-healthy population subjected to regular lifestyle evaluations and health checkups for decades to prospectively identify individuals who develop disease and follow their molecular trajectories. Here, a maximum number of participants should be enrolled, and their samples preferentially subjected to low-cost “omics” studies, with sufficient quantities kept for post hoc confirmation and/or later profiling using advanced methods that decrease in cost. On the other end, samples could be obtained only in the context of carefully executed clinical interventions (such as administration of drugs, chemically defined food supplements, or genetically defined microbial communities) to evaluate hypotheses and/or modes of action. In this case, the enrolled sample size would be limited, but the depth of the “omics” analyses increased. However, in both scenarios, obtaining longitudinal samples from patients is critical, and both designs should further consider discovery and validation cohorts if attempting to confirm biomarker(s) utility to reduce the risk of reporting spurious correlations.

Overall, contingent on strategic resource allocation and careful trial design, there are strong prospects for identifying a new generation of multi-omics, multi-species biomarkers that identify inflammation- and immunity-relevant biomarkers, providing information for stratification of patients with melanoma, personalization of prophylactic or therapeutic interventions, and, ultimately, identification of novel drug targets.

## TRANSLATING BIOMARKER DISCOVERIES INTO CLINICAL PRACTICE

Before “omics”-based biomarkers can make a real impact on patient outcomes, biomarkers need to be adapted to methods that can readily be implemented into clinical practice. For broad adaptation and compliance of the use of biomarkers in clinical practice, aspects such as patient comfort and compliance, high reproducibility and precision, cost, turnaround time, and actionable results for physicians need to be considered. Regulatory authorities have also established mandatory guidelines and standards that require compliance.

Among many factors to be considered, we highlight the following considerations.

Collection of biological samples from the colon. Although collection of samples or biopsies from the small intestine may be optimal, invasive methods will add risks for patients, costs, and reproducibility challenges.<sup>98</sup> Therefore, in a routine clinical setting, non-invasive methods such as collection of fecal samples with commercially available kits may be preferred. A second consideration related to the collection of fecal samples is the observed day-to-day variance in the microbial composition of fecal samples,<sup>99</sup> which may be circumvented by three or more collection time points at baseline.

DNA extraction is a key step. Several methods and kits exist for DNA extraction, each with certain biases. However, when repeatedly using the same kit, improved reproducibility can be obtained.<sup>100</sup> Other steps to improve precision and reproducibility include automation, simplification, targeting, and statistical robustness. Establishing highly automated procedures reduces the person-to-person and day-to-day variation that is observed in microbiota analyses.<sup>101</sup> Greater reproducibility can be achieved by reducing excessive steps that introduce variability. An optimal biomarker diagnostic test to be implemented in the clinic would benefit from a method that utilizes a predefined set of targets, such as various PCR approaches (e.g., qPCR, dPCR, RT-PCR) because they have precedent for regulatory approval. The evolution of bioinformatic approaches, databases, and tools will shape the interpretation of the raw data from DNA sequencing of microbiota samples.

## INTRATUMORAL MICROBES AND MECHANISMS

A growing body of evidence has demonstrated the presence of intratumoral microbes, with studies using either metagenomics or culturomics suggesting the presence of intracellular,<sup>14,38,102,103</sup> cancer type-specific,<sup>14,104–106</sup> spatially heterogeneous,<sup>14,103,107</sup> metabolically active,<sup>14,38,49,108,109</sup> pro-metastatic,<sup>102,103,110,111</sup> and immunoreactive<sup>38,60,112–115</sup> communities of tumor-resident bacteria, fungi, and viruses,<sup>116</sup> including in melanoma.<sup>14,38,49,104,105</sup> While murine experiments can be well controlled, the methodology used to detect intracellular bacteria in human tumors is vulnerable to

contamination (especially with culturomics) and poor curation of microbial-specific DNA sequence databases (with metagenomics), as discussed in the following section.

Microbes can translocate into solid tumors via various routes.<sup>49,110,114,115,117,118</sup> Several reports indicate that for gastrointestinal tumors, such as pancreas and colon carcinoma, direct interaction between bacteria and healthy cells can trigger genetic alterations and inflammation leading to cancer.<sup>119</sup> For example, orally administered fluorescent *Enterococcus faecalis* can be identified in pancreatic cells from normal mice.<sup>117</sup> Conversely, another group demonstrated that following FMT from feces of patients with pancreatic cancer, only a minority of bacteria found in orthotopic mouse pancreatic carcinomas were of donor origin, although FMT did influence the composition of the recipient-derived intratumoral microbiota.<sup>114</sup> Intravenous injection of *F. nucleatum* caused its enrichment in rectal cancer from mice, suggesting that bacteria can travel to various organs via the bloodstream.<sup>120</sup> Accordingly, in mice, *L. reuteri* trespasses the colon epithelial barrier and then colonizes subcutaneous melanoma cells to locally produce I3A and enhance the efficacy of ICIs.<sup>49</sup> In melanoma, more complex mechanisms for translocation have arisen when considering therapeutic-induced epithelial damage and/or the host immune response, such as immunotherapy-mediated (CD47-based or ICI) translocation of gut bacteria to tumors.<sup>60,62</sup> However, the mere presence of a melanoma tumor may trigger gut translocation via  $\beta$ -adrenergic-mediated stress ileopathy that increases gut permeability.<sup>21</sup> Major interest exists to delineate the synchronicity, or lack thereof, of metastatic processes and microbial translocation, with preliminary data suggesting both can happen.<sup>102,118</sup> It also remains unclear whether metastasis-seeding microbes can translocate back to primary tumors or between multiple metastases, with only anecdotal evidence suggesting similarities between multiple melanoma metastases within the same patient.<sup>38</sup> Systematic decoupling of microbial origins, seeding preferences, and timelines will require dense, longitudinal, multi-regional sampling in murine models, and, when possible, tumors from patients.

Intratumoral bacteria can impact cancer progression and response to cancer therapy via multiple mechanisms of action. At the level of the cancer cell, microbes can potentially modify every aspect of cellular fitness: genomic integrity,<sup>121</sup> transcriptional states,<sup>122</sup> proteomes,<sup>38,112</sup> and metabolism.<sup>14,108</sup> Single-cell data of infected cancer cells indicate higher expression of pathways related to epithelial-to-mesenchymal transition and p53 signaling than non-infected cells.<sup>103</sup> At the level of the TME, intratumoral microbes may modulate immune tone through innate<sup>115,123</sup> and adaptive<sup>38,41,112</sup> pathways, or both,<sup>117</sup> as well as through immunoactive metabolites.<sup>49</sup> A few studies have suggested that intratumoral microbiota compositions are predictive of ICI responses,<sup>14,105,124,125</sup> which remain to be confirmed. The exact distribution of which microbes are passengers versus complicit actors or drivers remains unclear.<sup>126</sup>

Controversies persist with respect to the methods used for evaluating the presence of intratumoral microbes, their quantitative estimation, as well as their biological and clinical relevance *in situ*. Quantitative PCR has estimated that among seven cancer types, approximately 0.68% (95% CI: 0.75, 1.46%) of the tumor cellular content is bacterial, with melanoma-specific density estimates at  $\sim 33$  bacteria/mm<sup>2</sup> or  $\sim 6.3 \times 10^6$  bacteria/cm<sup>3</sup> on average (using bootstrapped values).<sup>14,127</sup> Fungal abundance estimates are one-to-two orders

of magnitude lower than bacteria,<sup>105</sup> but the abundances of other domains (e.g., viruses, phages, archaea) remain unexplored. Theoretically, more than one bacterial cell can infect a single cancer cell,<sup>38,103</sup> but the total number of unique species per tumor, bacterial or fungal, drops (<50) when decontamination strategies are applied.<sup>14,38,105</sup> Three studies claimed the uniqueness of intratumoral microbiota and the possibility to diagnose cancers based on metagenomic deep sequencing of circulating DNA,<sup>14,104,106</sup> but concerns have been raised based on strikingly high numbers of intratumoral bacteria<sup>128</sup> and methodological disputes, including the separation of bacterial and human DNA (e.g., bacterial DNA sequences deposited in official databanks can be contaminated by human DNA) and batch correction algorithms generating spurious artificial intelligence-based classifications.<sup>129</sup> Overall, we will need to develop more robust microbiological and molecular methods to exclude contaminant sequences (either human or contaminating microbes) for more precise and reliable characterization of intratumoral microbiota. Such an effort could be developed at the consortium level, analogous to The Cancer Genome Atlas (TCGA),<sup>130</sup> even if solely focused on melanoma (Figure 3) and would require biobanking of multiple specimen types (feces, saliva, blood, urine, primary tumor, metastatic tumors) with experimental contamination controls over time, including before, during, and after treatment when possible. Multi-omic and multi-domain (e.g., bacteria, fungi, viral) assessments would guide hypothesis generation that could be subsequently tested in validation subsets, external cohorts, and/or mouse models. Such an endeavor will facilitate the application of new technologies that study host-microbe interactions at the single-cell and spatial levels, providing critical information on microbial proximity to tertiary lymphoid structures, metastatic clones, and spatial patterns directly related to response or resistance to ICIs.

## DIET AND PREBIOTICS AS MICROBIOTA MODULATORS

Gut microbiota are largely determined by environmental exposure, especially what we ingest, i.e., diet and medications.<sup>12,18,131</sup> Many bacteria associated with ICI response have known dietary associations, such that specific dietary patterns (e.g., plant-based versus western diet) are associated with increased or decreased relative abundances of these candidate taxa.<sup>131–135</sup> Thus, there has been a strong interest and rationale in microbiota-centered interventions (MCIs) and to conjunctively examine diet and microbiota in cohorts of ICI-treated melanoma patients.

Recent observational studies in melanoma patients have demonstrated, in the context of ICI administration, that sufficient dietary fiber intake is associated with improved PFS compared to insufficient fiber intake.<sup>13</sup> Consumption of a Mediterranean-style diet or higher omega-3s is also associated with an increased likelihood of ICI response in melanoma patients.<sup>18,136</sup> These observational studies are potentially limited by confounding factors, but the biological plausibility of this diet-microbiota-immune axis rests on the idea that microbial breakdown of dietary fiber and other prebiotic foods results in the production of immunomodulatory metabolites.<sup>13,137,138</sup>

Importantly, causality is supported by preclinical studies in which dietary fiber manipulation in murine models rapidly shifts the microbiota and can potentiate the effect of ICIs in specific pathogen-free, tumor-bearing mice.<sup>13,20</sup> Notably, this phenomenon is not seen in

germ-free mice, suggesting that ICI response augmentation is microbiota dependent.<sup>13</sup> The effects of more specific/restrictive dietary interventions on antitumor immunity and ICI response, including ketogenic diet and caloric restriction, have also been verified in mice.<sup>139,140</sup> In humans, a fasting-mimicking diet decreases immunosuppressive myeloid cells while increasing CD8<sup>+</sup> T cells and NK cells.<sup>141</sup> Although prior studies of macronutrients or caloric restriction focused on altering tumor metabolism, these findings suggest that dietary interventions may also exert immunomodulatory effects via microbiota, or microbial or host metabolite effects, on immunocytes. They also set the stage for dietary intervention studies to improve outcomes in ICI-treated patients with cancer, but several issues remain to be addressed. Moreover, standardization of the definition of each diet, as well as consideration of cultural and geographic distribution, will be important when performing homogeneous studies.

Behavioral changes including following and maintaining a specific diet over months are challenging, particularly in patients who are physically and emotionally overwhelmed by an active malignancy and treatment-related gastrointestinal complications.<sup>131,142</sup> Sustaining beneficial dietary changes may also be important, as changes to the metabolome/microbiota are reversed after an individual reverts to their habitual diet.<sup>131,142</sup> However, the critical time for dietary microbiota modulation may only be at the priming phase prior to ICI initiation.<sup>131</sup> In addition, the microbial response to dietary intervention varies among individuals, complicating straightforward prediction of results.<sup>131,143</sup> In this sense, the value of a personalized diet warrants further exploration, specifically considering predictors of antitumor response to diet<sup>143,144</sup> in relation to habitual diet and the person's microbiota.<sup>131</sup>

An alternative to full-scale dietary changes is to identify specific nutrients or metabolites that mediate the desired antitumor effects from dietary studies. These compounds, in turn, could be used directly as “drugs” while obviating challenging behavioral modifications. For example, one could therapeutically administer 3-hydroxybutyrate to mediate effects of the ketogenic diet in mouse ICI models.<sup>140</sup> Similarly, the prebiotic berry camucamu, which is rich in polyphenols including castalagin, favorably alters the gut microbiota composition and the TME, and is now being clinically investigated.<sup>145</sup> A recent preclinical study discovered that *L. reuteri* converts dietary tryptophan into the AhR agonist indole-3-carbaldehyde, which was necessary and sufficient to enhance ICI efficacy in preclinical melanoma via activation of AhR in CD8<sup>+</sup> T cells and driving Tc1 immunity.<sup>49</sup> Although a tryptophan-enriched diet further enhanced the antitumor effect of *L. reuteri*, increasing dietary tryptophan in the presence of complex microbiota and in the absence of exogenous *L. reuteri* administration also enhanced ICI efficacy in preclinical melanoma. This observation implies that other tryptophan-catabolizing bacteria besides *L. reuteri* contribute to antitumor immunity; this hypothesis should be further evaluated.

The prebiotics inulin and mucin produce antitumor immune responses in melanoma-implanted mice, but not in germ-free mice.<sup>146</sup> However, a different study showed that soluble fiber such as inulin may induce liver cancer in mice with a disrupted gut microbiota, highlighting that specific fiber sources and context may matter; moreover, it suggests that we need to be careful when deciding which fiber to use in cancer patients, as their gut microbiota has been significantly disrupted and may not respond the same as healthy

gut microbiota.<sup>147</sup> It is possible that some dietary fiber may promote pathogens when no competing beneficial bacteria exist. It is also plausible that with a reductionist approach (i.e., only compounds), we will overlook some of the potential beneficial immunomodulatory effects of whole food, given that fiber in the human diet is not consumed in isolation. Overall, though, the Mediterranean and plant-rich diets associated with ICI response in patients are rich in polyphenols and other compounds that can modulate both microbiota and immunity.<sup>13,28,140,145,148–151</sup>

It remains challenging to fully replicate diet-microbiota interactions in preclinical models due to the relative complexity of the diets of free-living humans versus laboratory mice. This limitation can obscure or compound the impact of complex diets on microbiota and their concomitant clinical implications. We thus need to better understand how dietary interventions that are successful in preclinical models can be recapitulated in the human diet. Moreover, it remains unclear what the endpoint of such dietary interventions should be (e.g., microbiota composition, metabolic output, bacterial species, taxa, or “guilds”). Indeed, gut bacteria respond to a high-fiber diet as guilds rather than taxa, suggesting that guilds may mediate the beneficial effects of dietary fiber on immunotherapy.<sup>152</sup> Sole reliance on tumor shrinkage as an endpoint may make some studies unfeasible.<sup>152</sup> The ultimate goal is improving ICI response, and establishment of a surrogate biomarker for an optimal response to ICIs following dietary intervention for use in early phase clinical trials will be critical.

Adding dietary modification or recommendations to official guidelines will require more data, and there is currently no strong recommendation from international associations such as ASCO or ESMO (Cancer Prevention Recommendations).<sup>153</sup> We do not fully understand how and if changing the diet the same way for all patients will provide a better cancer outcome or whether an intervention should involve a whole dietary change, prebiotics, or synbiotics.<sup>131</sup> Several clinical trials are underway to answer important questions about diet and response to cancer immunotherapies (e.g., [NCT03950635](#), [NCT04645680](#), [NCT05303493](#), and [NCT04866810](#)). It is likely that patient cohorts will need to be identified for select interventions based on a unique marker set, allowing better monitoring of possible changes upon altered diet-microbiota composition. Clinical trials of dietary interventions remain challenging, including exact measurement of dietary intake data from patients, diet/nutrient “dose,” intervention provision (controlled feeding study versus behavioral intervention), cultural differences around particular food types, and participant preferences and willingness to comply.<sup>144</sup> However, despite these challenges, ultimately demonstrating that diet or specific nutrients can impact ICI response has the potential to be widely scalable, cost-effective, and impactful for patients globally.

## **MCIs IN CANCER: LIVE BIOTHERAPEUTIC PRODUCTS, CONSORTIA, FMT, AND GENETICALLY MODIFIED BACTERIA**

Before gut-based microbial therapy of cancer can be widely used, several key issues need to be addressed (Figure 4). First, the field needs consensus on what constitutes “beneficial” microbiota. Criteria may be treatment dependent, disease dependent, context/host dependent,<sup>154</sup> or agnostic, and functional considerations may be more important than



taxonomic ones. Conversely, knowing what constitutes “adverse” microbiota would also be valuable, as some microbial signatures are associated with poor outcomes and specific adverse events (e.g., *Streptococcus* spp.).<sup>12</sup> In other words, the concept of “dysbiosis” needs to be clearly defined, and an optimal definition may need to consider microbe-microbe interactions in addition to host-microbe interactions.

In that direction, this group of experts recently described Gut OncoMicrobiome Signatures (GOMS) across different cohorts of patients with cancer, histotypes, and geographical sites, and showed that GOMS are shared among individuals with seemingly unrelated disorders and characterized by an imbalance between health-related and chronic inflammatory disease-related commensals.<sup>22</sup> A meta- and mega-analysis of stools obtained from patients with cancer treated with ICIs showed that GOMS associated with ICI resistance comprised microorganisms affiliated with chronic inflammation and immunosuppression and oral genera, and were shared across various pathological disorders.<sup>22,155</sup> Conversely, GOMS associated with ICI response encompassed genera enriched in health-related states. Moreover, several organisms involved in fermentation of dietary fibers, immunogenic commensals, and *Prevotella copri* SGB1626 were associated with cancer-free status. Responder GOMS for patients with lung and kidney cancer had higher predictive values than melanoma responder GOMS.<sup>22</sup> In addition to GOMS, we constructed a co-abundance network depicting relative abundance interrelationships correlated with patient survival using shotgun metagenomic stool samples at baseline, enabling calculation of an individual ICI response probability score (Derosa, Abstract 259MO, presented at ESMO Asia Congress 2022).

Mapping metagenomic data obtained from published melanoma patient cohorts onto a microbiota map derived from an American Gut Project database of more than 10,000 fecal samples from across the US led to the identification of distinct gut enteric signatures or microbiotypes of different geographical areas, which accounted for most of the between-cohort differences.<sup>12</sup> Therefore, enteric microbiotypes can be identified in large datasets despite being not entirely discrete and still variable by taxonomic, functional, and ecological properties. Such classifiers may be useful in the context of microbiota-based diagnostics and therapeutic interventions.

Ideally, a user-friendly test for dysbiosis would consist of PCR or accelerated shotgun metagenomic sequencing using routine tests compatible with clinical management constraints.<sup>156</sup> Current clinical proxies for dysbiosis include MAdCAM-1, REG3, calprotectin, and lipocalin-2 in fecal samples.<sup>52,157,158</sup> However, some discrepancies between microbial signatures associated with ICI response may be due to geographically distributed enterotypes of ecologically balanced commensal communities that are not fully characterized.<sup>12</sup> Once the “good” and “bad” poles of the gut microbiota are defined, the field can more precisely modulate microbiota in a way that is expected to be favorable, followed by understanding of how subsequent treatments or diet will influence those microbiota.

Despite preliminary findings of encouraging clinical outcomes of FMT in patients with refractory or first-line melanoma, questions with respect to safety, scalability, and reproducibility of FMT remain; evaluation in large randomized trials is needed. Donor

selection based on specific criteria and extensive microbial testing to ensure safety are limitations for large-scale implementation. As a result, standardized microbial consortia composed of defined bacterial populations should be tested in future trials.<sup>159</sup> Nevertheless, ongoing FMT trials ([NCT04951583](#) and [NCT0498884](#)) will provide the opportunity to understand the determinants of patient-donor compatibility and to unravel the mechanisms through which the microbiota modulates the desirable and deleterious effects of ICIs.

For FMT, selection of the donor and/or recipient requires careful consideration, including with regard to the emergence of viral infections.<sup>160</sup> Regarding the recipient, we need to develop technologies to rapidly (within 2 weeks) identify patients who already carry a favorable microbiota (and hence do not require FMT) as well as those affected by dysbiosis (and hence may benefit from FMT),<sup>15</sup> knowing that some bacterial species may interfere with FMT engraftment.<sup>160</sup> Regarding the donor, multiple microbial signatures are associated with a clinical response to ICIs, and the response may be context dependent and hence be influenced by the type of cancer. FMT from healthy donor(s) may establish a healthy ecological network and minimize or eliminate harmful bacteria in the recipient.<sup>30</sup> The composition of the optimal gut microbiota obtained from healthy donors remains to be elucidated. At this point, it is not clear whether “universal donors” exist or whether each recipient requires a different donor for the best possible outcome. At this level, the (considerable) cost of identifying suitable FMT donors is being evaluated in phase II clinical trials ([NCT04951583](#) and [NCT0498884](#)).<sup>161</sup> Moreover, whether all harmful microbiota need to be removed remains an open question, as long as restoration of a gut microbiota “balance” can be achieved in the recipient; nonetheless, we acknowledge that in immunocompromised individuals, transmission of any harmful microbiota can cause mortality, and exclusion of donor-derived drug-resistant bacteria carriage appears mandatory.<sup>162</sup> Moreover, cultivation of microbes in bioreactors may generate spontaneous mutations, and gene flipping may potentially change their function.<sup>163</sup>

To identify the best possible “lead strains” for live biotherapeutic product (LBP) drug development, ecological competence, compatibility, and dominance must be considered. Optimization of culture conditions may produce a stable, reproducible consortium with desired characteristics.<sup>164,165</sup> A number of consortium-level approaches have been or are currently under investigation ([NCT03817125](#), [NCT04208958](#), [NCT03686202](#), [NCT04988841](#), and [NCT04107168](#)). In metastatic melanoma resistant to PD-1 blockade, a Phase Ib clinical trial is being prepared for the multi-strain LBP MB097 that initially showed antitumor efficacy *in vitro* and *in vivo* (Robinson, Abstract P074, presented at AACR Virtual Special Conference 2021). Whether the LBPs generated with these approaches produce clinical responses in patients with cancer remains to be demonstrated.

Alternatively, monoclonal bacterial strains of high potency in conjunction with PD-1 and/or CTLA-4 blockade in solid tumors are currently being studied in Phase I/IIa trials. Two clinical studies in patients with metastatic renal cell carcinoma reported that CBM588, a strain of *Clostridium butyricum*, in combination with either nivolumab/ipilimumab or cabozantinib/nivolumab provided superior clinical benefits compared with nivolumab/ipilimumab or cabozantinib/nivolumab alone (Ebrahimi, Abstract LBA104, presented at ASCO 2023).<sup>166</sup> However, interestingly, the studies did not reach their primary endpoint of

increasing abundance of the probiotic bacteria. Given the small sample size of both studies, the results will require further validation in larger randomized trials. In addition, patients devoid of *Akkermansia* spp. are being treated with this LBP (NCT05865730) because avastar mice transferred with *Akkermansia* spp.-negative stools failed to respond to PD-1 blockade unless supplemented with oral *Akkermansia* spp.<sup>15</sup>

The role of antibiotic pretreatment for both FMT and LBP remains unclear, as well as the precise definitions of engraftment success or failure. Notably, antibiotics may increase engraftment but have been associated with reduced ICI efficacy in patients not undergoing microbiota modulation.<sup>167–169</sup> Even with engraftment success, certain factors can prevent response, including immune resistance due to tumor-intrinsic factors, lack of necessary taxa in the donor sample, and failure to mount an efficient immune response regardless of microbiota.<sup>170</sup> The many tests that need to be performed in a timely fashion represent a significant limitation to the development of FMT trials in immuno-oncology.

Careful clinical trial design for these interventions requires not only a clear definition of dysbiosis, but also demonstration that nonresponse or resistance to treatment is in fact due to dysbiosis. Appropriately, the primary outcome for initial studies is safety and engraftment. This would include alternate administration, e.g., single versus repeat dosing of microbiota modulation and design variations (e.g., classical FMT via colonoscopy vs. stool pills) to determine the optimal administration schedule. Second, randomized, large trials stratified by measures of dysbiosis will be needed. For example, patients who are nonresponders to ICIs due to dysbiosis could be randomized to microbiota modulation (via FMT, single LBP, or consortia LBP) with re-introduction of ICIs, or to the physician's choice treatment. One might anticipate that nonresponse due to low tumor mutational burden, low pre-existing T cell-mediated immune responses, or a cold TME may overlap with nonresponse due to dysbiosis.<sup>171,172</sup> Patients who are nonresponders for reasons other than dysbiosis should be treated according to physician's choice. Caution is needed, as FMT or LBPs will likely have consequences on the tumor microbiota that, to date, have not been well characterized. As an alternative design, investigators may consider targeting the treatment-naïve population, for instance randomizing ICI plus FMT versus ICI plus placebo in advanced melanoma (NCT04988841). Importantly, MCIs should be accompanied by robust pharmacodynamic studies (see Inflammation and “omics” section) to identify which physiological pillar or combination of pillars—immune, metabolic, epithelial barrier fitness, taxonomic, or functional—best correlate with clinical benefit for each MCI-based strategy.

Recent studies point to the potential utility of genetically engineered probiotics with a defined mode of action.<sup>173–175</sup> For example, an *E. coli* Nissle 1917 strain engineered to secrete immune checkpoint blockade nanobodies and cytokines can boost the antitumor responses in mouse models.<sup>176</sup> Moreover, *E. coli* Nissle 1917 can be programmed to target tumor microenvironments and deliver CAR antigens and enhance CAR-T cell tumor cell killing.<sup>177</sup> *E. coli* Nissle 1917 has also been engineered to generate higher levels of L-arginine, which metabolically reprograms TMEs and enhances the efficacy of ICI antitumor activity.<sup>45</sup> *Staphylococcus epidermidis* can be genetically manipulated to express tumor antigens and to prevent or treat cancers expressing such antigens, including melanoma.<sup>178</sup> However, whether this anti-melanoma effect obtained by skin painting with *S.*

*epidermidis* is mediated by local cutaneous or distant gastrointestinal effects is not yet clear. The identification of bacterial immunomodulatory factors may provide new opportunities for commensal or probiotic engineering. Thus, the peptidoglycan hydrolase SagA from *Enterococcus faecium* can be transferred into *Lactococcus lactis* to generate a probiotic strain that stimulates innate immune responses via NOD2 activation and promotes ICI antitumor activity in mice.<sup>26</sup> These examples highlight how engineered LBPs may improve ICI cancer therapy, if concerns about the safety of such genetically modified organisms (GMOs) can be solved. Importantly, additional genetic modifications (to modulate stress resistance, improve tissue/cell-type-specific targeting, and obtain inducible expression of transgenes) may improve the bioactivity and biocontainment of such GMOs. In this regard, advances in synthetic biology and CRISPR-Cas systems should offer novel opportunities for the optimization of probiotics.<sup>175,179</sup>

## MOVING THE FIELD FORWARD

Four major areas will help advance the field: standardization, technology development, collaborative resourcing, and fieldwide definitions. Standardization in sample collection, sample sharing, storage, extraction methods, sequencing (depth and instrumentation), analysis methods, and data storage is urgently needed. In turn, clinically relevant metadata and primary unannotated data, as well as structured, multi-omic data, need to be deposited in publicly available databases, so that bioinformatics capabilities can be pooled for large-scale meta-analyses. Accomplishing this is difficult and costly but necessary to properly inform guidelines and ensure reproducibility.<sup>180</sup> However, much can be learned from how cancer genomics accomplished a similar task with the TCGA and International Cancer Genome Consortium projects.

Technologically, efforts to improve sample biobanking, culturomics, microbial imaging (e.g., fate mapping/tracing), histology, and other systems that identify the presence of microbes, their function, and whether they are alive or dead will expedite progress. Technology will be key to move beyond descriptive science and into functional territory. Advances that facilitate data sharing across nations while ensuring data integrity and protection will also be essential for conducting meta-analyses. Developing preclinical model systems to determine the core principles underlying effective interventions and their mechanisms of action will expedite these efforts.

Collaboratively, researchers and clinicians need to engage other experts including biostatisticians, bioinformaticians, data managers and data scientists, and dieticians as part of the clinical trial design team. Mechanisms to retain contributions of trained individuals to the groups need to be identified. Support from nonprofits and foundations is needed to fund biobanking and cooperative group trials, increase sample sizes, and establish standard operating procedures for sample collection, storage, and data sharing. Sustained and coordinated efforts to define microbiota in relation to the disease, stage, treatment (e.g., longitudinal follow-up), and geography are mandatory. Additional challenges include the deployment of multi-omics, which is costly and may not be possible in large trials. Conversely, multi-omics in small cohorts is feasible, but sample size and statistical power can be challenging if validation cohorts and a collaborative spirit are not present. Continued

patient advocacy is critical to encourage patients to donate samples and participate in MCI trials, whether based on nutritional or microbial interventions.

Lastly, clear definitions are needed to explain new terminology in this field. Terms such as “responders/non-responders,” “dysbiosis,” “high-fiber diet,” “enterotypes/microbiotypes,” and “guilds” need to be unified across studies. As stated before, a surrogate marker of response to microbial therapy of cancer is needed and may practically be context dependent (e.g., tumor type, donor, treatment). Importantly, because a single therapy or introduction of a single microbial species or microbiota-related product is unlikely to work for every patient, biomarker stratification is critical to select the appropriate patient subset for the most suitable MCI at the right time of disease development (e.g., first-line treatment versus treatment when refractory to ICIs or CAR-T cell therapy). Steps toward translating biomarker discoveries to clinical utility will be paramount for success in this space. Development of model systems to determine the principles underlying effective interventions, and increased understanding of what mouse models tell us about human patients, are expected to expedite these efforts.

## CONCLUSIONS

The impact of the gut microbiota on melanoma systemic and cancer immunosurveillance is well established and will continue to have major clinical consequences. However, delineating the full functional repertoire (or metabolites) of gut, skin, urine, and intratumoral microbes; having accurate tests for gut dysbiosis; and rationally designing microbiota-centered interventions all represent significant challenges. Building international consortia, collaborations, and large, shareable databases will accelerate research and development on these topics. Collectively, a thorough understanding of melanoma microbiota has the potential to be harnessed to benefit patients worldwide.

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## DECLARATION OF INTERESTS

B.R. received grand funding from Davolterra and Kanvas and honorarium from Merck, BMS and AstraZeneca. He is also the co-founder of Curebiota. C.K.B. is an employee and stockholder of AbbVie. M.B. sits on the scientific advisory board of Micronoma. A.B. was an employee of Genentech and is a holder of Roche stock. N.C. is CSO of MaaT Pharma. D.D. is a consultant for: ACM Bio, Ascendis Pharma, Clinical Care Options, Gerson Lehrman Group, Merck, Medical Learning Group, Xilio Therapeutics; CE Speakers' Bureau: Castle Biosciences; and Intellectual Property: US Patent 63/124,231, "Compositions and Methods for Treating Cancer", Dec 11, 2020 and US Patent 63/208,719, "Compositions and Methods For Responsiveness to Immune Checkpoint Inhibitors (ICI), Increasing Effectiveness of ICI and Treating Cancer", June 9, 2021. L.D. sits on the scientific advisory board of EverImmune, holds patents covering the treatment of cancer and the therapeutic manipulation of the microbiota by LBP, and has been consulting for Ipsen and Sanofi. H.C.H. has filed a patent application (PCT/US2020/019038) for the commercial use of SagA-bacteria to improve checkpoint blockade immunotherapy, which is licensed by Rise Therapeutics to develop immunological-based biologics. M.I. is the founder and shareholder of Bio-Me. G.K. is a consultant for ReiThera; is on the Board of Directors of the Bristol Myers Squibb Foundation France; is a scientific co-founder of EverImmune Ltd., Osasuna Therapeutics, Samsara Therapeutics, and Therafast Bio; and is the inventor of patents covering therapeutic targeting of aging, cancer, cystic fibrosis, and metabolic disorders. G.K.'s brother, Romano Kroemer, was an employee of Sanofi and now consults for Boehringer-Ingelheim. F.M. reports honoraria from Therakos/Mallinckrodt, BMS, Sanofi, Jazz Pharmaceuticals, Gilead, Novartis, and Astellas, all outside the scope of this work. S.P. received travel reimbursement from CRISPR Therapeutics, and Ipsen. Z.R. is the founder of and a Scientific Advisor to Pangea Biomed. B.R. is the co-founder of Curebiota. G.D.S.-P. is an inventor on a US patent application (PCT/US2019/059647) submitted by The Regents of the University of California and licensed by Micronoma that covers methods of diagnosing and treating cancer using multi-domain microbial biomarkers in blood and cancer tissues; a founder of and reports stock interest in Micronoma; and has filed several additional US patent applications on cancer bacteriome and mycobiome diagnostics that are owned by The Regents of the University of California. R.F.S. reports consulting fees from Astellas, AstraZeneca, AVEO, BMS, EMD Serono, Editas, Exelixis, Gilead, Eisai, Janssen, Loxo, Lilly, Mirati, Pfizer, Silverback, and Seattle Genetics; research support (to institution) from Ascendis, ALX Oncology, Astellas, AstraZeneca, Bayer, BMS, CytomX, Eisai, Genentech/Roche, Gilead, Immunocore, Jounce, Loxo, Lilly, Merck, Moderna, Mirati, Novartis, Pfizer, Pionyr, Pyxis, Scholar Rock, QED Therapeutics; equity in AbbVie; and patents: Neo-antigens in Cancer, PCT/US2020/031357. M.v.d.B. has received stock options from Seres Therapeutics, Notch Therapeutics, and Pluto Therapeutics; royalties from Wolters Kluwer; consultant for and honorarium from or participated in advisory boards for Seres Therapeutics, Rheos Medicines, Ceramedix, Pluto Therapeutics, Thymofox, Garuda, Novartis (Spouse), Synthekine (Spouse), BeiGene (Spouse), and Kite (Spouse); IP Licensing with Seres Therapeutics and Juno Therapeutics; and holds a fiduciary role on the Foundation Board of DKMS (a nonprofit organization). R.K.W. is a consultant for Takeda Pharmaceuticals. H.Z. sits on the scientific advisory board of EverImmune and is a consultant for MaaT Pharma. L. Zhao is a co-founder of Notitia Bio-technologies Company. J.M. received honoraria from Roche, BMS, and Merck. L. Zitvogel is a co-founder of EverImmune; the President of EverImmune scientific advisory board; holds patents covering the treatment of cancer and the therapeutic manipulation of the microbiota by LBP; has held research contracts with bioMérieux, Daiichi Sankyo, Glaxo Smith Kline, Incyte, Lytx, Kaleido, PiLeJe, Transgene, 9 Meters, Tusk Pharma, Merus, Roche, and Innovate Pharma; is on the scientific advisory board of Hookipa; and was on the Board of Directors of Transgene.

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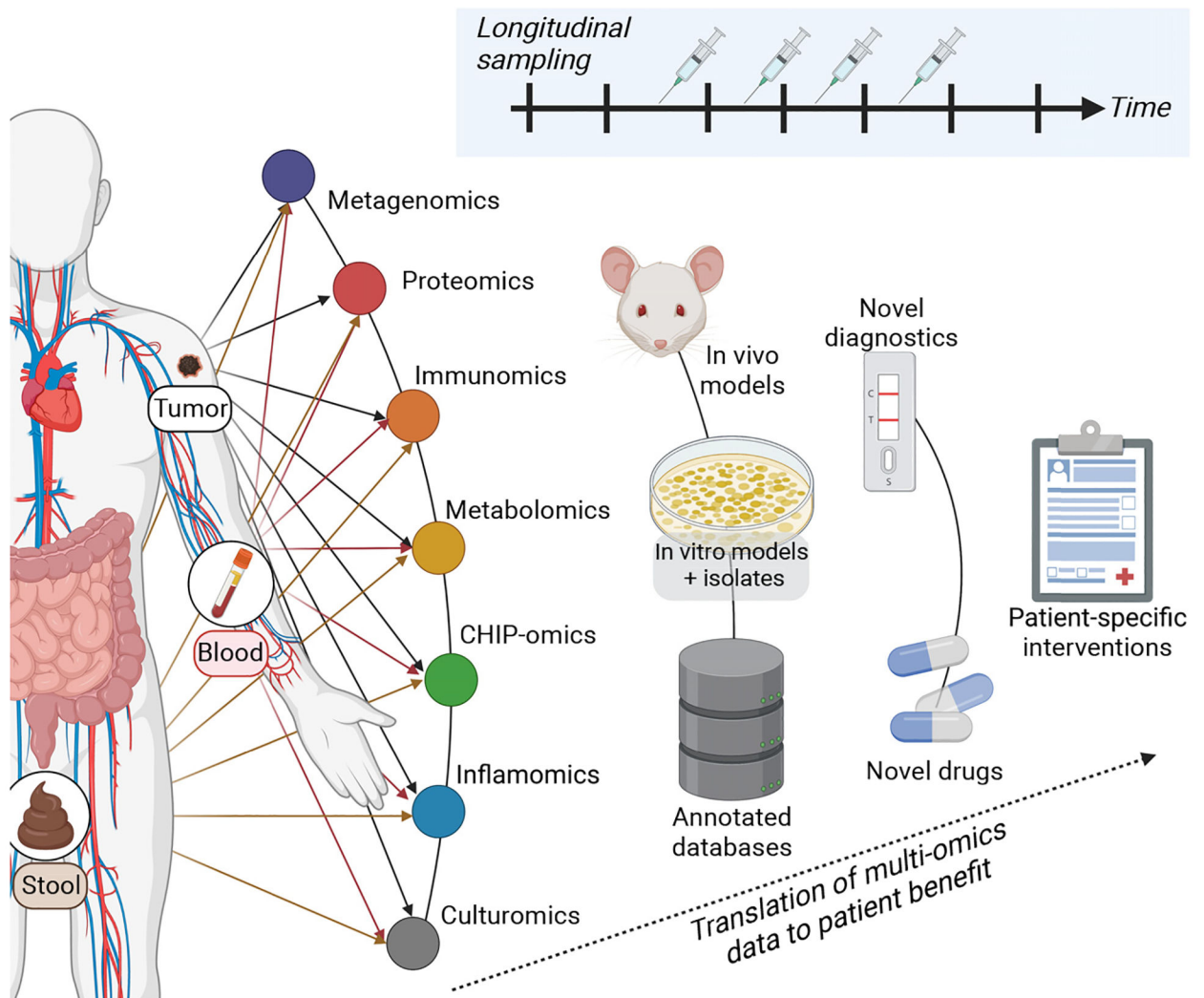


**Figure 1.**

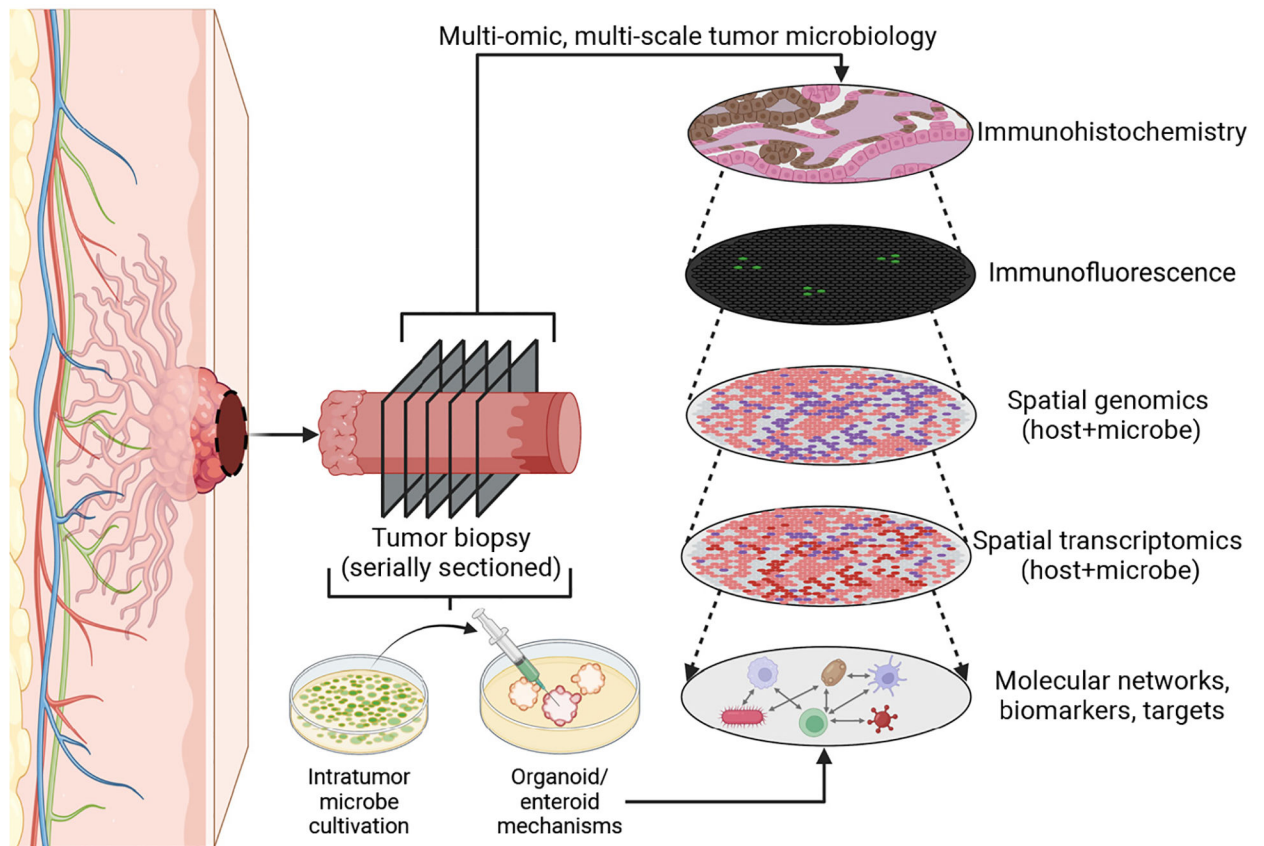
Potential mechanisms of the gut microbiota on innate and adaptive immunity

Intestinal microbes (center) act through at least three modes of action (on immunity, metabolism, and vascular, epithelial and/or endothelial barriers) involving host-microbe interactions, leading to exposure or secretion of various mediators that reshape the TME directly (after translocation to distal tumors) or indirectly via the intestinal tract. We have listed published mechanisms of action most often observed in preclinical mouse tumor models. The relevance of these mechanisms in human cancer immunotherapy still needs to be ascertained.

PV-1, marker of disrupted gut vascular barrier; MAdCAM-1, Mucosal vascular addressin cell adhesion molecule 1; RGMB: repulsive guidance molecule b



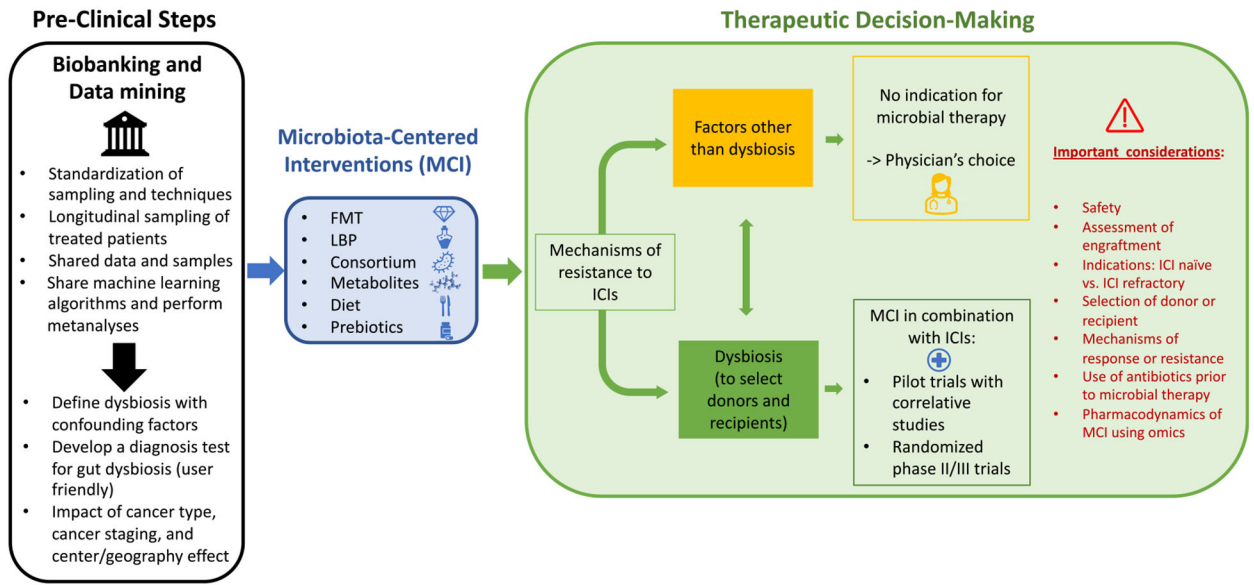
**Figure 2.** Multi-omic collections for advancing microbiota science in melanoma  
 Diverse specimen types (e.g., tumor, blood, stool, and saliva) are densely and longitudinally collected before, during, and after treatment, followed by multi-omic data generation and analyses. These data are systematically annotated and deposited into secure, shareable databases and serve as the foundation for subsequent *in vitro* and *in vivo* studies, which in turn guide applications for novel diagnostics, prognostics, and therapeutics. Eventually, after clinical trials, these tests and interventions provide rationale for patient-specific interventions and stratifications.



**Figure 3.**

**Systematic, multi-scale tumor microbiology**

Fresh tumor biopsies are serially sectioned with contamination controls (e.g., operating room environment). Gathered tissue sections are then subjected to imaging and sequencing approaches, which are then combined to determine molecular networks, biomarkers, and putative drug targets. In parallel, microbial cultivation is performed using freshly resected tissue specimens, and the resultant isolates can be injected into organoids and/or enteroids to validate mechanistic hypotheses generated from the multi-scale, multi-omic data.



**Figure 4.**  
Microbiota-centered interventions in cancer

Accumulating evidence over the last decade has enabled deconvolution of the taxonomic composition of stools from patients with cancer (at least in distinct malignancies), enabling better definitions of gut dysbiosis and identification of patterns associated with resistance to immunotherapy, independent of PD-L1 tumor expression. Coordinated efforts in biobanking and translational research across investigators, centers, and continents will allow expansion of this progress to other malignancies for better stratification of patient cohorts and personalization of microbiota-centered interventions.

LBP, liver biotherapeutic product; FMT, fecal microbiota transplant.