Understanding the relationship between breast cancer, immune checkpoint inhibitors, and gut microbiota: a narrative review

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Contributions: (I) Conception and design: TL Ng, J Leigh; (II) Administrative support: None; (III) Provision of study material or patients: None; (IV) Collection and assembly of data: G Hrubesz, J Leigh; (V) Data analysis and interpretation: G Hrubesz, J Leigh; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

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Background and Objective: The composition of gut microbiota plays an important role in predicting and influencing outcomes of cancer treated with immunotherapy. Our objective is to summarize the role of gut microbiota and immunotherapy in breast cancer.

Methods: A systematic search from inception until July 2024 of key search terms including immunity, breast neoplasm, gastrointestinal microbiome/microbiota, fecal microbiota transplantation, pro- and prebiotics, antibiotics and immunotherapy using EMBASE, MEDLINE and CENTRAL was conducted. The results were screened by two reviewers independently and synthesized and presented descriptively.

Key Content and Findings: Thirteen studies (5 clinical, 8 pre-clinical) met the eligibility criteria and were published from 2020–2024. Clinical studies showed that the composition and diversity of gut microbiota was associated with patient response to immunotherapy. In pre-clinical studies, dysbiotic states induced by obesity, antibiotics, and diet were associated with immunosuppression and influenced response to programmed cell death-ligand 1 (PD-L1) inhibitors. Microbiota-modulating treatments such as probiotics showed the ability to enhance response to immunotherapy, indicating their potential use as adjunct therapies in breast cancer treatment.

Conclusions: The composition of gut microbiota could help predict the chance of response to immunotherapy, and modulating gut microbiota has the potential to enhance the efficacy of chemo-immunotherapy in breast cancer. However, the available data relating to breast cancer are limited. Larger prospective studies are required to further elucidate their role as a biomarker and treatment.

Keywords: Gut microbiota; dysbiosis; breast cancer; immunotherapy

Introduction

Breast cancer is the most commonly occurring cancer and leading cause of cancer-related death for females worldwide (1). Although population-based mammography screening has led to earlier detection of newly diagnosed breast cancers and an increasing chance of cure (2,3), there is still a huge unmet need for novel therapeutics against breast cancer to improve outcomes for patients. One recent change in the treatment of breast cancer has been the incorporation of immunotherapy.

Immunotherapy has become a major pillar of cancer therapy and has been successfully implemented in many types of solid and liquid tumors. They can range from technologies like vaccines and oncolytic viruses, which are relevant in antigen presentation and cancer immune activation (2,3), to monoclonal and bispecific antibodies that modulate inhibitory and stimulatory immune check points (4,5), to gene or cell therapies that involve

customization of chimeric antigen receptor (CAR) T cells or *ex-vivo* expansion of desired immune cell products (adoptive T cells) or molecules (e.g., cytokines) relevant in anticancer activity (6-8). However, the most used type of immunotherapy for solid tumor treatment are immune checkpoint inhibitors (ICIs), humanized monoclonal antibodies that target various cellular immune checkpoints and their ligands at the tumor-immune interface. Cells acquire cancerous properties through genetic and epigenetic modifications that allow them to proliferate uncontrollably and invade surrounding tissues (9,10). The term cancer immunosurveillance refers to the coordinated response of innate and adaptive immunity against cancer. Immune checkpoints are a potential rate-limiting step in enabling T cell infiltration and tumor cell kill. Under physiologic conditions, the immune system maintains an equilibrium between its ability to clear foreign pathogens and its capacity for immune regulation. For example, programmed cell death 1 (PD-1), which is expressed on the surface of many immune cells, acts as a molecular "brake" to prevent T cells from destroying healthy cells (11,12). When T cells infiltrate the tumor microenvironment, tumor cells evade "detection" by these infiltrating lymphocytes by overexpressing inhibitory checkpoint ligands like PD-L1, which when bound to its receptor PD-1, sends inhibitory signals to PD-1 expressing cells (e.g., CD8+ T cells) and prevent an anti-tumor response (9-13). Other clinically approved ICIs target cytotoxic T lymphocyte antigen 4 (CTLA-4), which is important earlier on in the adaptive immune cascade when the requisite antigen-mediated lymphocyte activation occurs prior to CD4+ helper and CD8+ T cell clonal expansion and migration (14,15). The currently approved agents aim to wake up the immune system by blocking the interaction between PD-L1, PD-1 and anti-CTLA-4 with their ligand or receptors (9,11,16).

For breast cancer, ICIs are only approved for the treatment of triple negative breast cancer (TNBC). TNBCs comprise approximately 15% of newly diagnosed breast cancers (17-21). Pembrolizumab has been approved in the neoadjuvant setting when it is given in combination with chemotherapy for ≥ cT2 and/or ≥ cN1 (stage I–III except pT1pN0) TNBC. It has also been approved for first-line treatment of metastatic TNBC in those with a PD-L1 score ≥10% (22-24).

While ICIs can be effective, response rates only range from 15% to 30% in most solid tumors (25-28). A recent meta-analysis of immunotherapy in metastatic breast cancer (mBC) found an overall response rate (ORR) of 19% to ICIs (29). Therefore, considering the costs of treatment-related toxicity and financial toxicity, an important avenue of research is the role of biomarkers, including the tumor- and host-related factors that predict the chance of patient response to ICIs. This continues to be an avenue of exploration to maximize clinical benefit for the patient population while sparing unnecessary toxicity (25,30,31).

Beyond PD-L1 expression score as a predictive biomarker of response in metastatic TNBC (32-35), other biomarkers such as tumor-infiltrating lymphocytes (TILs) and T cell receptor (TCR) diversity have also been explored as predictors of ICI efficacy in breast cancer (18,36-39). However, the accuracy and reliability of these biomarkers have been inconsistent in practice, limiting their clinical utility (24-26). Thus, more research is needed to identify practical tumor- and patient-specific biomarkers.

Patient factors including the diversity of the gut microbiome can influence responses to ICI therapy (40,41). The gut microbiota consists of the various microorganisms found within the digestive tract, including bacteria, viruses, fungi and protozoa. Technically, the gut microbiome refers to the metagenome of these organisms, but is often used interchangeably with microbiota as it is referring to the taxonomic classification of microbial species and overall composition (38). Gut microbiota have been linked directly to many humans diseases, including cancer (42-45). Importantly, it can influence cancer development and progression, and it can also influence response to cancer treatment (46-48). For example, gut microbiota can stimulate antitumor immune responses through the regulation of CD8+ T cells and tumor-associated myeloid cells (49-51). Additionally, reduction of gut microbiota diversity through the use of antibiotics has been shown to reduce response to cancer immunotherapy (52-54). Correlations between the gut microbiome and efficacy of immunotherapy have been studied in various cancer types, including lung cancer, prostate cancer and melanoma (55-57).

In breast cancer, gut microbiota have been shown to influence response to systemic therapies including chemotherapy and anti-human epidermal growth factor receptor 2 (HER2)-targeted therapies (49,58-60). For example, increased alpha diversity, which is a measure of microbiota diversity in an individual sample, has been shown to predict responsiveness to the anti-HER2 monoclonal antibody trastuzumab in HER2+ breast cancer (61,62). Additionally, patients who did not respond to trastuzumab showed lower alpha diversity and higher abundance of

Table 1 Search strategy summary

Items	Specification
Date of search	June 4, 2024
Databases and other sources searched	EMBASE, MEDLINE, CENTRAL
Search terms used	Innate immunity, adaptive immunity, monocytes, macrophages, myeloid-derived suppressor cells, T-lymphocytes, immune checkpoint inhibitors, immunotherapy, breast neoplasms, gastrointestinal microbiome/microbiota, fecal microbiota transplantation, probiotics, prebiotics, antibacterial agents, proton pump inhibitors, hydrocortisone
Timeframe	Inception to June 4, 2024
Inclusion criteria	Population: clinical studies with adult males or females with confirmed breast cancer of any stage, animal models of breast cancer and/or *in vitro* models of breast cancer treated with immunotherapy
	Concept: the relationship between gut microbiota compositions and response to immunotherapy and/or overall outcomes
	Context: original articles and abstracts in English language, any country of origin
Exclusion criteria	Studies with <5 patients excluded
Selection process	Dual reviewer selection based on title and abstract and full text screening; single reviewer full text analysis

certain species of *Bacteroides* (62,63). Another study by Li *et al.* also showed higher alpha diversity being associated with response to neoadjuvant breast cancer chemotherapy (64). Other studies have shown that the relative abundance of specific gut microbiota taxa or consortia is also associated with chemotherapy response in breast cancer (64,65). The role of gut microbiota in modulating the efficacy of immunotherapy, specifically, ICIs, in breast cancer, however, is less well characterized.

Given the increased recognition of the role that the gut microbiome plays in ICI response, our objective is to summarize current data on the link between gut microbiota and ICI efficacy in breast cancer. Specifically, we discuss the role of gut microbiota signatures and dysbiosis in predicting ICI response and the use of novel microbiota-modulating treatments to enhance response to ICI therapy in breast cancer. We present this article in accordance with the Narrative Review reporting checklist (available at https://tbcr.amegroups.org/article/view/10.21037/tbcr-24-14/rc).

Methods

We used EMBASE, MEDLINE, and CENTRAL databases to conduct a search using the following terms: immunity (innate and adaptive), immune checkpoint inhibitors, immunotherapy, breast neoplasms, gastrointestinal microbiome/microbiota, fecal microbiota transplantation, pre- and probiotics and antibiotics. We reviewed all studies published until June 4, 2024. The search strategy is summarized in *Table 1* and the full search strategy is available in Appendix 1.

Our initial search strategy of the relevant databases yielded 311 citations. However, after a subsequent expansion of our search strategy, an additional 1,644 publications were found. These articles were screened by two reviewers (G.H., J.L.), using title and abstract, according to the criteria defined in *Table 1*. Studies that did not meet inclusion criteria were excluded, including duplicate articles. This resulted in 137 articles eligible for full text screening conducted by two reviewers (G.H., J.L.). Review articles, ongoing clinical trials without published results and studies not including immunotherapy were removed. However, the reference lists of review articles were searched for potentially relevant studies. In total, there were 16 abstracts and articles that met all inclusion criteria. These were critically appraised, and data were extracted by a single reviewer (G.H.).

Results

Based on our systematic search strategy, two-stage citation screening, and after an updated manual search of the

literature on July 6, 2024, a total of 16 abstracts and articles met the inclusion criteria—13 studies were available as published conference abstracts only and 3 studies were available as full-text peer-reviewed manuscripts. Some of these abstracts were presenting updated data to an ongoing study. In total, there were 13 unique clinical and pre-clinical studies that were included in our review. These studies were published between 2020–2024, and originated from research groups in the United States, United Kingdom, Italy, China and the Republic of Korea.

Of the 13 eligible studies, 5 (38%) were clinical trials (n=202), of which 4 studies reported microbiome-related outcomes as a post-hoc exploratory endpoint and one trial had pre-planned sample collection, but the analysis was also done retrospectively (*Table 2*). Stool samples were not routinely collected in all study participants and the breast cancer subtypes enrolled in those trials were heterogeneous. To summarize, two clinical trials (n=70) included estrogen-receptor positive (ER+) breast cancer only, one trial (n=10) included TNBC only and two trials included a mix of TNBC and ER+ breast cancer (n=88). The remaining 8 (62%) studies were conducted in the pre-clinical setting and were predominantly based on mouse models of breast cancer (*Table 3*). Four studies used TNBC mouse models (n=40), one study looked at ER+ breast cancer and the two remaining studies examined HER2+ breast cancer (n=15) and an ER+, PR+ breast cancer mouse model (n=39), respectively. In terms of scope, two of the studies addressed the impact of dysbiosis on immunotherapy efficacy in breast cancer mouse models, four studies assessed the use of novel microbiota agents to increase immunotherapy efficacy *in vitro* and in a mouse model, and two studies explored the role of diet and obesity in immunotherapy efficacy in a mouse model.

Discussion

Given that the incorporation of immunotherapy into the treatment of breast cancer is relatively new, there is a limited understanding as to which biomarkers predict response outside of PD-L1. Several studies in other disease sites such as melanoma lung, liver, gastric and prostate, have identified the potential use of gut microbiome signatures to predict patient response to immunotherapy (57,81-89). We sought to better characterize the role of gut microbiome signatures for immunotherapy in breast cancer.

Gut microbiome signatures as predictors of immunotherapy response in ER+, HER2– breast cancer

Three studies addressed the relation of gut microbiome signatures and response to immunotherapy in ER+, HER2– mBC.

Barroso de Sousa *et al.*, reported on the gut microbiome correlates of a previously completed randomized phase II trial (NCT03051659) of eribulin monotherapy (E) *vs.* eribulin plus PD-L1 inhibitor pembrolizumab (E+P) in pre-treated ER+ mBC (66). A subset (eribulin: n=11; eribulin plus pembrolizumab: n=12) of these patients had fecal samples collected at baseline (BL), post two cycles (C2), and at end of treatment (EOT). Among the 23 patients (23 BL, 22 C2, 5 EOT), subjects with progression-free survival (PFS) and overall survival (OS) below the median had comparable alpha-diversity at baseline to those above the median in both treatment groups. In this initial analysis, which used 16S(v3-4) RNA, the findings were limited by a small sample size and limited depth of strain evaluation. Nonetheless, they descriptively reported an increase in *Faecalibacterium* from baseline amongst patients who had a partial response (PR) to eribulin while levels remained unchanged in those who had stable disease (SD). The gut microbiome composition within this small subgroup with available fecal samples was possibly prognostic for a patient's survival outcomes but did not seem predictive of response to eribulin or pembrolizumab. The authors subsequently performed metagenomic shotgun sequencing on baseline fecal samples that were collected from 26 patients prior to randomization for more in-depth microbiome coverage and strain elucidation. With this, they found that higher alpha diversity was associated with longer OS across all patients and in the E+P subgroup. This was not the case in the eribulin monotherapy arm (67). At the taxonomic level, longer OS was associated with baseline lower relative abundance of *Blautia wexlerea* and higher relative abundance of *Odoribacter splanchnicus*, a common short-chain fatty acid producing gut bacterium, even after controlling for age, ECOG status and prior lines of therapy. Overall, this suggests that the gut microbiome could be of prognostic value whereas the predictive value in relation to treatment-specific response and outcomes require further study (66,67).

The single-arm phase II KELLY trial (NCT03222856) similarly tested the safety and efficacy of eribulin plus pembrolizumab in patients with ER+, HER2– unresectable

Table 2 Summary of clinical studies

Author	Year	Country	Publication type	Study design	Clinical trial number	BC type	No of patients	Interventions*	Microbiome sample source and analysis	Outcome measures
Barroso-Sousa et al. (66,67)	2023 & 2020	United States	Abstract	Interventional (post-hoc analysis)	NCT03051659	ER+	26	Group 1 (n=15): eribulin + pembrolizumab; Group 2 (n=11): eribulin	Sample: fecal; Analysis: 16S RNA sequencing + metagenomic shotgun sequencing; Database: MetaPhlAn3	Association between microbiome diversity, taxonomic composition and clinical outcomes; PR, CR; SD, PD; OS
Teng et al. (68)	2022	United Kingdom	Article	Interventional (post-hoc analysis)	KELLY trial NCT03222856	ER+ HER2−	44	Pembrolizumab + eribulin	Sample: fecal; Analysis: 16S RNA sequencing + metagenomic shotgun sequencing; Database: LeFSe, MEGAHIT	Safety and efficacy of chemotherapy with ICI; microbiome composition; CB; SD; drug-related microbial toxicity
Wong et al. (69); Yuan et al. (70)	2021	United States	Abstract	Interventional (post-hoc analysis)	(I) NCT02971761; (II) NCT02778685	(I) TNBC; (II) ER+	(I) 12; (II) 10	(I) Pembrolizumab + enobosarm; (II) Palbociclib + pembrolizumab + letrozole[5]	Sample: fecal; Analysis: 16S RNA sequencing + metagenomic sequencing; Database: Illumin MiSeq	Association of microbiome with response to triple therapy; microbiome composition; PR, CR; SD, PD
Chun et al. (71)	2023	United States	Abstract	Interventional (pre-planned retrospective analysis)	AMTEC trial NCT03801369	TNBC	10	Olaparib + durvalumab	Sample: fecal; Analysis: 16S RNA sequencing	Correlation of gut microbiome diversity with tumor biomarkers (PD-L1, tumor immune cell density, TILs, MutSig3 and IFN gene signatures; clinical response to therapy
Kulkarni et al. (72)	2024	United States	Article	Interventional (post-hoc analysis)	I-SPY2 trial NCT01042379	TNBC, ER+, HER2−	66	Pembrolizumab + paclitaxel + doxorubicin + cyclophosphamide +/− antibiotic use during treatment	–	Residual cancer burden; pathologic complete response rates

*, eribulin = chemotherapy (microtubule dynamics inhibitor); pembrolizumab = ICI (PD-1 inhibitor); enobosarm = hormone therapy (non-steroidal selective androgen receptor modulator); palbociclib = targeted therapy (CKD4/6 inhibitor); letrozole = hormone therapy (aromatase inhibitor); olaparib = targeted therapy (PARP inhibitor); durvalumab = ICI (PD-L1 inhibitor); paclitaxel = chemotherapy (mitotic inhibitor); doxorubicin = chemotherapy (anthracycline); cyclophosphamide (alkylating agent). BC, breast cancer; ER+, estrogen-receptor-positive; PR, partial response; CR, complete response; SD, stable disease; PD, progressive disease; OS, overall survival; HER2, human epidermal growth factor receptor 2; ICI, immune checkpoint inhibitor; CB, clinical benefit; TNBC, triple-negative breast cancer; PD-L1, programmed cell death-ligand 1; TIL, tumor-infiltrating lymphocyte; IFN, interferon; PD-1, programmed cell death 1.

Table 3 Summary of pre-clinical studies

Author	Year	Country	Publication type	Study design	BC model	BC type	Number of subjects	Interventions	Microbiome sample source and analysis	Outcome measures
Rosean et al. (73)	2020	United States	Abstract	Interventional	Mice	HR+	Not reported	Intervention: anti-PD-1 Intervention: PGE2 inhibitor	–	Efficacy of anti-PD-1 therapy when combined with PGE2 inhibitors in dysbiosis
Pingili et al. (74)	2021	United States	Article	Interventional	Mice	ER+, PR+	39	Group 1 (n =12): high-fat diet + anti-PD-1 Group 2 (n=11): high-fat diet + IgG control Group 3 (n=9): low-fat diet + anti-PD-1 Group 4 (n=7): low-fat diet + IgG control	Sample: fecal Analysis: 16S RNA sequencing Database: GreenGenes	Composition of the microbiome; tumor incidence; tumor progression; response to ICI
Bohm et al. (75)	2023	United States	Abstract	Interventional	Mice	TNBC	Not reported	Group 1: obese on high fat diet Group 2: formerly obese following surgical weight loss Intervention: antibiotic therapy followed by FMT Intervention: anti-PD-1 immunotherapy or IgG control	Sample: cecal contents Analysis: 16S RNA sequencing	Tumor burden; tumor progression; immunotherapy response
Clear et al. (76)	2022	United States	Abstract	Interventional	Mice	TNBC	EMT-6: n=5–7/ group; E0771: n=8–10/group	Group 1: low-fat control diet Group 2: high-fat Western diet Group 3: mediterranean diet Intervention 1: IgG or anti-PD-L1 antibodies Intervention 2: FMT	Sample: fecal Analysis: metagenomic sequencing	Impact of diet on ICI response; tumor volume; tumor weight; ICI responsiveness

Table 3 (*continued*)

Table 3 (*continued*)

Author	Year	Country	Publication type	Study design	BC model	BC type	Number of subjects	Interventions	Microbiome sample source and analysis	Outcome measures
Clear et al. (77)	2024	United States	Abstract	Interventional	Mice	TNBC	Unknown	Intervention 1: IgG or anti-PD-L1 or anti-PD-1 antibodies Intervention 2: A. muciniphila or short-chain fatty acid supplementation	–	Tumor progression; tumor volume; tumor cell populations
Ferrari et al. (78)	2021	Italy	Abstract	Interventional	Mice, human cell lines	HER2+, IDC	15	Group 1: microbial derivatives + anti-PD-1 Group 2: microbial derivatives + vehicle control	–	HLA expression; activation and cytotoxic levels of CTLs; response to ICI
Kim et al. (79)	2021 & 2022	Republic of Korea	Abstract	Interventional	Mice	TNBC	Not reported	Group 1: tumor control Group 2: anti-PD-1 Group 3: RAPO Group 4: anti-PD-1 + RAPO	Sample: fecal Analysis: 16S RNA sequencing	Tumor volume; PD-L1 IHC score; PD-L1 gene expression; proportion of M2 macrophages and mRNA expression; response to ICI; pro- and anti-inflammatory cytokine expression
Li et al. (80)	2024	China	Article	Interventional	Human cell lines; mice	TNBC	40	Group 1 (n=8): control (no tumor) Group 2 (n=8): PBS treatment Group 3 (n=8): anti-PD-1 antibody Group 4 (n=8): fucoidan Group 5 (n=8): fucoidan + anti-PD-1 antibody	Sample: fecal Analysis: 16S RNA sequencing	Efficacy of anti-PD-1 therapy when combined with fucoidan

BC, breast cancer; HR+, hormone-receptor-positive; PD-1, programmed cell death 1; PGE2, prostaglandin E2; ER+, estrogen-receptor-positive; PR+, progesterone-receptor-positive; ICI, immune checkpoint inhibitor; TNBC, triple-negative breast cancer; FMT, fecal microbiota transplantation; PD-L1, programmed cell death-ligand 1; HER2, human epidermal growth factor receptor 2; IDC, invasive ductal carcinoma; HLA, human leukocyte antigen; CTL, cytotoxic T-lymphocyte; RAPO, Bifidobacterium longum RAPO; IHC, immunohistochemistry; PBS, phosphate-buffered saline.

BC or mBC (68). In the subsequent CALADRIO sub study, Teng *et al.* analyzed both fecal and saliva samples collected from 44 of these patients at baseline, post three cycles of treatment, and at EOT. They found that treatment with eribulin plus pembrolizumab did not cause significant oral or gut microbiota changes from baseline. However, clinical benefit was associated with an abundance of gut-associated *Bacteriodes fragilis* and oral *Streptococcus* and increased gut microbiota richness at baseline (68).

Preliminary results from 16SRNA analysis of fecal samples collected from 10 ER+, HER2– mBC patients during a phase I/II clinical trial testing the safety and efficacy of the combination of letrozole, palbociclib and pembrolizumab (NCT02778685) found that patients with PR to treatment had 'healthy' gut signatures dominated by *Bacteroides* and short chain fatty acid-producing *Firmicutes* (69). In a subsequent analysis of samples from the same clinical trial, metagenomic sequencing was performed on fecal samples from 11 patients collected at baseline and during treatment, and found that a higher relative abundance of *Gemmiger formicillis* was associated with response to therapy whereas an increase in relative abundance of *Bacteroides vulgatus* was associated with resistance to therapy (69).

Gut microbiome signatures as predictors of ICI response in metastatic TNBC

Currently, TNBC is the only subtype of breast cancer with approved indications (neoadjuvant chemo-immunotherapy for stage II/III TNBC and first-line unresectable/metastatic TNBC) for using ICI as part of standard care treatment. There are two clinical studies that explored gut microbiome signatures and response to immunotherapy.

Chun *et al.*, performed a correlative analysis of gut microbiome on patients with metastatic TNBC with baseline stool sample and tissue biopsy collected (n=10) prior to starting olaparib monotherapy for a 28-day cycle, followed by on-treatment stool collection and on-treatment tissue biopsy (paired pre-treatment and on-treatment; n=8) before adding in durvalumab in cycle 2 (NCT03801369) (71). Using 16S RNA analysis, they reported that the average baseline alpha diversity did not vary with treatment response. However, the average alpha diversity on-treatment after one cycle of olaparib monotherapy was higher in patients who subsequently derived clinical benefit (PR/SD) from the olaparib and durvalumab combination. This suggests that fecal microbiome alpha diversity prior to starting

durvalumab, a PD-L1 inhibitor, may predict likelihood of response to the combination. On-treatment microbiome alpha diversity was inversely related with tumor PD-L1 expression (P=NS) and was positively associated with high TILs in the paired on-treatment biopsy. However, there was no association between alpha diversity and immune cell density by multiplex IHC, inflammatory signature, IFN gene signature, or MutSig3 score (71).

In an exploratory analysis, gut microbiome diversity was assessed in patients with androgen receptor positive (AR+) metastatic TNBC who were treated with pembrolizumab and a selective androgen receptor modulator, Enobosarm in a phase II clinical trial (70). They found that patients who had disease progression had dysbiotic gut microbiomes and displayed a relative abundance of *Prevotella copri*, an organism associated with inflammation. Patients who had PR to treatment had relatively healthy gut microbiota dominated by *Bacteroides*, *Faecalibacterium prausnitzii* and other short chain fatty acid producers (70).

Taken together, these studies show the potential of using gut microbiome signatures to predict response to ICI therapy in TNBC. Positive responses to immunotherapy appear to be associated with increased alpha-diversity of the gut microbiome prior to starting immunotherapy. Additionally, abundance of certain microbiota, such as *Bacteroides*, may be associated with improved survival in patients, while microbiota such as *Prevotella* could be associated with early disease progression and should be further explored.

Metabolic and dietary factors related to gut dysbiosis and ICI efficacy

Gut dysbiosis, characterized by a disruption to the microbiome relative to that found in a healthy individual and resulting in changes in their functional composition and metabolic activities, has been shown to influence response to immunotherapy in patients with cancer (46,90). Gut dysbiosis can be induced or influenced by extrinsic interventions like antibiotics, medications, and diet but also intrinsic, patient-specific factors like body composition and systemic metabolism (91,92). Five pre-clinical studies and one clinical study investigated the impact of gut dysbiosis on breast cancer immunotherapy.

Rosean *et al.* previously showed that meloxicam combined with PD-L1 blockade was effective in an endocrine-resistant ER+ breast tumor model by targeting prostaglandin E2 (PGE2) production via COX-2

inhibition (93). This mechanism is important since PGE2 is a mediator of systemic immune suppression in mBC. However, this therapeutic combination was ineffective in mice with antibiotic-induced gut dysbiosis. They found that mice with commensal dysbiosis had more recruitment of suppressive myeloid populations and irreversible T cell dysfunction (73). Importantly, breast glands from dysbiotic mice showed enhanced COX-1 activity, and since COX-1 also regulates PGE2 synthesis, this suggests a secondary mechanism for PGE2 synthesis and resistance to meloxicam therapy. Supporting this hypothesis, targeting COX-1 mediated PGE2 synthesis resulted in decreased tumor burden only in dysbiotic mice. These findings support that dysbiosis-induced COX-1 mediated tissue inflammation is sufficient to drive immune suppression and treatment resistance (73,93).

Obesity is another known factor that modifies the gut microbiome and induces gut dysbiosis (94,95). Obesity has also been associated with increased risk of early recurrence in patients with ER+ disease (96). Pingili *et al.* investigated the role obesity plays in ICI response in a syngeneic TNBC mouse model using E0771 cells (74). They found that high-fat diet induced obesity led to greater immunosuppression and tumor growth. However, anti-PD-1 therapy was able to induce tumor regression not only in lean mice but also in obese mice by downregulating immune suppression and upregulating antitumor immunity. In obese mice, the presence of breast tumor was associated with decreased relative abundance of *Ruminococcus* and an increased relative abundance of *Bacteriodales* whereas treatment with anti-PD1 reduced the abundance of *Enterobacteriaceae* and *Bacteriodales* while it led to an increase in *Akkermansia*, *Bifidobacterium*, *Lactobacillus*, *Aidercreutzia*, *Odoribacter*, *Mogibacteriaceae*, and *Ruminococcus* (74).

In addition, Bohm *et al.* investigated the role of bariatric surgery in modulating the gut microbiome and its impact on anti-PD-1 immunotherapy in a TNBC mouse model (75). The commensal microbiota of study mice were ablated with broad spectrum antibiotics prior to fecal microbial transplant (FMT) using cecal contents from obese mice on high fat diet or from formerly obese mice following surgical weight loss. Results showed that FMT from weight loss donor mice was associated with reduced tumor burden and improved anti-PD-1 immunotherapy response. FMT from weight loss donors resulted in elevated infiltration of CD8+ T cells into the tumor microenvironment. Additionally, microbes from mice with weight loss surgery combined with anti-PD-1 treatment decreased circulating

levels of lithocholic acid, a secondary bile acid that drives immunosuppression (75). Thus, gut microbiota can improve response to anti-PD-1 therapy in breast cancer after bariatric surgery.

Diet is another factor known to be important in microbiome modulation. Clear *et al.* investigated the role of diet in potentiating immunotherapy response in a syngeneic mouse model using EMT-6 and EO771 (TNBC) bearing mice (76). They found that both mice fed with either a high-fat Western diet or Mediterranean diet experienced a significant reduction in tumor volume and weight when treated with PD-L1 inhibitor compared to mice fed with a low-fat control diet. Additionally, Western and Mediterranean-fed mice displayed a 25–45% increase in proportional abundance of gut *Akkermensia muciniphila*, which degrades mucin to produce short-chain fatty acid metabolites. Furthermore, fecal microbiota transplant enriched with *Akkermensia muciniphila*, when given in conjunction with PD-L1 therapy, enhanced responsiveness to PD-L1 blockade. In a later study, Clear *et al.* investigated if *Akkermensia muciniphila* or mucin short-chain fatty acid supplementation could directly enhance ICI therapy in EO771 tumor-bearing mice (77). Results showed that mice supplemented with *Akkermensia muciniphila* demonstrated a significant reduction in tumor volume and increased levels of immune cell populations in the tumor microenvironment. Supplementation with short-chain fatty acids and anti-PD-1 with chemotherapy also showed enhanced response to treatment. These results suggested that relative abundance of *Akkermensia muciniphila* could potentially predict, or even enhance ICI efficacy in breast cancer, as has been associated with anti-PD-1 response in other cancer models (88). Overall, the results of these studies indicate that dysbiosis, which can be induced through multiple factors including antibiotics, diet and metabolic processes such as obesity, can influence the efficacy of immunotherapy in breast cancer.

Antibiotics can also play a role in inducing gut dysbiosis and have been shown to impact ICI efficacy in cancer treatment (97-100). Kulkarni *et al.* investigated the impact of antibiotic exposure on residual cancer burden in HER-negative early-stage breast cancer patients treated with pembrolizumab as part of the I-SPY2 trial (72). Overall, the results showed that antibiotic use during immunotherapy treatment was associated with higher mean residual cancer burden and lower pathologic complete response rates. This data suggests that antibiotic treatment may negatively impact ICI treatment outcomes in early-stage breast cancer, possibly by changing gut microbiome composition (72).

Use of microbiota agents to increase ICI efficacy

Given the known interplay between the microbiome and systemic treatment response, there has been an interest in identifying microbial interventions that could enhance this treatment effect. One particular agent of interest has been the use of probiotics (101,102). Ferrari *et al.* tested two proprietary microbial derivatives on murine and human primary immortalized cells lines, including those from breast cancer, and found that *in vitro* treatment with microbial derivatives led to human leukocyte antigen (HLA) upregulation (78). Concordantly, this led to microbial derivative-dependent increase in cytotoxic T-lymphocyte (CTL) tumor cell recognition. In 4T1 (TNBC)-bearing Balb/c mice, intraperitoneally injected microbial derivatives in combination with anti-PD-1 significantly reduced tumor growth when compared to anti-PD-1 given with vehicle control. Microbial derivative-treated mice expressed higher levels of MHC class I compared to mice treated with vehicle control. This study suggests that microbial derivatives could be used in combination with ICIs to enhance anti-cancer immune responses (78).

Kim *et al.* investigated the effects of oral supplementation of *Bifidobacterium longum* RAPO on anti-PD-1 therapy in a 4T1 mouse model of TNBC (79). They found that tumor volume was significantly inhibited in the anti-PD-1 plus RAPO group when compared with the control, RAPO only, and anti-PD-1 only groups. Treatment with anti-PD-1 + RAPO was also associated with increased levels of spleen NKT cells and tumor NK cells. Treatment with anti-PD-1 + RAPO decreased the proportion of pro-tumor M2 macrophages and reduced mRNA expression of M2 markers IL10 and Arg1, while increasing anti-tumor cytokines IFNγ and TNFα compared with anti-PD-1 treatment alone. Additionally, the relative abundance of genus *Bifidobacterium*, *Akkermansia* and *Lachnospiraceae* was enriched in the anti-PD-1 + RAPO group compared with other groups. Additionally, Kim *et al.* analyzed tissue samples from RAPO and anti-PD-1-treated mice using qRT-PCR to determine if RAPO could reduce the risk of immune-related adverse events associated with anti-PD-1 treatment by altering the expression of pro- and anti-inflammatory cytokines (103). Functional analysis showed that expression of pro-inflammatory cytokines IL1β, IL6 and TNFα was significantly decreased, while the expression of anti-inflammatory cytokine IL10 was increased in lung, liver and kidney tissues in the anti-PD-1+RAPO group compared to the anti-PD-1 group. Similarly, TNFα expression was decreased in heart and colonic tissue, while IL10 was increased in the anti-PD-1+RAPO group compared to the PD-1 group. Finally, anti-PD-1+RAPO was associated with decreased levels of pro-inflammatory enzyme, myeloperoxidase, in the liver. This data suggests that combination therapy of B. longum RAPO with anti-PD-1 therapy could be used to improve anti-PD-1 immunotherapy response in TNBC (79). Additionally, this data suggests that RAPO could even reduce the risk of immune-related adverse events related to anti-PD-1 treatment by reducing pro-inflammatory cytokines and increasing anti-inflammatory cytokines in crucial organs, such as the heart, lung, kidney, liver and colon (103). Thus, specific microbiota agents, such as Bifidobacterium longum RAPO, could be used as adjuvants to enhance immunotherapy efficacy and reduce immunotherapy-associated side effects in breast cancer.

Li *et al.* investigated the role of dietary supplement, fucoidan, and its impact on the efficacy of anti-PD-1 therapy in a TNBC mouse model (80). Fucoidan is a polysaccharide isolated from brown algae and marine invertebrates (104). As a dietary supplement, fucoidan has been reported to exert anti-tumor and immunomodulatory effects and enhance response to chemotherapeutic agents (105,106). Additionally, fucoidan is a potential prebiotic that can increase the diversity of gut microbiota and increase short-chain fatty acid production (107-109). The study showed that fucoidan enhanced the anti-tumor effect of anti-PD-1 blockade by decreasing tumor size, weight and growth. Correlating to this observed anti-tumor effect, 16S sequencing of the gut microbiota showed that fucoidan, when combined with anti-PD-1 antibody, increased the abundance of *Firmicutes*, *Bifidobacterium and Faecalibaculum*, which are believed to be beneficial for anti-PD-1 therapy, and decreased the abundance of *Bacteroidetes*, which is not favorable for ICI therapy. These changes were associated with an increase in short-chain fatty acid content in the cecum. In addition, intervention with fucoidan and anti-PD-1 antibody increased abundance of *Lactobacillus*, which has been shown to increase efficacy of immunotherapy in pre-clinical and clinical trials (110,111). Additionally, treatment with antibiotics prior to fucoidan and anti-PD-1 antibody treatment impaired the antitumor effects of fucoidan and anti-PD-1 immunotherapy. Thus, the gut microbiota is likely necessary to support the antitumor effect of fucoidan. Thus, the oral administration of fucoidan has the potential to sensitize breast cancer to anti-PD-1 immunotherapy in the clinic (80).

Future directions

Many clinical studies have investigated the association between patient gut microbiome and response to cancer therapy. However, our understanding of the exact roles and mechanisms of the microbiome in relation to breast cancer immunotherapy remains limited. In this review, we summarized some of the key available data regarding gut microbiome as a prognostic and predictive marker of immunotherapy response in patients, and in pre-clinical models, the role of gut dysbiosis and ICI efficacy, as well as proof-of-concept that microbiome-modulating interventions have the potential to improve immunotherapy response in breast cancer.

The exploratory clinical data presented indicates a potential role for using gut microbiome signatures to predict ICI benefit in breast cancer, as summarized in *Table 4*. In particular, increased baseline alpha diversity seemed to correlate with improved response to PD-1/PD-L1 immune checkpoint blockade (67-71). Due to the small sample size of patients with stool correlatives available and due to variation in gut microbiome sequencing technology (16S RNA and metagenomic sequencing) and bioinformatic approaches, most of the reported findings are purely exploratory and hypothesis generating.

Additionally, a number of pre-clinical studies identified that dysbiosis induced alterations in the immune system had context-dependent importance in regulating breast cancer growth (73-77,112). Modifiable drivers of gut dysbiosis, such as diet, weight management and antibiotic use, could be used to predict and potentially alter tumor response to immunotherapy (72-77). Prospective clinical studies are required to elucidate the practical application of dietary and weight management interventions. The use of microbial derivatives, probiotics, and fecal transplantation in pre-clinical breast cancer models (76-80,103) highlight that microbiome-modulating interventional trials in humans may be on the horizon.

The understanding of the interaction between the gut microbiome and tumor-related immunity and control in breast cancer remains in its infancy. As a result, one of the limitations of this review is the scarcity of published studies regarding this topic. Much of the data included in this review consisted of preliminary data from published abstracts only, which do not have complete information and thus present a higher risk of bias. All the studies included in this review explored the use of immunotherapy in advanced ER+ breast cancer and advanced TNBC, but not HER2+

breast cancer (22,24,113), reflecting the current landscape of immunotherapy development in breast cancer. Clinical data from human therapeutic trials were limited by small sample size and sometimes incomplete correlative sample sets, and the samples were analyzed retrospectively. The real-world applicability of gut microbiome signatures or gut microbiome directed interventions will need to be validated using randomized prospectively validated studies.

In terms of future directions, the interrelationship between gut microbiota and host immunity is evidently complex. As the technology and cost of metagenomic and metabolomic sequencing and bioinformatics continue to improve, it is hopeful that we may understand the mechanistic underpinnings and functional consequences of the gut microbiome on cancer immunity at the population and individual level. It is unknown yet whether the gut microbiome is the most direct resource to predict cancer response or if some downstream immune-metabolomic factor is more direct and important. It is also yet unknown if the correlation between gut microbiome species/signature and immunotherapy response is dependent on the cancer type and other host factors.

Future studies of the gut microbiome and cancer immunity should also address the role of age-related gut microbial and immune system changes. For example, low-grade inflammation and immunosenescence are hallmarks of aging in the elderly (114-116). As the gut microbiome has an important role in cancer initiation, progression, and metastases, and because there is a general trend towards increased gut dysbiosis in advanced stage disease compared to early-stage disease, we need to account for cancer stage when interpreting the results of gut microbiome studies (117,118). It is most likely that immunotherapy responses modulated by gut microbiota are going to be heterogeneous across cancer subtypes, and therefore promising gut microbial species or consortia with potential therapeutic or prognostic relevance need to be validated individually across different cancers.

Conclusions

The data presented in this review highlights the importance and potential of the gut microbiome in predicting and influencing the efficacy of ICI therapy in breast cancer. There is preliminary evidence to suggest that the microbiome composition can be used to predict response to immunotherapy, and that microbiome dysbiosis resulting from factors such as antibiotics, diet, obesity, and probiotics, modulates these responses. Preliminary data also support

Table 4 Summary of gut microbiota analysis in clinical studies

Author	Model	BC type	Interventions*	Microbiome sample source	Analysis	Database	Associated with positive response to ICI therapy	Associated with poor response to ICI therapy
Barroso-Sousa et al. (66,67)	Patients	HR+	Group 1 (n=15): eribulin + pembrolizumab; Group 2 (n=11): eribulin	Fecal	16S RNA sequencing; metagenomic shotgun sequencing	MetaPhlAn3	Faecalibacterium; Odoribacter splanchnicus; Increased alpha diversity	Blautia wexlerea
Teng et al. (68)	Patients	HR+ HER2-	Pembrolizumab + eribulin	Fecal + saliva	16S RNA sequencing		Bacteriodes fragilis; Increased alpha diversity	
Wong et al. (69); Yuan et al. (70)	Patients	(I) TNBC; (II) ER+	(I) Pembrolizumab + enobosarm; (II) Palbociclib + pembrolizumab + letrozole	Fecal	16S RNA sequencing; metagenomic shotgun sequencing	LeFSe, MEGAHIT	Bacteroides; Firmicutes (metabolite: SCFAs); Gemmiger formicillis; Faecalibacterium prausnitzii (metabolite: SCFAs)	Bacteroides vulgatus; Prevotella copri
Chun et al. (71)	Patients	TNBC	Olaparib + durvalumab	Fecal	16S RNA sequencing		Increased alpha diversity	
Pingili et al. (74)	Mice	TNBC	Anti-PD-1	Fecal	16S RNA sequencing	GreenGenes	Akkermensia; Bifidobacterium; Lactobacillus; Aidercreutzia; Odoribacter; Mogibacteriaceae; Ruminococcus	Enterobacteriaceae; Bacteroidales
Bohm et al. (75)	Mice	TNBC	Bariatric surgery + anti-PD-1	Cecal contents	16S RNA sequencing			Lithocholic acid
Clear et al. (76)	Mice	TNBC	Anti-PD-L1	Fecal	Metagenomic sequencing		Akkermansia muciniphilia (metabolite: SCFAs)	
Kim et al. (79)	Mice	TNBC	Anti-PD-1 + RAPO	Fecal	16S RNA sequencing		Bifidobacterium; Akkermansia; Lachnospiraceae	
Li et al. (80)	Mice	TNBC	Fucoidan + anti-PD-1 antibody	Fecal	16S RNA sequencing		Firmicutes; Bifidobacterium; Faecalibaculum actobacillus; SCFAs	Bacteroidetes

*, eribulin = chemotherapy (microtubule dynamics inhibitor); pembrolizumab = ICI (PD-1 inhibitor); enobosarm = hormone therapy (non-steroidal selective androgen receptor modulator); palbociclib = targeted therapy (CKD4/6 inhibitor); letrozole = hormone therapy (aromatase inhibitor); olaparib = targeted therapy (PARP inhibitor); durvalumab = ICI (PD-L1 inhibitor). BC, breast cancer; ICI, immune checkpoint inhibitor; HR+, hormone-receptor-positive; HER2, human epidermal growth factor receptor 2; TNBC, triple negative breast cancer; ER+, estrogen-receptor-positive; SCFAs, short chain fatty acids; PD-1, programmed cell death 1; PD-L1, programmed cell death-ligand 1; RAPO, Bifidobacterium longum RAPO.

the development of microbial-based therapeutics (e.g., probiotics) as an adjunct to cancer therapy. While this is a promising field, this topic in breast cancer remains in its infancy. Larger prospective studies are required to better understand these relationships before incorporating them into clinical practice.

Acknowledgments

Footnote

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