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Hot Topic

Navigating the complex relationship between human gut microbiota and breast cancer: Physiopathological, prognostic and therapeutic implications

Francesco Schettini [a,b,*,1], Federica Gattazzo [c,d,e,1], Sabrina Nucera [a,f,1], Elisa Rubio Garcia [g], Ruben López-Aladid [g], Lorenzo Morelli [c], Alessandra Fontana [h], Paolo Vigneri [i,j], Climent Casals-Pascual [g,k,l,m], Valerio Iebba [n], Daniele Generali [n,o,*]

[a] Translational Genomics and Targeted Therapies in Solid Tumors, August Pi I Sunyer Biomedical Research Institute (IDIBAPS), Barcelona, Spain
[b] Department of Medical Oncology, Hospital Clinic of Barcelona, Barcelona, Spain
[c] Università Cattolica del Sacro Cuore, Piacenza-Cremona, Italy
[d] Gustave Roussy Cancer Campus, Villejuif, France
[e] Institut National de la Santé et de la Recherche Médicale (INSERM) U1015, Equipe Labellisée-Ligue Nationale contre le Cancer, Villejuif, France
[f] Department of Human Pathology "G. Barresi", University of Messina, Messina, Italy
[g] Department of Clinical Microbiology, Biomedical Diagnostic Center (CDB), Hospital Clinic of Barcelona, Barcelona, Spain
[h] Department for Sustainable Food Process-DiSTAS, Università Cattolica del Sacro Cuore, Piacenza-Cremona, Italy
[i] Department of Clinical and Experimental Medicine, University of Catania, Catania, Italy
[j] Medical Oncology Unit, Istituto Clinico Humanitas, Misterbianco, Catania, Italy
[k] Barcelona Institute for Global Health (ISGlobal), Barcelona, Spain
[l] Faculty of Medicine and Health Sciences, University of Barcelona, Barcelona, Spain
[m] CIBER Enfermedades Infecciosas (CIBERINFEC), Instituto Salud Carlos III, Madrid, Spain
[n] Department of Medicine, Surgery and Health Sciences, University of Trieste, Trieste, Italy
[o] Multidisciplinary Unit of Breast Pathology and Translational Research, Cremona Hospital, Cremona, Italy

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ABSTRACT

The human body represents the habitat of trillions of symbiotic microorganisms, collectively known as human microbiota, approximately half of which residing in the gut. The development of next-generation sequencing techniques has boosted the profiling of human microbiota in recent years. A growing body of evidence seems to support a strict relationship between the disruption of the mutualistic relationship between the microbiota and the host (i.e., dysbiosis) and the development of several diseases, including breast malignancies. Breast cancer still represents the most frequent cause of cancer-related death in women. Its complex relationship with gut microbiota is the object of a growing body of evidence. In fact, the interaction with the host immune system and a direct impact of gut microbiota on estrogen, lipid and polyphenols metabolism, seem to potentially affect breast tumor development, progression and response to treatments. In this review, in an attempt to help oncologists navigating this rapidly-evolving research field, we provide an essential overview on the taxonomy, main analytical techniques and terminology most commonly adopted. We discuss what is currently known regarding the interaction between gut microbiota and breast cancer and potential efforts to harness this complex interplay for therapeutic purposes, and revise main ongoing studies. We also briefly provide an overview on breast cancer intratumoral microbiota and its potential role beyond gut microbiota.

Introduction

Human microbiota comprises a diverse community of symbiotic microorganisms (~10–100 trillion), including bacteria, viruses, fungi, and archaea. Each individual possesses a unique microbiota, which becomes established in the intestinal habitat at birth and evolves with the

* Corresponding authors at: Department of Medicine, Surgery and Health Sciences, University of Trieste, Strada di Fiume 447, 34149 Trieste, Italy (Daniele Generali); Translational Genomics and Targeted Therapies in Solid Tumors, August Pi i Sunyer Biomedical Research Institute (IDIBAPS), Enric Granados St. 86-88, 4th floor, 08008, Barcelona, Spain (Francesco Schettini).
E-mail addresses: schettini@recerca.clinic.cat (F. Schettini), dgenerali@units.it (D. Generali).
[1] Equally contributed as co-first authors.

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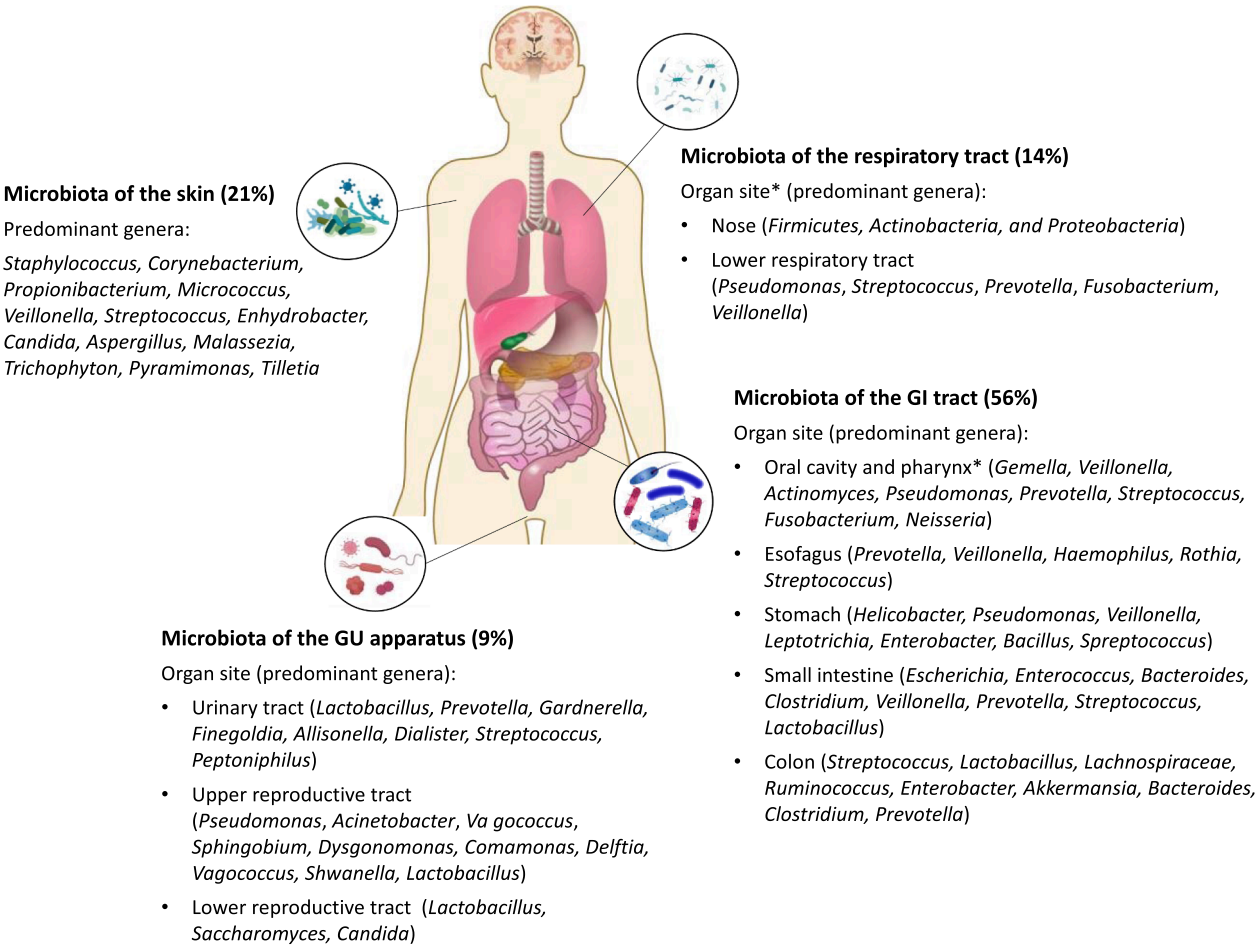

Fig. 1. Overview of the human microbiota composition. Legend. GU genitourinary; GI gastrointestinal. This image was created with Reactome (https://reactome.org) and Microsoft PowerPoint for MacOSX.

host. This community plays a crucial role in various functions, including the synthesis of essential substances, digestion, absorption of nutrients, maintenance of innate and cell-mediated immunity, and other metabolic processes essential for maintaining host health[1]. The interactions between the host and external environment, especially with factors such as diet, lifestyle changes, drugs, and pathogens have the potential to disrupt the mutualistic relationship between the microbiota and the host, resulting in a condition known as dysbiosis, meaning an imbalance in gut microbiota characterized by a decrease in microbial diversity and increase in proinflammatory species[2]. This condition has been demonstrated to play a role in the pathogenesis of various diseases, including cancer[3]. With the growing recognition of the gut microbiota influence on carcinogenesis, research has shifted to evaluating its potential role in prognosis and response to anticancer treatments. Studies involving patients with various types of solid tumors have demonstrated the significant impact of the microbiota on treatment response, as well as its involvement in the development of treatment-related side effects and resistance[4–6]. Since breast cancer is the most common cancer worldwide and the most frequent cause of cancer-related death in women[7], the interest in exploring its potential relationship with human microbiota has quickly grown in the last few years. Despite encouraging results from preliminary studies, the evidence in this regard is still limited[8–10]. Nevertheless, elucidating the complex relationship between breast cancer and microbiota could represent the missing piece of the puzzle for a more comprehensive understanding of this lethal disease[7], paving the way for the development of more effective prophylactic and therapeutic strategies.

This review aims to offer an overview of the state-of-the-art in current knowledge of gut microbiota in breast cancer pathogenesis, prognosis and prediction of response to treatments and to clarify basic analytical and taxonomical concepts for familiarizing with, when approaching this rapidly-growing research field. Furthermore, a brief overview of the current knowledge concerning the characteristics and research potential of breast cancer intratumoral microbiome will be presented, following the recently increased interest of the scientific community on this topic.

Taxonomy and gut microbiota composition

For practicality, we hereby briefly remind the cellular taxonomic hierarchy, which subdivides living organisms in groups based on shared characteristics, named taxa. These groups are given a taxonomic rank, favoring the aggregation in a more inclusive group of higher rank. This classification system, originally introduced by the botanist Carl Linnaeus, results in a taxonomic hierarchy. Older approaches were phenomenological, meaning that taxa were created on the basis of similarities in appearance, organic structure and behaviour. The advent of Darwin's evolutionary theory first, and then cladistic methodology in the 1970s and later development of polymerase chain reaction (PCR) and subsequent methodological improvements in genomic analyses, have led to significant conceptual and methodological changes[11]. Nonetheless, novel discoveries and theoretical changes have been mostly adapted to the established classification system for practicality [12].

Higher-ranked groups are currently represented by three so-called superkingdoms, or domains (i.e. *Eukarya*, *Bacteria* and *Archea*). Each

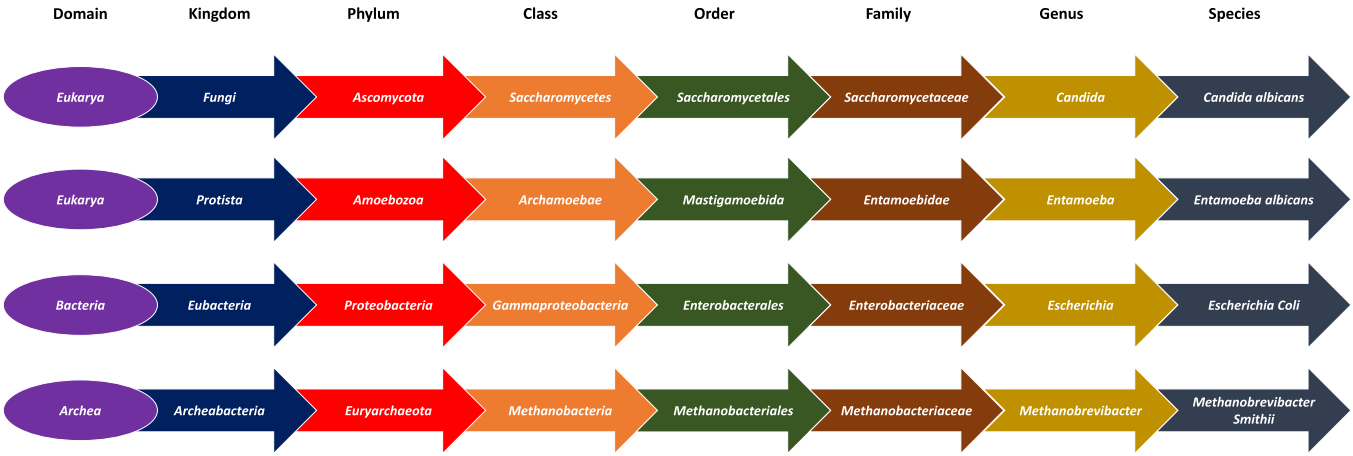

Fig. 2. Practical example of microbial taxonomization by considering for each kingdom one of the most prevalent species at the human gut level. Legend. Protista are more frequently pathogens rather than commensalia, while fungi are rarer in the gut microbiota than in other compartments like skin and lower genitourinary tract in women. The most prevalent species in human gut microbiota pertain to the *Eubacteria* kingdom.

domain, includes one or more kingdoms. The kingdoms *Fungi, Animalia, Plantae* and *Protista* belong to the *Eukarya* domain; bacteria belonging to the *Bacteria* domain are included within the *Eubacteria* kingdom, whilst bacteria belonging to the *Archea* domain, are part of the *Archaebacteria* kingdom. Kingdoms are then progressively divided in phyla, classes, orders, families, genera and species. At present, especially at the domain and kingdom level, this classification is debated. However, this subject has no practical implications from a medical perspective, thus being out of the scope of this review.

At times, the term "microbiota" is used interchangeably with "microbiome" which, in contrast, refers to the collection of genomes of all microorganisms present in the environment[13]. These life forms colonize specific districts of the human body, such as the gut, skin and mouth, establishing a vital symbiotic relationship. The gut microbiota, comprising microorganisms that inhabit the gastrointestinal (GI) tract, constitutes the predominant portion of the microbial population of the host, accounting for more than 50% of the total microorganisms coexisting in the human body (Fig. 1). The four main bacterial phyla are represented by *Firmicutes, Bacteroidetes, Actinobacteria* and *Proteobacteria*, all pertaining to the *Eubacteria* kingdom[14]. Of note, the *Archeabacteria* kingdom represents only 1–2% of human gut microbiota, and that of *Fungi* represents < 1% of microbiota components. The *Eukarya* microbes (kingdom of *Protista*) were generally considered parasites, though this is no longer considered to be always true[15–17] (Fig. 2). For example, several studies have revealed that some *Entamoeba* species (e.g. *E. coli, E. dispar* and *E. hartmanni*) are normal commensals in human gut microbiota, as they have been found both in healthy and ill individuals. They are frequently detected in healthy human coprological-parasitological analyses, differently from other species such as *E. histolytica*, which is commonly associated with amoebic disentery [18]. Moreover, gastrointestinal autoimmune disorders as ulcerative colitis, have been linked to low prevalence of protists[19]. More in general, a multifaceted role for *Eukarya* gut microbes, involving immune modulation and interaction with gut bacteria to impact nutrient cycling and absorption, is currently recognized and under further investigation [16,20,21].

Methods to study the gut microbiome

The development of next-generation sequencing (NGS) technology in the last couple of decades has facilitated the study of the composition, diversity, and functionality of various microorganisms comprising the gut microbiota through microbiome analysis[22].

Microbiome analysis requires an adequate sampling method, as the accuracy of the samples profoundly influences the value of studies. Currently, there are various sampling methods available, such as collection of fecal samples, mucosal biopsies, and luminal brushing,

Table 1
Different sampling techniques for the study of gut microbiota and main limitations

Methods	Main Limitations
Fecal sampling	Does not allow for an accurate representation of the different spatial distribution of distinct bacterial communities in the GI tract; microbial components uncertainly reflect direct interaction with mucosa; unequal distribution of microbiome in fecal samples; need for minimization of systematic bias that can be introduced in preprocessing steps if immediate specimen storage at −80° is not feasible
Endoscopic sampling	
Intestinal biopsy	Effects of bowel preparation in microbial load and composition; invasive; unavoidable contamination; insufficient biomass yield; limitation of sampling sites; costly and time-consuming; not suitable for healthy controls.
Luminal brush	Same bowel preparation effects than intestinal biopsy; invasive; inevitable contamination; costly and time-consuming
Laser capture microdissection	Same as biopsy; need for sample preprocessing with risk of nucleic acid degradation; insufficient sample amount; preprocessing procedural steps may not be feasible in large-scale studies; not suitable for healthy controls
Sampling from aspirated intestinal fluid	Bowel preparation effects; invasive; unavoidable contamination; uncertainty of sampling sites; time-consuming; patient discomfort
Sampling from surgery	
Ileostomy	Preoperative preparation effects on microbiome composition and distribution; significant and durable surgical-induced alterations in the composition of gut microbiota; unclear whether results based on ileostomy effluent are suitable for people with normal anatomical structures; impossible in healthy individuals
Other surgical procedures on the gastrointestinal tract	Preoperative preparation effects on microbiome composition and distribution; significant and durable surgical-induced alterations in the composition of gut microbiota; impossible in healthy individuals
Ingestible sampling devices	Potential contamination of samples with intestinal fluid from non-collected sites; costly; technical complexities

among others. However, each of these methods may present limitations that could affect the precision of the studies[23] (Table 1). In this perspective, the analysis of the fecal microbiome is commonly used in clinical studies as a proxy for studying the gut microbiome because it represents an easy, non-invasive, and reproducible method. However, fecal microbial populations are not fully representative of those present in the contents or mucosa of the entire GI tract, as microbial populations vary along the length of the GI tract, with distinct communities present in the stomach, small intestine, colon, and rectum[24] (Fig. 1). Additionally, even within the same fecal sample, microbial populations may be unevenly distributed due to factors such as transit time, microbial adhesion to particulate matter, and spatial heterogeneity within the gut lumen. Furthermore, the collection and storage of fecal samples can influence the stability and composition of microbial communities. Despite these limitations, fecal microbiome analysis remains a valuable tool for studying the gut microbiota in a wide range of clinical and research settings[23]. However, careful sample collection, processing and storage are required, as all of these steps can affect posterior analytical results. More specifically, stool samples storage in domestic frost-free freezer (with temperatures ranging −20° to −2°C) before collection at the study center affects bacterial taxa relative expression if samples are stored for more than 3 days, as well as unprocessed sample being left for more than 15 min at room temperature. Also, subsampling without prior homogenization (e.g. bead-beating, manual stirring with spatula) of the fecal sample impairs posterior determination of microbiota composition. Homogenization timing (preferably no more than 10 min) has itself an impact on sample diversity. The general recommendation is to usually preserve stool samples at −20°C within 15 min after collection and for no more than 3 days. The samples should be then transferred to a laboratory on dry ice or frozen gel packs (4°C), properly homogenized, processed and stored at −80°C[25–27]. Nevertheless, since rapid freezing is often unfeasible, reagents have been developed to allow for room temperature storage for up to approximately 14 days [28], but the preservation media itself can affect the relative abundance of different phyla[29]. One of the best preserver is based on sodium citrate, EDTA and ammonium sulfate in ddH2O and sulfuric acid to adjust its pH to 5.2 (RNAlater™); it acts as both DNA and RNA stabilizer and inactivates nucleases when specimens are thawed before extraction [29,30]. To note, differently from rapid freezing, preservers likely kill all microorganisms, preventing possible molecular analyses at a later time; this should be taken into account in study protocols involving also culture-independent molecular approaches. Noteworthy, long-term (up to 5 years) stool storage at −80°C of samples collected in RNAlater™ has limited effects on the microbiota composition of human feces[31].

When processing samples, it is always crucial to minimize external contamination by using sensible precautions (e.g. wearing gloves, sterilized materials) and commercially available kits or laboratory reagents that have been thoroughly tested to be free of contaminating nucleic acids[32]. Depending on the subsequent NGS-based analytical approach, samples are processed by cellular lysis and DNA and/or RNA extraction. The intensity of the lysis can result in bias towards a particular taxonomic group. Usually, a combination of chemical, physical and mechanical means is recommended to lyse cells efficiently[32]. Moreover, several extraction methods exist and the choice impacts the metagenomic profile of human gut microbiota, as well[33,34]. All these pre-analytical steps and their potential impact on fecal microbiota profiling highlight the complexity of microbiota studies and the importance of harmonizing protocols in this relatively novel and fast-growing research field.

NGS-based approaches

Among NGS-based approaches, the identification of 16S ribosomal RNA (rRNA) plays a crucial role in acquiring information on microbial taxonomy. This gene-sequencing technique explores the 16S rRNA gene, which comprises approximately 1,500 nucleotides and contains regions highly conserved among all bacteria, interspersed with variable regions between bacterial "phylotypes". Through PCR amplification and sequencing, several sequencing reads are generated and then regrouped into meaningful clusters, defined as operational taxonomic units (OTUs), by using a fixed threshold of similarity (i.e., usually 97% for species, 95% for genera, >90% for order and family and >80% for phyla and class). OTUs are then used as a representative consensus sequence, adopted to taxonomize bacteria, mostly by comparing experimentally-obtained OTUs with sequences previously deposited in specific databases. While suitable for analyzing large numbers of samples, this method has limited taxonomic resolution and does not provide functional resolution, as it does not encompass the entire genomic content of a sample. Furthermore, some OTUs can arise from errors/artifacts. Additionally, this method does not consider non-bacterial components of the gut microbiota, such as fungi[35]. More recently, a methodological shift from sequence identity-based clustering producing OTUs, to denoising methods producing amplicon sequence variants (ASVs) has been introduced, though not yet universally adopted. The ASV method infers the genomic sequences in the sample at the single nucleotide level. Hence, in the case of ASVs, no representative consensus sequences are generated; instead, exact genomic sequences are obtained, which might differ by as little as one nucleotide. This approach determines which exact sequences were read and how many times each exact sequence was read, then sequences are filtered according to a threshold value for confidence. This methodology provides an exact sequencing of high statistical confidence with potentially higher resolution than OTU-based methods, but at the cost of potential loss of real sequences that were present at very low levels.

Overall, the sensitivity and specificity as good or better than OTU methods, a better capability of discriminating ecological patterns, reusability and reproducibility of ASVs across studies and datasets, and no limitations related to incomplete reference databases, makes them the currently most suitable atomic unit of microbiome analysis[36,37]. Nonetheless, OTUs-based approaches are still a valid choice in large, population-based studies where the expected taxa are already well-defined and well-documented in the reference databases and also when attempting to study low-abundance sequences, as OTUs are generally considered to be much more likely to retain rare sequences than ASVs[36,37].

Recent advances in sequencing technologies have enabled metagenomics to revolutionize the study of microbial communities, providing enhanced resolution, more accurate taxonomic profiling, and the discovery of novel genes. However, these processes come with higher costs and time, require a sufficient quantity and high quality of samples, and necessitate more complex analyses[38]. Shotgun Metagenomic Sequencing represents a non-targeted sequencing approach that enables the sequencing of all microbial genomes within the sample. It provides taxonomic and functional profiling of the microbial community at a higher resolution than 16S rRNA-based sequencing[39]. One limitation of metagenomics is its inability to offer insights into the activity or gene expression of microorganisms. The development and utilization of techniques such as metatranscriptomics and metaproteomics have addressed this constraint, enabling the acquisition of information on the functional activity of microorganisms. The functional profiling of microbiota, in addition to the classical descriptive approach, helps providing a more detailed understanding of the molecular activities occurring within the complex and mixed population of a microbial community and their interaction with the host[40].

Metatranscriptomics is an RNA-sequencing-based approach capable of complementing metagenomics data with more detailed information regarding which genes are transcribed and to what extent. As a consequence, metatranscriptomics can reveal details about bacterial populations that are transcriptionally active providing valuable information on the metabolic activities of microbes and allowing for the study of their association with specific environmental conditions (e.g. different therapeutic settings, different breast cancer subtypes etc.). This is

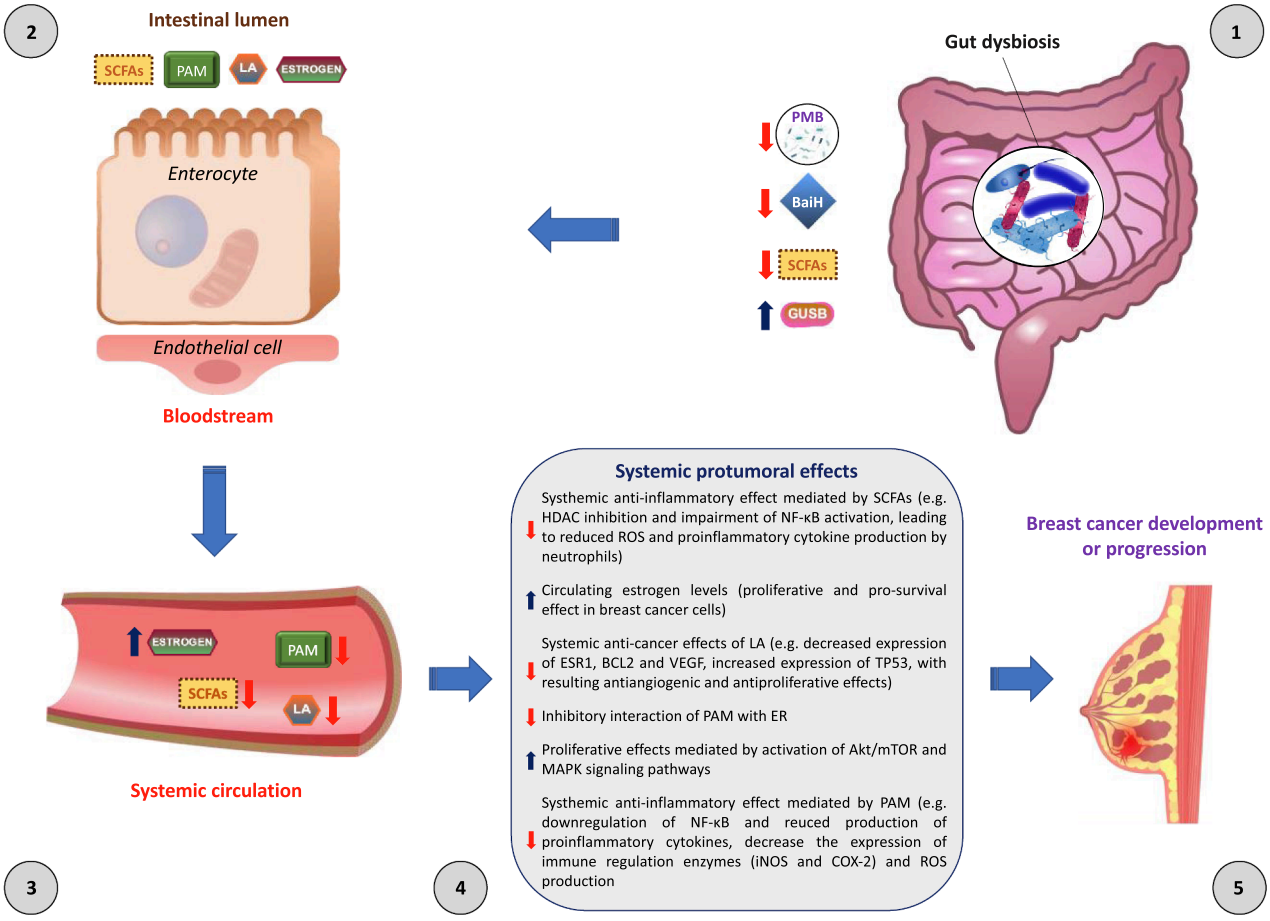

Fig. 3. A scheme of the complex and multifaceted interaction among gut microbiota, estrogen, SCFAs and polyphenols metabolism. Legend. 1: Dysbiosis may result in an increase in β-glucuronidase-producing bacteria, increasing the metabolization of glucuronized estrogens. In addition, a reduced presence of bacteria producing short-chain fatty acid (i.e. butyrate, acetate, propionate) has been observed in patients with breast cancer. Also, a reduced abundance of bacteria producing 7α/β-hydroxysteroid dehydroxylase has been observed, resulting in reduced production of lithocolic acid. Gut dysbiosis might also affect the proportion of polyphenolic-metabolizing bacteria, with a direct impact on the production of polyphenolic active metabolites. 2: The metabolites produced by the dysbiotic gut microbiota are released in the intestinal lumen and reabsorbed by enterocytes in order to be released in the bloodstream, thus producing systemic effects. 3: The result of gut dysbiosis as described in point 1 is a reduction of circulating SCFAs and LA and an increase in estrogens, along with an altered proportion of PAM (sometimes more, sometimes less, depending on the metabolites and the polyphenols introduced with diet). 4: Circulating metabolites have several systemic protumoral effects based on a direct interaction with intracellular proliferation-related or inflammation-related signaling pathways, cell cycle checkpoints, estrogen receptors and other transcriptional factors. 5: breast cancer tumorigenesis or progression of already-formed tumors, as well as resistance to anti-cancer treatments can be promoted as the ultimate consequence of gut dysbiosis. PMB polyphenolic-metabolizing bacteria; GUSB β-glucuronidase; BaiH 7α/β-hydroxysteroid dehydroxylase; SCFAs short-chain fatty acids; PAM polyphenolic active metabolites; LA lithocholic acid; ER estrogen receptor; ROS reactive oxygen species.

critical for understanding different microbial communities' role in cancer pathogenesis, prognosis and prediction of response to different treatments. The main challenges are represented by difficulties in obtaining sufficient quantities of RNA, the short half-life of RNA, and limitations in available databases[41].

Other techniques

Metaproteomics is a functional method that examines proteins and peptides produced by mixed bacterial communities at a specific point in time, offering more comprehensive functional information compared to metatranscriptomics. However, it has lower resolution and can discriminate only a few thousand proteins or peptides out of the potentially millions present simultaneously in the complex microbiota of a sample. Metaproteomics shares similar challenges with metatranscriptomics[42].

Quantitative techniques, such as Fluorescence In Situ Hybridization (FISH) and Real-Time PCR, are employed to assess and measure specific aspects within biological samples. FISH uses fluorescently labeled rRNA-targeted oligonucleotide probes to visualize specific microbial groups within a sample. However, this technique can only detect a limited number of organisms simultaneously[43]. Real-time PCR measures the amount of target DNA present in a sample. This technique can quantify not only the total number of bacterial cells in a sample but also the populations present in different bacterial groups. However, it only allows the recognition of known bacterial groups[44]. Another technique involves the use of DNA microarrays. In brief, a microarray consists of DNA probes attached to a solid surface. The mRNA molecules to be identified are extracted from the cells, converted into cDNA (targets) using the reverse transcriptase enzyme, and then labeled with fluorescent substances. Quantification is achieved by detecting the fluorescence signal. Main limitations of this approach are represented by the high cost, lack of detection of multiple bacterial components simultaneously and limited capability to detect unknown microbes[45].

Detecting microbial diversity

The study of microbial diversity is primarily conducted when the microbiome is analyzed using NGS techniques, due to its compositional characteristics. This analysis implies the need to estimate the diversity of

the microbial communities present in different samples, as well as the microbial heterogeneity in the composition of a specific sample. Therefore, microbiota studies usually report both measures of α-(within-sample) and ß-diversity (between-samples) [46]. The former can be estimated with different measures reflecting the richness (number) or distribution (evenness) of a microbial sample or aiming to reflect a combination of both. The latter is a measure of the similarity or dissimilarity of two communities. As for α-diversity, many indices exist, each reflecting different aspects of community heterogeneity. Significant distinctions revolve around how indices account for fluctuations in rare species, whether they solely acknowledge presence/absence or also integrate abundance, and their approach to interpreting shared absence [46]. Bray-Curtis dissimilarity is the usually favored metric for ß-diversity analysis, as it encompasses both the magnitude (total abundance *per* sample) and structure (abundance of individual taxa) of microbial communities. It is widely used in Microbiology to analyze differences in microbiome composition between different samples or conditions[46].

Gut microbiota and breast cancer pathogenesis: endocrine, immunologic and metabolic interactions

Although the relationship between gut microbiota and breast cancer has not been completely elucidated, a complex interaction between microbiota, estrogen, chronic inflammation, lipid and polyphenol metabolism supports the hypothesis of a specific microbiome role in cancer development and progression (Fig. 3). The majority of the available studies focused on pre- or postmenopausal status, taking into account the direct link between breast cancer risk and circulating estrogen levels, considering the involvement of the gut microbiota in estrogen metabolism[47]. Crucial to this activity is the community of intestinal bacteria capable of metabolizing estrogens (e.g. the estrobolome); specifically, bacteria possessing β-glucuronidases and β-glucosidases, which remove glucuronic acid from conjugated estrogens, promoting their reabsorption in the bloodstream[47]. The onset of dysbiosis may result in an increase in β-glucuronidase-producing bacteria and, consequently, an increase in estrogen levels in circulation, potentially influencing the development of breast cancer[48,49]. Current evidence is characteried by mixed results, with differences according to patients' menopausal status. The studies from Hu *et al.* and Zhu *et al.* did not identify significant differences in terms of α-diversity comparing microbiota of premenopausal patients with healthy controls [8,50]. However, the former study revealed compositional differences and identified two genera with potential prognostic value that characterized the premenopausal state in patients and healthy controls (*Desulfovibrio* belonging to Proteobacteria and *Pedicoccus* belonging to Firmicutes, respectively) [50]. Although Zhu *et al.* metagenomic analysis revealed no significant differences at the premenopausal level, the taxonomic profile and genetic functional capacity of postmenopausal patients differed significantly from healthy controls[8]. Particularly, in the postmenopausal cohort, significantly higher α- and β-diversity were reported in patients when compared to healthy controls, as well as a significant difference in the relative abundance of 45 species[8]. In contrast, Goedert *et al.* had previously demonstrated that the postmenopausal fecal microbiota had significantly lower α-diversity in patients compared to healthy controls, although some taxonomic differences in patients at the level of the Firmicutes phyla (higher levels of Clostridiacea, *Feacalibacterium* and Ruminococcaceae and at lower levels of *Doreae* and Lachnospiracea) were observed[51]. In line with this evidence, Aarnoutse *et al.* confirmed that richness and diversity did not differ between postmenopausal hormone receptor-positive (HR+)/HER2-negative(−) patients with breast cancer compared with healthy individuals, and taxonomic composition was not associated with postmenopausal breast cancer[52]. Another study also investigated a cohort of patients with early HR+/HER2- breast cancer albeit not limited to the postmenopausal state. The α-diversity was significantly lower in the patients vs. controls, with a higher relative abundance of Firmicutes and lower relative abundance of Bacteroidetes. At the genus level, *Clostridium* cluster IV and XIVa and *Lachnospira* were significantly more abundant, while *Coproccus, Odoribacter* and *Butyricimonas* were less abundant in breast cancer patients' fecal samples than controls[53]. Several studies focused on a specific tumor subtype, while other studies investigated potential differences in the gut microbiota between estrogen receptor (ER), progesterone receptor (PgR) and HER2 status and cancer stages[54,55]. No significant α-diversity or phyla-level discrepancies were found in the different stages as well as in the ER+/ER- or PR+/PR- statuses, except in HER2+ breast cancer, characterized by a lower intra-sample diversity and level of Firmicutes compared to HER2-disease. However, specific taxa have been identified for each stage and receptor status with potential diagnostic value[54]. Luu *et al.* highlighted that the *Clostridiaceae* and *Lachnospiraceae* families (specifically *C. coccoides* and genus *Blautia*) were present with higher proportional abundance in stage II/III than in earlier stages, suggesting the potential association between microbial composition and tumor progression[55]. Interestingly, a lard diet regimen modulated the gut microbiota in mice, by increasing the levels of *Lachnospiraceae*[56], suggesting a putative role of a high-fat dietary regimen in promoting cancer progression *via* microbiota modulation.

Gut microbiota exerts profound effects on both the innate and adaptive immune systems by influencing the development and function of gut-associated lymphoid tissues (GALTs). Additionally, microbes and their products play a crucial role in activating and maintaining innate hematolymphoid cells (ILCs 1, 2, and 3), natural killer (NK) cells, cytotoxic and non-cytotoxic cells, as well as lymphoid helper cells[57]. Dysbiosis has the potential to compromise the integrity of the intestinal barrier, allowing the passage of the microbial population and their particles, such as lipopolysaccharide (LPS), into circulation, promoting a systemic inflammatory response. Moreover, the compromise in barrier integrity may lead to an imbalance between regulatory T cells and inflammation-associated Th17 cells, further contributing to intensifying the inflammatory response. Chronic inflammation is known to play a role in the development and progression of cancers[58,59]. A link with breast cancer has been suggested by the observation that high circulating levels of biomarkers of chronic inflammation like C-reactive protein (CRP) and TNFα, were associated to an increased risk of breast tumors. To note, CRP has been identified in nipple aspirate fluid of healthy women and has been directly associated to breast cancer risk [60]. Also, some evidences suggest that, when taken regularly, the anti-inflammatory drug aspirin might reduce the incidence of breast cancer [60]. Crown-like structures (CLS), microscopic foci of dying adipocytes surrounded by macrophages, are a tissue hallmark of chronic inflammation[60]. High proportion of CLS has been found independently and directly associated to higher risk of breast cancer development. CLS may be present in normal breast tissue, and their proportion seem to be directly associated with increasing BMI. In fact, CLS can be observed in the normal breast adipose tissue of obese women[60]. One of the main causes of chronic inflammation is precisely obesity, a condition associated to higher risk of developing breast cancer[61]. Hypertrophic adipose tissue secretes a large number of proinflammatory cytokines and adipokines, e.g. TNFα, IL-1, IL-6, IL-8, resistin and leptin, and attracts macrophages. Many of these cytokines promote the activation of the transcriptional factor NF-κB. Moreover, adipocytes express a functional and proinflammatory toll-like receptor (TLR) signaling system, which can promote the activation of the TLR–MD-2–NF-κB pathway, as well. The ultimate consequence is, among others, the promotion of aromatase expression and activity, increasing estrogen production and favoring the development of endocrine-sensitive breast tumors[60,62]. Regardless of obesity, many chronic inflammatory components are frequently observed in breast cancer microenvironment, namely T, B and NK lymphocytes, macrophages, neutrophils and cancer-associated fibroblasts; all these cells can produce a range of proinflammatory cytokines and growth factors, as those previously mentioned, which might either promote or unfavor breast cancer development and progression[60,62].

Table 2
Summary of main published studies in breast cancer.

Study /Reference	Sample size	Population characteristics	Sample Source	Analysis Methods	Treatment Regimen	Aim	Main Findings
Vernaci, G. et al[75]	25	eTNBC	Fecal samples	16S rRNA gene sequencing	Anthracycline + Taxane Anthracycline + Taxane + Platinum	To assess the feasibility of gut microbiome analysis in patients undergoing NACT To evaluate impact on treatment response and association with clinicopathologic factors To describe longitudinal changes before and after exposure to CT	Higher α- diversity and richness in the pCR group at BL No difference in α-diversity at the species level between the 2 groups at BL. No significant correlation between α-diversity and clinical-pathological features. No significant differences on β-diversity between the two groups at BL. Higher *B. eggerthii* on pCR group. Stability in composition at the phyla and species levels in both groups No change in median *Firmicutes/ Bacteroidetes* ratio after CT in either group.
Abuhadra, N. et al[76]	85	eTNBC	Fecal samples	16S genomic DNA sequencing	Adriamycin/ Cyclophosphamide	To investigate the association between gut microbiome and response to NACT	No difference in α-diversity or ß-diversity between pCR and RD group Higher species richness of *Bifidobacterium longum* in pCR group. Higher richness of the *Bacteroides thetaiotaomicron* species and of the *Lachnospiraceae* genus in RD group.
Terrisse, S. et al[10]	76	eBC	Fecal samples	Shotgun metagenomics	Anthracycline/Taxane/ HT	To analyze associations between baseline or post-CT (neo/ adjuvant) fecal microbiome and plasma metabolomics with BC prognosis and with therapy-induced side effects.	No difference in α-diversity between the favorable and unfavorable prognosis groups. ß-diversity as a stratification factor. Higher α-diversity after CT. Higher health-related species after CT. β-diversity post-CT predicted neurological side effects
Guan, X et al [78]	31	mBC	Fecal samples	DNA extraction 16S rRNA gene sequencing	Metronomic capecitabine (500 mg, three times daily) Standard capecitabine (1,000–1,250 mg/m2, twice daily, days 1–14 every 3 weeks)	To evaluate the composition and the function of Gut microbiome associated with metronomic capecitabine compared to conventional dosage	No statistical difference in α-diversity Lower ß-diversity in metronomic group Presence of *Slackia* associated with worse PFS. Presence of *Blautia obeum* associated with better PFS.
Di Modica, M et al [85]	24	HER2 + eBC	Fecal samples	DNA extraction 16S rRNA gene sequencing	Adriamycin/ cyclophosphamide/ taxane/trastuzumab	To investigate the role of gut microbiota to the response to neoadjuvant trastuzumab through regulation of the preexisting or trastuzumab-conditioned tumor immune microenvironment.	Higher α-diversity in R than in NR patients. *Bacteroidetes* was more abundant in NR patients. Clostridiales (i.e., *Lachnospiraceae*), *Bifidobacteriaceae*, *Turicibacteraceae*, and *Bacteroidales* (i.e., *Prevotellaceae* family) was enriched in R patients. β-diversity segregated patients according to the response to treatment
Li, Y. Et al [86]	23	eBC	Fecal samples	Metagenomic DNA sequencing	Anthracycline/Taxane/ cyclophosphamide/ Trastuzumab	To investigate the relationship between gut microbiome and responses to NACT e underlying mechanisms.	Low diversity of fecal microbiome in NR group *Bacteroides* genus enriched in NR group *Firmicutes* decreased in NRgroup Greater representation of *Coprococcus*, and

(*continued on next page*)

Table 2 (*continued*)

Study /Reference	Sample size	Population characteristics	Sample Source	Analysis Methods	Treatment Regimen	Aim	Main Findings
							Ruminococcus correlated with higher levels of CD4 + T cells in blood. *Coprococcus, Dorea*, and uncultured *Ruminococcus sp*. correlated with CD4 + TILs Higher CD4 + and CD8 + TILs in R group
Barroso-Sousa, R. et al[82]	23	HR+mBC	Fecal samples	16S rRNA gene sequencing	Eribuline +/- Pembrolizumab	To study fecal microbiome profiles and clinical outcomes	Shifts in the abundance were characteristic of pts receiving E but not of those receiving E+P. Higher *Faecalibacterium* in the E-treated group
Schettini, F. et al[90]	14	HR+/HER2- mBC	Fecal samples	DNA extraction 16S rRNA gene sequencing	CDK4/6i + ET	To explore the potential association among faecal microbiome, immune circulating cells with a focus on NLR, and therapeutic efficacy of first/second line CDK4/6-inhibitors + ET	Higher levels of NLR significantly associated with worse PFS No significant differences in α e β-diversity between two groups *B. Longum* and *R. Callidus* significantly more abundant in R *C. Innocuum* and *S. Odontolytica* present exclusively in NR. Identification of two clusters of bacterial species, SIG 1–2, SIG1 harbouring 75 % of NR-related bacterial species, and SIG2 regrouping 76 % of R.
Zidi, O. et al (PMID: 33919750)	8	HR+eBC	Fecal samples	NMR spectroscopy	FEC	To identify and characterize specific fecal metabolite profiles in BC patients following chemotherapy treatment	Fecal metabolic profile influenced from second cycle of therapy. SCFAs increased after CT ↑ Specific aminoacids increased in R patients

Legend: eTNBC early triple negative breast cancer; BL baseline; NACT neoadjuvant chemotherapy; CT chemotherapy; pCR patologic complete response; RD residual disease; eBC early breast cancer; BC breast cancer; mBC metastatic breast cancer; PFS Progression Free Survival; NR non responders; R responders; E Eribuline; E/P Eribuline +/- Pembrolizumab; CDK4/6i Cyclin-dependent-kinases 4/6 inhibitors; ET endocrine therapy; NLR neutrophil/lymphocyte ratio; SIG Species Interacting Groups; SCFAs Short-chain fatty acids; FEC 5-fluorouracil-epiribucine-cyclophosphamide; pts patients.

Chronic inflammation is also responsible for the production of reactive oxygen species (ROS), which can cause genotoxic damage to epithelial cells and contribute to cancer development and progression[60,62].

Despite controversial results, many studies revealed in breast cancer fecal samples a reduction in bacteria involved in short chain fatty acid (SCFA) synthesis[8,50,53,55], as well as an increase in bacteria involved in estrogen metabolism[8,53,55]. SCFAs (e.g. propionate, acetate and butyrate) play a protective role in the process of carcinogenesis, as observed *in vitro*, through inhibition of apoptosis and invasion of breast cancer cell lines[63,64] and inhibition of epithelial-to-mesenchymal transition (EMT) [65]. Propionate and butyrate and, less potently, acetate seem to modulate negatively the inflammatory response by diminishing the production of nitric oxide and proinflammatory cytokines (i.e. CINC-2αβ and TNF-α) by interfering with neutrophils' activity. This anti-inflammatory effect, more pronounced with butyrate, appeared to be mediated by histone deacetylase (HDAC) inhibition and by deactivating the NF-κB pathway, pivotal for the expression of proinflammatory genes [66].

Gut microbiota has been also hypothesized to indirectly promote tumorigenesis by impairing primary bile acid metabolism. Particularly, anaerobic bacteria can deconjugate and dehydroxylate primary bile acids (i.e. cholic and chenodeoxycholic acid), leading to the formation of secondary bile acids, mostly represented by deoxycholic acid and lithocholic acid (LA). LA has demonstrated a potential anti-cancer activity across various tumor types, including breast cancer[67], through the inhibition of the vascular endothelial growth factor (*VEGF*) gene expression, a reduced expression of the anti-apoptotic *BCL2* gene, an increased expression of the oncosuppressor *TP53*, as well as a decrease expression in estrogen receptor alfa gene (*ESR1*). A resulting anti-proliferative and anti-mitotic effects have been observed, along with disruption of the EMT [66]. To note, in early stage breast cancer patients, as compared to healthy women, reduced serum LA levels were detected, in association with a reduced abundance of the 7α/β-hydroxysteroid dehydroxylase (baiH) gene in fecal DNA, key for LA generation [68].

Gut microbiota can also metabolize polyphenols, generating active metabolites that seem to exhibit an anti-cancer effect by inhibiting cell proliferation and suppressing the inflammatory response[69]. Certain metabolites can also exert a protective function by directly interacting with estrogen receptors[66]. However, some compounds seem to impair tamoxifen efficacy in breast cancer cell line models[70]. Gut dysbiosis may result in a unfavorable proportion of polyphenol-metabolizing bacteria (essentially, *Clostridium* sp., *Eubacterium* sp., *Bifidobacterium* sp. and *Lactobacillus* sp.) and either favor breast cancer development or reduce the efficacy of anticancer treatments[66,70].

Fecal microbiota and prediction of response to breast cancer treatments

Individual responses to treatments can be influenced by several factors. It has been demonstrated that the microbiota can regulate the metabolism of certain cancer treatments, thereby influencing the

therapeutic response and the onset of related side effects. Moreover, these treatments, in a bidirectional manner, can induce alterations in bacterial diversity and richness. At times, a limited microbial diversity has been detected before starting treatment, with association to a different range of outcomes[71,72]. Overall, several studies have revealed the microbiome's potential in predicting treatment response, modulating efficacy, and determining the onset of side effects[4,6,73]. In the context of breast cancer, evidence in this regard is still limited [10,74] (Table 2). Here we resume the main already-available results.

Association with treatment response and side effects

In the context of triple-negative breast cancer (TNBC), a recent study evaluated the composition of the fecal microbiome from patients undergoing neoadjuvant therapy and assessed the impact on therapy response and association with clinico-pathological factors. Patients were divided into two groups based on whether they achieved a pathological complete response (pCR) or not. The results showed that patients who achieved a pCR exhibited greater α-diversity and richness compared to the other group at baseline. Additionally, *Bacteroides eggerthii* was more abundant in patients who achieved pCR[75]. Another study involving patients with TNBC undergoing neoadjuvant treatment found no significant difference in α or β diversity of fecal samples between patients achieving a pCR and those with residual disease (RD). However, some significant differences in microbial species were highlighted between the two groups[76].

In a small cohort of the large observational prospective CANTO (CANcer TOxicities) trial, no significant differences in α-diversity were observed in patients with different prognosis. However, ß-diversity seemed to be able to discriminate breast tumors with different size, grade, lymph-node metastasis, and TNM stage. Additionally, several commensals pertaining to the *Bacteroides* species, *Streptococcus* genera, the *Veillonella* genus, the Lachnospiraceae, Enterobacteriaceae and Clostridiaceae family members were associated with poor prognostic tumor characteristics, namely larger tumors at diagnosis, axillary node involvement and tumor stage ≥II[10]. Furthermore, adjuvant chemotherapy resulted in an increased α-diversity and a shift in microbiota composition[10]. Finally, specific gut commensals were associated with the development of neurologic side effects[10]. These metagenomic results defined breast cancer-specific Gut OncoMicrobiome Signatures (GOMS) with prognostic and predictive value associated with tumor staging, chemotherapeutic response, and treatment-related toxicities [77].

Interestingly, a study conducted on fecal samples from women with metastatic breast cancer demonstrated differences in the microbiota of patients receiving metronomic dosing of capecitabine, as compared to the standard dosing group. Furthermore, a higher presence of *Blautia obeum* associated with better progression-free survival (PFS), whereas worse PFS associated with the presence of the *Slackia* genus[78].

Finally, immunotherapy with anti-PD1/PD-L1 immune-checkpoint inhibitors (ICI) has revolutionized the treatment of solid tumors in recent years[79], though, at present, this strategy has limited applicability in breast cancer[80,81]. In a small cohort of patients with HR+/HER2- metastatic breast cancer treated with eribulin alone or in combination with the ICI pembrolizumab, fecal microbiome analysis revealed no difference between the two groups at baseline. However, patients receiving eribulin alone and obtaining a partial response, compared to those with stable disease, showed an increase in the *Faecalibacterium* genus after two treatment cycles. Also, a decrease in *Akkermansia* could be detected in those with larger median overall survival[82].

All these studies, while impaired by small cohorts and scarce mechanistic data, support the concept the fecal microbiome is an emerging promising biomarker of response to a diverse range of anticancer agents and a potential predictor of toxicities.

Immunomodulatory effects and association with treatment response

Gut microbiota is emerging as a critical regulator of the immune homeostasis. The modulatory effect that seems to exert on the immune microenvironment might impact on the response to diverse anti-cancer treatments[83]. Considering the crucial role of the immune system in the therapeutic efficacy of the anti-HER2 monoclonal antibody trastuzumab in HER2 + breast cancer[84], Di Modica *et al.* investigated the potential correlation between gut microbiota and response to trastuzumab. In a murine model of HER2 + breast cancer treated with trastuzumab, an antibiotic-induced alteration of fecal microbiota composition reduced intratumoral recruitment of CD4 + T lymphocytes and granzyme B-expressing cells, specifically NK cells, suggesting an impairment of trastuzumab-dependent cell-mediated cytotoxicity (ADCC). At the lymph-node level, antibiotics reduced dendritic cell (DC) activation and the plasmatic levels of IL12p70, an interleukin critical for the efficacy of trastuzumab[85].

Interestingly, in mice not treated with antibiotics, IL12p70 significantly increased upon trastuzumab administration, likely reflecting the activation status of nodal DC. In these mice, the neutralization of IL12p70 through an anti-IL12p70 antibody impaired trastuzumab antitumoral activity, with a parallel significant decrease in NK cells recruitment at the tumor level. Conversely, the administration of a recombinant IL12p70 in mice treated with antibiotics restored trastuzumab efficacy. Subsequently, the investigators observed in a clinical cohort of women with HER2+ breast cancer undergoing neoadjuvant trastuzumab-based treatment, a significant reduction in α-diversity in the fecal samples of non-responders (NR), as compared with responders (R), with a different taxonomic composition between the two groups. The fecal microbiome β-diversity clustered patients according to response to the anticancer treatment and the transfer of fecal microbiota from R and NR into mice bearing HER2+ tumors recapitulated the response to trastuzumab observed in patients. Overall, this study elegantly suggests that fecal microbiota composition might predict response to trastuzumab and that the manipulation of gut microbiota could be exploited to enhance therapeutic response to this agent[85].

Another study involving patients with breast cancer undergoing neoadjuvant chemotherapy, revealed lower α-diversity in the NR patient group, along with a significant difference in β-diversity between R and NR patients [86]. Interestingly, some bacterial species were overrepresented in patients with increased blood CD4+ lymphocytes counts and positively correlated to CD4+ tumor-infiltrating lymphocytes (TILs). In this study, R patients showed a higher level of peripheral blood CD4+ T cells and higher absolute numbers of CD4+ and CD8+ TILs, suggesting that the interaction between gut microbiota and host CD4+ T lymphocytes may be critical for chemosensitivity and response to neoadjuvant chemotherapy[86].

Lasagna *et al.* compared the potential association between fecal microbiota, proinflammatory markers and levels of TILs in a cohort of postmenopausal patients with HR+/HER2- breast cancer receiving adjuvant aromatase inhibitor treatment (controls) or who had developed endocrine resistance (cases). The study reported no significant differences in composition or diversity between cases and controls with the exception of the *Veillonellaceae* family, which is involved in the metabolism leading to increased levels of circulating estrogen and which was more abundant in the case group [87]. The patient cohort was also subdivided according to the percentage of TILs, but no significant differences in fecal microbial composition was observed, as opposite to other studies[87,88]. Interestingly, a negative association between TILs levels and circulating levels of IL17 was reported, being IL17 a proinflammatory cytokine favouring breast cancer cell proliferation, invasion, and metastasization[87].

In HR+/HER2- metastatic breast cancer, cyclin-dependent-kinases 4/6 inhibitors (CDK4/6i) and endocrine therapy (ET) represents the standard 1st/2nd-line treatment[89]. In this clinical setting, Schettini *et al.* investigated the potential association between fecal microbiota

Table 3
Ongoing Clinical Trials Investigating the Relationship Between Breast Cancer and Microbiota.

Clinical Trial (NCT)	Type of study	Population	Biospecimen	Aim	Primary Outcome	Status
Breast Cancer and its relationship with the Microbiota − MICROMA (NCT03885648)	Observational Case-control cross-sectional	BC, stage I and II patients	Blood, breast tissue, stool and urine samples	To evaluate the contribution of microbiota together with their alteration by environmental contaminants to breast cancer risk.	Metagenomic study of the breast microbiota Metagenomic study of the intestinal microbiota	Active, not recruiting
Gut microbiome components predict response to neoadjuvant therapy in HER2 + BC patients: a prospective study (NCT05444647)	Observational Cohort Prospective	Early stage HER2 + BC patients	Stool and blood samples	To investigate the probability of pCR and the relation with gut microbiota	pCR	Recruiting
Gut and intratumoral Microbiome effect on the NACT-induced immunosurveillance in TNBC (NCT03586297)	Observational Cohort prospective	Early TNBC patients	Tumor and normal adjacent non-tumor tissue, stool and blood samples	To correlate gut and intratumoral microbiome composition and antitumor immune responses with pCR.	pCR	Recruiting
Gut and tumor microbiome in patients with advanced HR+and HER2- BC or advanced melanoma undergoing PD-1 Checkpoint Inhibitor Therapy (NCT06126003)	Observational Cohort prospective	Advanced HR+/ HER2- BC, Advanced melanoma	Tumor tissue, stool and blood samples	To study how the gut microbiota and tumor microenvironment influence cancer and response to treatment	Interaction between the gut microbiome, tumor microbiome and serum immune profile	Not yet recruiting
Predictive Biomarkers of Response to Checkpoint Inhibitors in TNBC: a Multiomics Platform − PORTRAIT (NCT05916755)	Observational Cohort prospective	TNBC stage II-III patients	Tumor tissue, stool, saliva and blood samples	To identify predictive biomarkers of response to NACT+ICI in eTNBC by correlating data from different layers of omics performed in different tissues and imaging, with pCR, EFS, and OS. Exploring multivariate predictive models of response to NACT+ICI	pCR EFS OS Identification of biomarkers to predict clinical outcomes	Recruiting
Adjuvant treatment for high-risk TNBC patients with the Anti-PD-L1 Antibody Avelumab: A-Brave (NCT02926196)	Interventional Phase III Randomized Open label	Early TNBC patients	Tumor tissue, stool and blood samples	To evaluate anti-PD-L1 antibody avelumab as adjuvant or post-neoadjuvant treatment for high-risk TNBC patients.	DFS DFS in PD-L1 + ve patients	Active, not recruiting
Neoadjuvant treatment of locally-advanced BC patients with Ribociclib and Letrozole − NEOLETRIB (NCT05163106)	Interventional, phase II, multicentre, single-arm, open-label	HR+/HER2- BC patients	Tumor tissue, stool and blood samples	To improve understanding of tumor responses and resistance in HR+/HER2- patients locally advanced breast cancer, focusing on the role of the immune system including the gut microbiome.	Change in levels of direct and indirect immunological biomarkers of targeted anticancer therapy	Recruiting
A study of the Gut Microbiome in HR+/HER2- BC with CDK4/6 Inhibitors − CICLIBIOME (NCT06171360)	Observational cohort prospective	HR+/HER2- BC patients	Blood, tumor tissue and stool samples	To study the interaction between the gut microbiome, circulating immune, metabolic and cytokine biomarkers and response to CDK4/6i	Pre-treatment gut metabolic profile and composition Pre-treatment circulating immune population and metabolic profile	Recruiting
Determinants of acquired endocrine resistance in mBC: A Pilot Study ENDO-RESIST (NCT04579484)	Observational cohort prospective	Metastatic BC ER+/ HER2- patients candidate to AI and CDK 4/6i	Stool and blood samples	To identify markers of endocrine resistance in ctDNA and the gut microbiome	TTF	Recruiting
Pilot observational study examining the effect of ET on aging − BETA (NCT05700006)	Observational cohort prospective	BC patients	Stool and blood samples	To evaluate the feasibility of collecting patient-reported data and stool and blood samples from patients aged 65 years and older treated with aromatase inhibitor therapy for breast cancer. Study the effects of AI therapy on aging biomarkers and the microbiome	Percentage of partecipants in the 65 + AI therapy group who complete 3 serial blood collections and 5 serial ePRO collections	Recruiting
Abemaciclib in treating patients with surgically resectable, CT Resistant, TNBC (NCT03979508)	Interventional, Phase II Non-Randomized Open label	TNBC patients	Blood, tumor tissue and stool samples	To study the efficacy abemaciclib in patients with TNBC resistant to treatment with CT alone, or in combination with pembrolizumab.	Proportion of patients who have a CD8/FOXP3 ratio < 1.6 in their residual tumors after neoadjuvant CT that convert to CD8/FOXP3 ratio >= 1.6	Recruiting

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Table 3 (*continued*)

Clinical Trial (NCT)	Type of study	Population	Biospecimen	Aim	Primary Outcome	Status
Intestinal Microbiota impact for prognosis and treatment outcomes in Early Luminal BC and Pancreatic Cancer patients (NCT05580887)	Observational cohort prospective	Early HR+/HER2- BC patients Locally advanced resectable and/or borderline resectable pancreatic cancer patients	Blood, tumor tissue and stool samples	To identify the gut microbiota pattern associated with poor and favorable treatment outcomes for further appropriate planning of treatment strategy.	Intestinal bacterial structure in BC and Pancreatic cancer (separately) patients with disease progression	Recruiting
Biospecimen and medical data collection and tumor biopsy in creating research tissue registry in patients with inflammatory or invasive BC (NCT00477100)	Observational cohort prospective	Inflammatory or invasive BC	Blood, tumor tissue and stool samples	To study the biospecimen and medical data collection in creating a research tissue registry in patients with inflammatory or invasive breast cancer to help doctors find better ways to treat and study inflammatory breast cancer in the future.	Collection of biospecimen and medical data to create a research tissue registry in patients with inflammatory or invasive breast cancer.	Recruiting
Clinico-biological data collection study of mBC − EPICURE SEIN (NCT03958136)	Interventional Parallel Assignment Non-randomized Monocentric open label	mBC patients	Blood, tissue tumor, urine and stool samples	To evaluate the use of a prospective database dedicated to breast cancer patients that contains clinical data as well as epidemiological, psychological, emotional, social, imaging, biological and biopathological data for the creation of new treatment modeling algorithms.	OS, Creation of complex prospective clinico-biological database in metastatic breast cancer specific metastatic biopsy intervention, Creation of complex prospective clinico-biological database in metastatic breast cancer search of algorithms combining multiple data (clinical, biological, imaging) in breast cancer management	Recruiting
ARGONAUT: Stool and Blood Sample Bank for Cancer Patients (NCT04638751)	Observational Cohort prospective	NSCLC, CRC, TNBC, or pancreatic cancer patients	Stool and blood samples	To develop precision microbiome medicines and for the identification of clinically actionable cancer-specific biomarkers to guide therapeutic decisions.	Determine whether the microbiome composition can predict progression-free survival and risk for colorectal cancer	Recruiting
Genetic analysis in blood and tumor samples from patients with advanced or metastatic HE+/HER2- BC receiving Palbociclib and ET (NCT03281902)	Observational Case-only prospective	HR+/HER2- BC patients	Tumor tissue, stool, urine and blood samples	To study genetic profiles changes in blood and tumor samples from patients with HR+/HER2- BC who are receiving palbociclib and endocrine therapy.	Identification of novel genomic variants and pathways associated with baseline, as well as change in serum TK1 levels (measured before enrollment, after two cycles, and at disease progression).	Active, not recruiting
Oral AI modify the Gut Microbiome (NCT05030038)	Observational Cohort prospective	BC patients	Stool and blood samples	To study the gut microbiota before and after receiving an AI	Changes in the gut microbiome from baseline after 4 and 12 weeks of treatment	Recruiting
The Gut Microbiome and Immune Checkpoint Inhibitor Therapy in Solid Tumors − PARADIGM (NCT05037825)	Observational Cohort prospective	NSCLC, MM, TNBC or RCC patients	Stool and blood samples	To evaluate the associations between the gut microbiota (composition and function), the host immune system and the efficacy of ICI treatment in multiple cancer types.	Changes in the microbiome composition from baseline to after cycle 2 of checkpoint therapy	Recruiting
Intestinal microbiota of BC patients Undergoing CT (NCT04138979)	Observational Case − control cross-sectional	BC patients		To evaluate changes in gut microbiota	Transcriptional changes in gut microbiota	Recruiting
Association between changes in the Gut Microbiome and CT-Induced Nausea in Stage I-III BC (NCT05417867)	Observational Case only prospective	BC Stage I-III patients	Stool and blood samples	To understand how changes in the composition of gut bacteria may be associated with the appearance of CIN in women undergoing CT for stage I-III BC	Feasibility of patient recruitment and retention, as well as specimen collection. Changes in gut microbiome composition profiles and metabolites in stool as well as blood from T1 to T2 associated with the occurrence of CIN. Differences in demographic, clinical characteristics and comorbidities between patients who do and do not report CIN	Recruiting
Assessing the impact of the Microbiome on BC	Observational Cohort prospective	DCIS or invasive BC patients	Stool and skin swab sample	To identify the microorganisms and determine whether breast	Evaluation of the fecal and breast skin microbiome profile	Active, not recruiting

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Table 3 (*continued*)

Clinical Trial (NCT)	Type of study	Population	Biospecimen	Aim	Primary Outcome	Status
Radiotherapy Toxicity (NCT04245150)				radiation side effects are associated with these microorganisms.	Correlation of the microbiome with the incidence of at least grade 3 skin toxicity using the CTCAE version 4.0 score	
Gut microbe composition, exercise, and BC Survivors (NCT04088708)	Interventional Randomized single blind	BC stage I, II, or III patients	Blood, hair and stool samples	To determine the effects of exercise on the gut microbiome in breast cancer survivors and determine how these changes may relate to psychosocial symptoms such as fatigue.	Composition of gut microbiota	Recruiting
Fecal microbiota transplantation in treating ICI induced-diarrhea or colitis in genitourinary cancer patients (NCT04038619)	Interventional Phase I Open label	Malignant genitourinary system, melanoma, lung cancer, ovarian cancer, uterine cancer, BC, cervical cancer patients	Tumor tissue, stool and blood samples	To assess the efficacy of FMT in managing diarrhea or colitis induced by ICI	Safety and tolerability of FTM. Efficacy of FMT for clinical remission/response of immune-related diarrhea/ colitis.	Recruiting
Sacituzumab Govitecan +/- Pembrolizumab in Metastatic TNBC (NCT04468061)	Interventional Randomized Phase I Open label	mTNBC	Tumor tissue, stool and blood samples	To test the safety and efficacy of an investigational intervention for patients with TNBC that has spread, or metastasized, to other parts the body and is PD-L1-negative.	PFS	Recruiting

Legend: BC breast cancer; mBC metastatic breast cancer; pCR pathological complete response; CT chemotherapy; TNBC triple negative breast cancer; OS overall survival; EFS event free survival; NACT neoadjuvant chemotherapy; ICI immune checkpoint inhibitors; DFS disease-free survival; CDK4/6i Cyclin-dependent kinase 4 and 6 inhibitors; AI aromatase inhibitors; ctDNA circulating tumor DNA; TTF time to treatment failure; ET Endocrine Therapy; NSCLC non small cell lung cancer; MM malignant melanoma; RCC renal cell carcinoma; CRC colon-rectal cancer; CIN chemotherapy-induced nausea; TK1 thymidine kinase; DCIS ductal carcinoma in situ; CTCAE common terminology criteria for adverse events; FMT fecal microbiota transplantation.

composition, circulating immune cells with particular attention to neutrophil-to-lymphocyte ratio (NLR) and the therapeutic efficacy of CDK4/6i + ET. No significant differences were observed between R and NR in terms of α/β-diversity at the phylum and species level. However, four bacterial species were discriminant for patients with more (*Bifidobacterium longum*, *Ruminococcus callidus*) or less durable responses to CDK4/6i + ET (*Clostridium innocuum*, *Schaalia odontolytica*), with network analysis evidencing also two major clusters of bacterial species differentially distributed between R and NR. Additionally, patients experiencing more prolonged responses to CDK4/6i-based regimens showed lower basal levels of NLR compared to poor CDK4/6i responders, with some bacterial species (e.g. *C. Innocuum*, *Oscillibacter ruminantium* and *Eubacterium hallii*) being directly associated with NLR, thus probably exerting a negative effect on response to CDK4/6 inhibition[90].

Investigation in prospective and adequately-powered studies is now required to confirm the complex potential relationship between gut microbiota, immune system and response to different anticancer agents [86].

Ongoing trials

Several studies are currently exploring, in cross-sectional or longitudinal fashion, the link between microbiome or specific microbial signatures and breast cancer diagnosis, prognosis, prediction of side effects or response to treatments, in a broad range of treatment settings and in different breast tumors subtypes. While the focus is mostly put on fecal microbiome, other tissue samples are being also collected. Another promising direction for further research involves assessing the manipulation of the intestinal microbiota to target dysbiosis and restore a balanced and beneficial microbial community. This includes fecal microbiota transplantation (FMT), the use of prebiotics, probiotics, antibiotics and lifestyle changes, which might help preventing breast cancer development, improving therapeutic responses or reducing side effects[91,92]. An overview of the most relevant ongoing studies is reported in Table 3.

Intratumoral microbiota in breast cancer: A brief overview

Finally, it is worth mentioning that the exploration of microbiota as a biomarker involves a comprehensive approach that is progressively extending beyond traditional stool sampling. Researchers are now incorporating the collection of diverse sample types, including but not limited to saliva, blood, stool, urine, and tissue biopsies, to provide a more holistic understanding of the relationship between human microbiota and health and disease. The collection of various samples facilitates the establishment of comprehensive databases encompassing a wide array of microbiota profiles across different body compartments, enabling to identify patterns, correlations, potential biomarkers and the development of sophisticated algorithms and machine-learning models.

To note, the study of tumor samples for the assessment of intratumoral microbiota is acquiring special relevance in latest years. While distinct bacterial populations specific to different solid tumors have been described, it is still unknown whether these microbial communities represent a predetermined niche or a stochastic and temporary colonization[93]. It has been recently suggested that intratumoral microbes might affect tumor microenvironment (TME) composition by interacting with immune cells, fibroblasts, endothelial cells, adipocytes and pericytes[93–95]. Preliminary evidence in several solid tumors already suggest a potential role for microbial-induced TME modulation and response to anti-cancer agents, including chemotherapy and immunotherapy[93–95]. Interestingly, in a seminal study, Nejman *et al.* observed that the solid tumor with the highest and most heterogeneous microbial composition was precisely breast cancer[96]. Differences were especially relevant with normal breast, and HR+ disease was characterized by a higher activation of bacterial metabolic processes in comparison to HR-negative disease[96]. Again in breast cancer, interactions between intratumoral bacterial species and chemotherapy efficacy were detected in small recent studies, mostly in mice models [93]. The true clinical impact of such interactions will need to be further elucidated in translational studies from real patients cohorts.

Conclusions

Although growing evidence indicates a potential link between microbiota and breast cancer, the complex mechanisms contributing to the development and progression of this disease remain elusive. Nevertheless, the potential utility of analyzing composition and diversity of fecal microbiota as a means to predict patient prognosis, response to treatments and toxicity, is becoming a promising research field. Furthermore, modulation of the gut microbiota represents an important and evolving research area with potentially profound implications. The study of intratumoral microbiome also holds promise, since microbial-induced TME modulation could affect response to anti-cancer agents. Only time will tell if the excitement currently surrounding this emerging research field will lead to a concrete opportunity for advancing Precision Oncology in breast cancer.

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Author contribution

Francesco Schettini, Sabrina Nucera, Federica Gattazzo and Daniele Generali conceived the manuscript. Sabrina Nucera and Federica Gattazzo performed the literature review. Francesco Schettini, Sabrina Nucera, Federica Gattazzo and Daniele Generali wrote the first manuscript draft. All authors discussed the article content, revised and approved the final version of the manuscript.

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