

UNIVERSITÀ CATTOLICA DEL SACRO CUORE

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Dottorato di ricerca per il Sistema Agro-alimentare

Ph.D. in Agro-Food System

Cycle XXXVII

S.S.D. MED/06 MED/07 CHIM/08



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# **The role of gut microbiota in the modulation of cancer therapy responses: from immune mechanisms to clinical implications**

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***Radici e ali.***

***Ma che le ali mettano radici e le radici volino.***

*Juan Ramón Jiménez*

## ABSTRACT

Growing evidence of the gut microbiome's impact on cancer has led to its recognition as a fundamental hallmark of oncological disease. Through the production of bioactive metabolites and immunomodulation of the tumor microenvironment (TME), the gut microbiome not only influences tumorigenesis and disease progression but also affects the outcome of anticancer treatments. This thesis investigated the role of gut microbial signatures as predictive biomarkers in HR+/HER2- metastatic breast cancer patients treated with CDK4/6 inhibitors and endocrine therapy. Metagenomic analysis of fecal samples identified specific bacterial species and microbial clusters differentiating responders from non-responders. A cross-correlation analysis further revealed an association between systemic immune markers, such as the neutrophil-to-lymphocyte (N/L) ratio, and the gut microbiota, highlighting its interplay with the immune system. To further investigate the immune mechanisms underlying host-microbiota interactions and their impact on cancer therapy, the clinical significance of pre-existing immune responses to specific commensal bacteria was examined in patients with various types of cancer treated with immunotherapy. In the NSCLC cohort, humoral and cellular immune responses against beneficial bacteria, such as *Akkermansia muciniphila*, combined with their depletion from the gut, were associated with poor therapeutic outcomes. A validation trial in refractory melanoma patients undergoing fecal microbiota transplantation (FMT) confirmed that eliciting a humoral immune response against beneficial bacteria negatively affected treatment efficacy. Conversely, in colorectal cancer (CRC) patients, humoral responses against pathobionts correlated with improved clinical outcomes. Although further mechanistic analyses are required, these findings suggest that seropositivity towards specific commensals, along with their absence or depletion from the gut, could significantly influence cancer therapy efficacy, as well as could be a new tool for the application of promising microbiota-centred interventions. The impact of gut microbiome on therapeutic responses, combined with a deeper understanding of immune mechanisms, offers new opportunities to manipulate the microbiota for improved cancer outcomes.

|                                                                                         |           |
|-----------------------------------------------------------------------------------------|-----------|
| <b>ABBREVIATIONS.....</b>                                                               | <b>4</b>  |
| <b>I. INTRODUCTION.....</b>                                                             | <b>6</b>  |
| <b>I.1 Gut microbiome: a new hallmark of cancer.....</b>                                | <b>8</b>  |
| <b>I.2 Gut microbiota and immune homeostasis.....</b>                                   | <b>11</b> |
| I.2.1 Innate immunity.....                                                              | 12        |
| I.2.2 Adaptive immunity.....                                                            | 14        |
| <i>I.2.2.1 Humoral immunity.....</i>                                                    | <i>14</i> |
| <i>I.2.2.2 Cellular immunity.....</i>                                                   | <i>18</i> |
| <b>I.3 Gut microbiota impact on anticancer therapy outcomes.....</b>                    | <b>25</b> |
| I.3.1 Tumor microenvironment immunomodulation through anti-commensal.....               | 27        |
| Immune activation in the intestinal mucosa                                              |           |
| <i>I.3.1.1 Adjuvanticity.....</i>                                                       | <i>27</i> |
| <i>I.3.1.2 Antigenicity.....</i>                                                        | <i>29</i> |
| I.3.2 Tumor microenvironment immunomodulation mediated by bacteria.....                 | 31        |
| and bacterial products translocated through the intestinal epithelium                   |           |
| <b>I.4. Gut microbiota-related biomarkers.....</b>                                      | <b>35</b> |
| I.4.1 Fecal metagenomics.....                                                           | 35        |
| I.4.2 Gut-vascular barrier (GVB) related biomarkers.....                                | 38        |
| I.4.3 Commensal-specific cellular and humoral responses.....                            | 41        |
| <b>I.5. Microbiota-centred interventions.....</b>                                       | <b>44</b> |
| I.5.1. FMT.....                                                                         | 44        |
| I.5.2 Probiotics and prebiotics.....                                                    | 47        |
| I.5.3 Dietary interventions.....                                                        | 49        |
| <b>II. RESEARCH CONTEXT AND OBJECTIVES.....</b>                                         | <b>52</b> |
| <b>III. MATERIALS AND METHODS.....</b>                                                  | <b>57</b> |
| <b>IV. RESULTS.....</b>                                                                 | <b>69</b> |
| IV.1 Gut microbiota signatures influence cancer treatment outcomes.....                 | 71        |
| <i>IV.1.1 Introduction.....</i>                                                         | <i>73</i> |
| <i>IV.1.2 Article#1.....</i>                                                            | <i>75</i> |
| IV. 2 Pre-existing anticomensal immune response: effects and clinical implications..... | 78        |
| <i>IV.2.1 Introduction.....</i>                                                         | <i>80</i> |
| <i>IV.2.2 Article#2 Not available online.....</i>                                       | <i>82</i> |
| <b>V. CONCLUSIONS AND PERSPECTIVES.....</b>                                             | <b>85</b> |
| <b>VI. BIBLIOGRAPHY.....</b>                                                            | <b>93</b> |

**VII. APPENDIX..... 117**

**APPENDIX#1: ARTICLE#3..... 119**

**APPENDIX#2: ABSTRACT#1 SENT TO ECCMID IN 2023..... 121**

**APPENDIX#3: ABSTRACT#2 SENT TO ASCO IN 2024..... 123**

**APPENDIX#4: MERIT AWARD FROM ASCO IN 2024..... 125**

**APPENDIX#5: ABSTRACT#3 SENT TO CICON IN 2024..... 126**

**ACKNOWLEDGMENTS..... 129**



## Abbreviations

|          |                                                     |           |                                           |
|----------|-----------------------------------------------------|-----------|-------------------------------------------|
| 3HB      | 3-hydroxybutyrate                                   | PSA       | Polysaccharide A                          |
| A2AR     | Adenosine A2A receptor                              | pTh17     | Pathogenic Th17                           |
| ABX      | Antibiotic                                          | PV-1      | Plasmalemmal vesicle-associated protein-1 |
| AhR      | Aryl hydrocarbon receptor                           | RA        | Retinoic acid                             |
| AID      | Activation-Induced Cytidine Deaminase               | RCC       | Renal cell carcinoma                      |
| AMIS     | Antibody-mediated immune selection                  | RFS       | Recurrence-free survival                  |
| APCs     | Antigen-presenting cells                            | SAA1/2    | Serum amyloid A proteins                  |
| CDI      | Clostridium difficile infections                    | SCFAs     | Short-chain fatty acids                   |
| CR       | Complete response                                   | SFB       | Segmented filamentous bacteria            |
| CRC      | Colorectal cancer                                   | SHM       | Somatic hypermutation                     |
| CSR      | Class-switch recombination                          | SIG       | Species-species interaction group         |
| CTLA     | Cytotoxic T-lymphocyte associated protein           | slgA      | Secretory IgA                             |
| CTX      | Cyclophosphamide                                    | sMAdCAM-1 | Soluble MAdCAM-1                          |
| DC       | Dendritic cell                                      | SPF       | Specific Pathogen Free                    |
| ENS      | Enteric nervous system                              | TAA       | Tumor-associated antigen                  |
| ESCC     | Esophageal squamous cell carcinoma                  | TD        | T-dependent mechanisms                    |
| FMD      | Fasting mimicking diet                              | TdLNs     | Tumor-draining lymph nodes                |
| FMT      | Fecal microbiota transplantation                    | Tfh       | Follicular helper T cells                 |
| GALT     | Gut-associated lymphoid tissue                      | TI        | T-independent mechanisms                  |
| GC       | Germinal center                                     | TILS      | Tumor-infiltrating lymphocytes            |
| GF       | Germ-free                                           | TLRs      | Toll-like receptors                       |
| GOMS     | Gut OncoMicrobiome Signatures                       | TLS       | Tertiary lymphoid structures              |
| GVB      | Gastrointestinal vascular barrier                   | TME       | Tumor microenvironment                    |
| GVHD     | Graft-versus-host disease                           | TMP       | Tail length tape measure protein          |
| HDAC     | Histone deacetylase                                 | Tregs     | Regulatory T cells                        |
| HEVs     | High endothelial venules                            | UC        | Urothelial carcinoma                      |
| IBD      | Inflammatory bowel disease                          | WT        | Wild-type                                 |
| ICB      | Immune checkpoint blockade                          |           |                                           |
| ILFs     | Intestinal lymphoid follicles                       |           |                                           |
| irAEs    | Immune-related adverse events                       |           |                                           |
| KD       | Ketogenic diet                                      |           |                                           |
| MAdCAM-1 | Mucosal vascular addressin cell adhesion molecule 1 |           |                                           |
| MIBC     | Muscle-invasive bladder cancer                      |           |                                           |
| MLCK     | Myosin Light Chain Kinase                           |           |                                           |
| mLN      | Mesenteric lymph nodes                              |           |                                           |
| NACI     | Neoadjuvant chemotherapy                            |           |                                           |
| NGS      | Next-generation sequencing                          |           |                                           |
| NK       | Natural killers                                     |           |                                           |
| NOD      | Nucleotide-binding Oligomerization Domain           |           |                                           |
| NSCLC    | Non small cell lung cancer                          |           |                                           |
| OMVs     | Outer membrane vesicles                             |           |                                           |
| PAMPs    | Pathogen-associated molecular patterns              |           |                                           |
| PC       | Plasma cells                                        |           |                                           |
| PDAC     | Pancreatic ductal adenocarcinoma                    |           |                                           |
| PD-1     | Programmed cell death-1                             |           |                                           |
| PD-L1    | Programmed cell death ligand-1                      |           |                                           |
| PFS      | Progression free survival                           |           |                                           |
| pIgR     | Polymeric immunoglobulin receptor                   |           |                                           |
| PPs      | Peyer's patches                                     |           |                                           |
| PPI      | Proton pump inhibitors                              |           |                                           |
| PR       | Partial response                                    |           |                                           |
| PRRs     | Pattern recognition receptors                       |           |                                           |



# **I. INTRODUCTION**

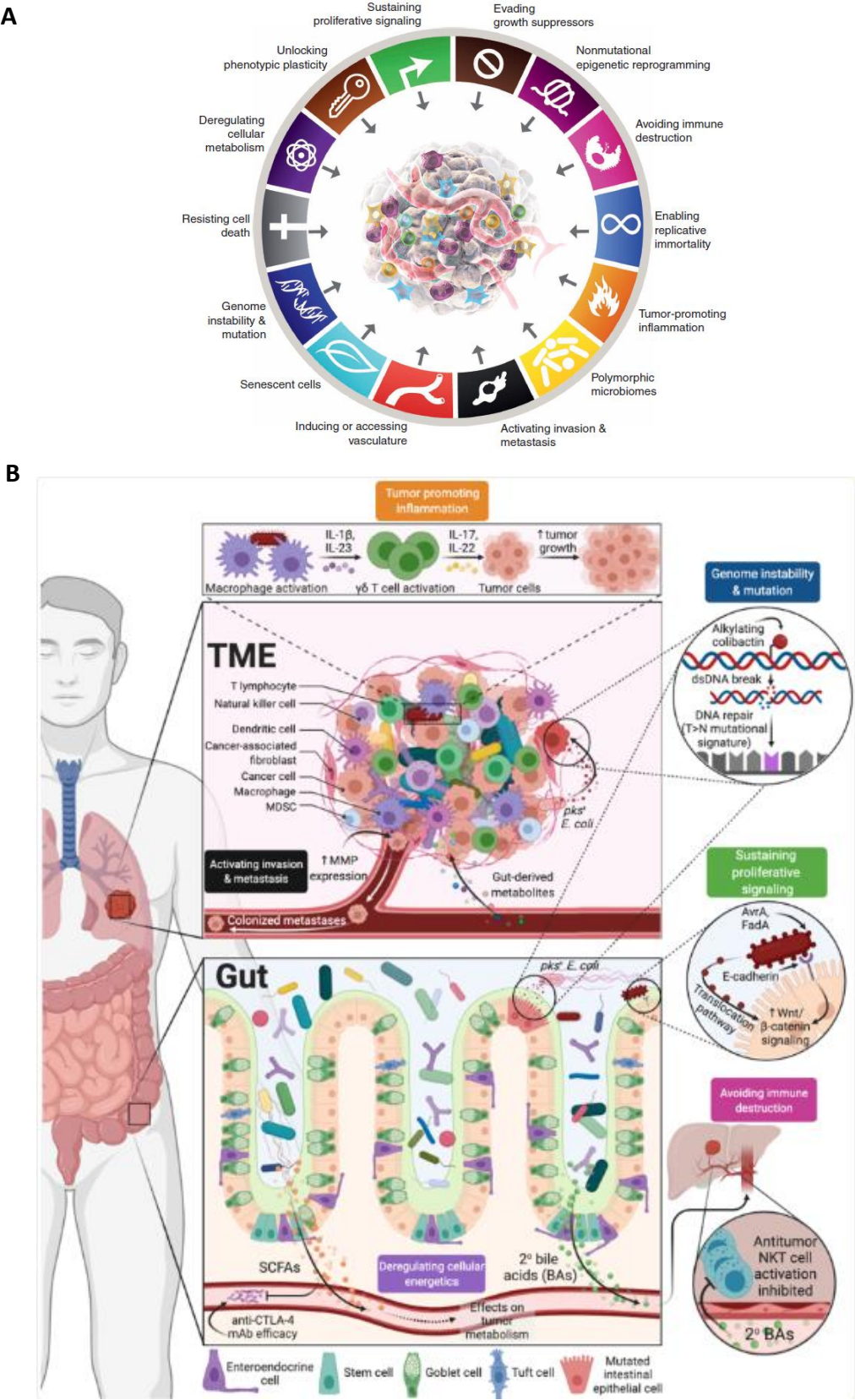


## I.1. Gut microbiome: a new hallmark of cancer

The microbiota is the complex and dynamic population of microorganisms such as bacteria, viruses, fungi, archaea, and protozoa that colonize the human body, establishing a symbiotic relationship with it. These microbes are present throughout the human body, particularly in sites that interact with the external environment: from external surfaces such as the conjunctiva, oral mucosa, and skin, to internal surfaces like the genitourinary and gastrointestinal tracts. Approximately  $4 \times 10^{13}$  microbial cells, encompassing  $\sim 3 \times 10^3$  species, inhabit the human body: of these cells,  $\sim 97\%$  are bacteria located in the colon,  $\sim 2\text{--}3\%$  are extra-colonic bacteria (proximal gut, skin, lungs, etc.), and  $\sim 0.1\text{--}1\%$  are archaea and eukaryotes (Almeida et al., 2019; Sender et al., 2016). The gut, particularly the colon, is the primary site of the human microbiota, whose main bacterial phyla include *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, and *Proteobacteria* (*Bacteroidetes* and *Firmicutes* account for 90% of bacteria in healthy individuals) (Dethlefsen et al., 2007; Forster et al., 2019). The overall genetic content of the gut microbiota contains approximately  $3 \times 10^6$  genes—about 150 times the length of the human genome (Zeng et al., 2022). The microbial genome is a component of the microbiome, which is defined as the entirety of a microbial community (microbiota) within a "reasonably well-defined habitat with distinct physico-chemical properties," representing its "theatre of activity". This concept thus includes not only the microbial genome but also microbial structural molecules and metabolites, as well as interactions with the environment and host organisms (Berg et al., 2020). A symbiotic relationship between the microbiota and its host begins as early as the prenatal period: just as the microbiota relies on the environment and nutrients the host provides for survival, the host, in turn, benefits from and depends on the microbiota and its functions. Indeed, through its metabolic activity, the microbiota is essential for digesting certain foods, synthesizing vitamins, and absorbing nutrients. It is also involved in hematopoiesis as well as cognitive and neurological development. Moreover, it prevents infections by pathogens and ensures the maintenance of innate and adaptive immune homeostasis (Roy & Trinchieri, 2017). Beyond its effects on local and intestinal immune physiology, the gut microbiota exerts systemic effects throughout the meta-organism (Belkaid & Naik, 2013), including on organs previously considered sterile, such as the cardiovascular, endocrine, and central nervous systems (Belkaid & Hand, 2014). The essential ecological characteristics of the gut microbiome are resistance, resilience, and diversity. Thanks to its resistance, although the relative abundance of each bacterial species may fluctuate over an individual's lifetime, the composition of the gut microbiota tends to maintain a certain level of stability. However, external perturbations such as pathogenic infections or antibiotic

therapy can temporarily alter the microbial composition, pushing the microbiota into an unstable but transient state: thanks to its resilience, the gut microbiota can restore its balance and return to its original state. Diversity is a key factor supporting microbiome resilience: a greater species richness within the microbiota ensures functional redundancy, allowing different populations to perform the same roles, thus stabilizing microbial functions even in the presence of perturbations. Beyond the diversity present in each individual (intra-individual diversity), the composition of the microbiota varies across individuals (inter-individual diversity), influenced by both host genotype and environmental factors. Among these, early microbial exposure, diet, age, geography, and antibiotics (ABX) use all play a crucial role in the establishment and maintenance of intestinal microbial diversity. Despite these ecological characteristics, some disturbances may sometimes lead to an alteration of the balance that is no longer transient but instead stable and typically associated with pathological states (Fassarella et al., 2021). An adverse change in the composition and functionality of the microbiota defines the condition known as “dysbiosis.” In contrast to “eubiosis”—associated with a state of health and a richly diversified but stable and balanced microbial ecosystem that provides protection to the host from pathogens and local or systemic inflammation—“dysbiosis” is typically characterized by reduced ecological diversity and an imbalance favouring potentially pathogenic bacteria (pathobionts) at the expense of beneficial commensals. Dysbiosis is considered a hallmark or “meta-hallmark” of multiple pathologies, including oncological diseases (Zitvogel et al., 2024). The cause-effect relationship between cancer and dysbiosis is likely bidirectional: while tumors can cause a condition of intestinal dysbiosis (Yonekura et al., 2022), dysbiosis itself may support the process of carcinogenesis and tumor progression (Asseri et al., 2023; Thomas et al., 2023). The gut microbiota may influence tumorigenesis through multiple strategies. Direct induction may be determined by so-called “oncomicrobes,” comprising 11 species labelled as human carcinogens by the International Association for Cancer Registries (IACR). Experimental evidence also includes in this category microbes that, through genotoxins such as colibactin (a DNA-alkylating agent produced by certain strains of *Escherichia coli*), trigger tumor mutagenesis. However, the vast majority of bacteria implicated in tumorigenesis belong to a category of “accomplices” that cannot directly induce cancer but can promote carcinogenesis through the production of bioactive metabolites and immunomodulatory mechanisms that act on the tumour microenvironment (TME) (Sepich-Poore et al., 2021). Increasingly robust and compelling evidence of the microbiome’s impact on cancer has led to the inclusion of microbial polymorphism among the fundamental hallmarks of cancer (Fig.1A). Furthermore, the microbiome can influence other distinctive features of cancer, such as pro-tumoral

inflammation, uncontrolled cell proliferation, or evasion of the anti-tumor immune system (Fig.1B) (Hanahan, 2022).



In particular, the immunomodulatory action of the tumor microenvironment (TME) promoted by the gut microbiota can, on one hand, weaken immune surveillance indirectly contributing to cancer development, and, on the other hand, strengthen anti-tumor immunosurveillance to the extent that it has been included among the factors defining the cancer-immune set point (Fig.2) (D. S. Chen & Mellman, 2017).

The microbiome thus becomes a critical piece of the puzzle in understanding how tumors develop, progress, and respond to therapy. Within this puzzle, it is essential to elucidate the role of the immune system, particularly the dialogue it establishes with the gut microbiota.

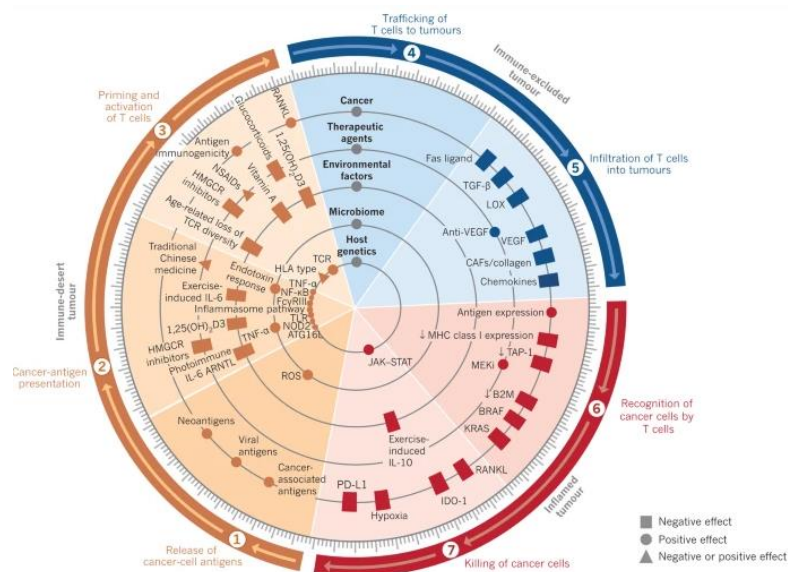


**Figure 2** Factors regulating the “cancer-immune set point” (D. S. Chen & Mellman, 2017)

## 1.2. Gut microbiota and immune homeostasis

The interactions between the gut microbiota and the host immune system are complex and bidirectional. While the microbiota itself plays a critical role in the education and proper functioning of the immune system, the immune system must learn to tolerate the commensal microbiota while responding appropriately to pathogens. This delicate balance between tolerance and reactivity leads to immune homeostasis. A specific subset of the immune system is dedicated to regulating this balance and a thorough analysis of this system allows a deeper understanding of the fundamental dialogue with the gut microbiota.

A central strategy adopted by the host to maintain a balanced relationship with the microbiota consists of minimising contact between microorganisms and the surface of epithelial cells, limiting tissue inflammation and microbial translocation (Belkaid & Harrison, 2017).

The intestinal mucosal barrier represents the host's primary line of defence and plays a crucial role in modulating immune tolerance. It is composed of the mucosal barrier (extracellular component), the epithelial monolayer (an enterocyte layer interspersed with specialized cells), and the underlying lamina propria (Fig. 3). The sub-epithelial structure of the lamina propria comprises immune cells, components of the enteric nervous system (ENS), and connective tissue. Notably, intestinal immune cells are organized into gut-associated lymphoid tissue (GALT), which includes lymphoid structures like lymphatic follicles, Peyer's patches (PPs), and mesenteric lymph nodes (mLN) (Di Sabatino et al., 2023). Each component of the mucosal barrier establishes a dialogue with the gut microbiota and plays a role in maintaining immune homeostasis. Analysing these interactions provides insight into the key mechanisms that support immune balance.

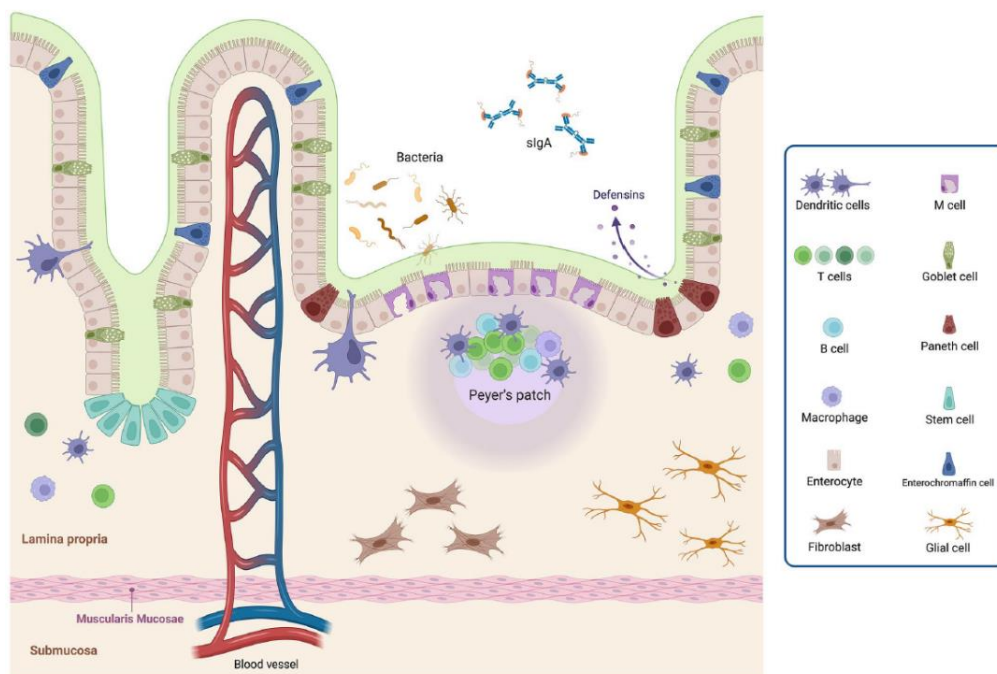


**Figure 3** Representation of the main components of the intestinal barrier: epithelial barrier, mucus barrier and lamina propria (Di Sabatino et al., 2023)

### 1.2.1 Innate immunity

In the gut, the first line of defence is innate immunity and includes epithelial cells, dendritic cells (DCs), macrophages and natural killers (NK). Epithelial cells play a crucial role thanks to specialised components such as goblet and Paneth cells that produce mucus and antimicrobial peptides, respectively. The mucus layer covering the intestinal villi is mainly composed of the highly glycosylated and gelling mucin MUC2, which, mixed with antimicrobial peptides, creates a “demilitarised zone” that intestinal bacteria cannot access. Antimicrobial peptides can be

constitutively present in mucus as in the case of  $\alpha$ -defensin or they can be secreted as a result of receptor-mediated recognition of microbial components as in the case of the RegIII peptide (Belkaid & Harrison, 2017). Indeed, epithelial cells act as sentinels by recognising pathogens and their pathogen-associated molecular patterns (PAMPs) through specific pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and Nucleotide-binding Oligomerization Domain (NOD). TLR stimulation directly impacts the integrity of the epithelial barrier: for instance, TLR2 stimulation preserves tight junctions, whereas TLR4 activation—inducing Myosin Light Chain Kinase (MLCK) expression—can compromise the barrier, leading to “leaky gut”. Similarly, NOD1/2 recognition of peptidoglycan (a bacterial structural component) initiates an intracellular signaling cascade that prompts cytokine and chemokine release to amplify and promote the immune response (Di Sabatino et al., 2023; Li et al., 2024). TLRs and NODs collectively play an active role in immune response monitoring and regulation, promoting the balance between antimicrobial defence and immune tolerance. PRRs contact with commensal bacteria triggers signaling cascades that drive macrophages and DCs toward a tolerogenic phenotype.

Macrophages respond to microbial metabolites such as short-chain fatty acids (SCFAs), which derive from fiber metabolism. Specifically, n-butyrate inhibits pro-inflammatory NO, IL-6, and IL-12 release, promoting tolerance toward commensal bacteria. Concurrently, n-butyrate ensures tolerance is balanced via autophagy mechanisms leading to microbial clearance (Chang et al., 2014; Schluter et al., 2020).

DCs are the antigen-presenting cells (APCs) for excellence, capable of initiating the adaptive response and supporting innate immunity. A proposed mechanism suggests that lamina propria DCs can transiently open epithelial tight junctions, extend dendrites beyond the epithelium, and directly sample bacteria. By expressing tight junction proteins like occludin, claudin-1, and zonula occludens-1, epithelial integrity is preserved (Rescigno et al., 2001). DC migration from the intestine to mesenteric lymph nodes (mLN) occurs exclusively through lymphatic vessels, ensuring their accumulation in the first draining lymph node encountered. This prevents commensal bacteria from entering systemic immune circulation, confining them to the intestinal compartment. Under homeostasis, this mechanism permits effective mucosal immune responses without triggering systemic immune reactions, avoiding potential aberrant responses (X. Li et al., 2022). This localized DC accumulation model has been revisited within the context of the “common mucosal immune system,” discussed in the next chapter (Galván-Peña et al., 2024).

Finally, NK cells, highly relevant in anti-tumor immunity, can also interact with commensals under homeostatic conditions. For example, probiotics like *Lactobacillus plantarum* effectively enhance IL-22 expression in NK cells. By secreting IL-22, NK cells support the maintenance of epithelial integrity, contributing to the protection of the intestinal barrier and preventing microbial translocation from the gut (Qiu et al., 2017).

## **1.2.2 Adaptive immunity**

### **1.2.2.1 Humoral immunity**

In addition to defending against pathogens, antibodies are tasked with recognizing and interacting with the microbiota, creating an immune balance that promotes tolerance to commensals and a targeted response against pathogens.

Among the five antibody isotypes, IgA, IgM, and IgG are known to be produced by plasma cells in the lamina propria under homeostatic conditions; however, IgA is by far the most abundant (Kubinak & Round, 2016). IgA<sup>+</sup> intestinal plasma cells (PCs) make up 80% of all PCs in humans (Fagarasan et al., 2010), and IgA represents a dominant fraction of antibody production, constituting over 70% of systemic immunoglobulin production (Macpherson & Uhr, 2004).

Antibodies support immune balance and the establishment of mutualism with commensal bacteria through various effector functions such as neutralizing microbial toxins, binding to surface epitopes to block microorganism adhesion to the host, agglutination which facilitates the elimination of pathogenic or excess bacteria (Roche et al., 2015), and chaining growing bacteria to limit their proliferation and invasiveness (Moor et al., 2017). In addition to providing a physical barrier, IgA has the ability to modulate the gene expression of intestinal microbes. Peterson et al.'s study in a gnotobiotic murine model observed that IgA against the capsular polysaccharide of the bacterium *Bacteroides thetaiotaomicron* was able to reduce the expression of the pro-inflammatory epitope without altering the abundance of the bacterium itself. However, a precise concentration window was required: too low or too high levels could trigger inappropriate inflammatory responses or bacterial antigenic variation strategies to escape the immune response, a phenomenon common in bacteria to maintain balance with the immune system (Peterson et al., 2007). Another example of genetic modulation by antibodies involves the downregulation of flagellin genes, resulting in reduced bacterial motility (Cullender et al., 2013). By binding to the CBir1 flagellin, IgA antibodies also prevent antigen absorption through the intestinal mucosa, thereby avoiding T-cell activation.

This process of "immune exclusion" helps prevent an excessive systemic immune response by retaining the antigen in the intestinal lumen and reducing further immune system activation (Cong et al., 2009).

The host's antibody responses can influence the colonization, diversity, and composition of bacterial communities in the gut, a phenomenon known as antibody-mediated immune selection (AMIS) (Kubinak & Round, 2016). This is demonstrated by the effect of humoral immunodeficiency on the microbiota: *Ighm*<sup>-/-</sup> mice, lacking B cells, show reduced microbial diversity compared to *Ighm*<sup>+/-</sup> mice. Specifically, IgA, by binding to specific components of the microbiota, favors greater microbial diversity and strengthens host-microbiota mutualism (Kawamoto et al., 2014). Consistent with this observation, individuals deficient in IgA show a fecal microbiota with a prevalence of pro-inflammatory taxa (Friman et al., 2002). For instance, *Proteobacteria*, a persistent phylum in the absence of IgA, has been associated with intestinal inflammation (Mirpuri et al., 2013).

Studies in mice knockout for the *Aicda* gene, which codes for the Activation-Induced Cytidine Deaminase (AID) enzyme, confirmed the role of IgA-mediated immune selection. This enzyme plays a crucial role in class-switch recombination (CSR) and somatic hypermutation (SHM) of immunoglobulin genes. In AID-deficient mice, hyperplasia of germinal centers (GCs) in Peyer's patches (PPs) and intestinal lymphoid follicles (ILFs) was observed due to B cell hyperproliferation. This hyperplasia was associated with dysbiosis, with a 100-fold increase in anaerobic bacteria compared to aerobic ones, and an altered distribution along the entire intestinal tract (Fagarasan et al., 2002). The use of a murine model with the AID G23S mutation, designed to express a mutated form of AID with reduced SHM activity, caused alterations in intestinal homeostasis and compromised mucosal defense: IgA production was insufficiently diversified to effectively address pathogens, making the mice more susceptible to infections (Wei et al., 2011).

The impact of the absence of secretory IgA (sIgA) on the microbiota has been studied using murine models lacking the polymeric immunoglobulin receptor (pIgR), which is essential for the transport of IgA and IgM antibodies through the epithelium to the apical surface. It was observed that pIgR-deficient mice had increased mucosal permeability, suggesting that the absence of IgA transport compromises mucosal barrier integrity (Johansen et al., 1999). Reikvam et al.'s study also found that pIgR-deficient mice had reduced microbial diversity and significantly altered bacterial composition, with a decrease in beneficial bacteria such as *Bifidobacterium spp.* and an increase in pathobionts like *Helicobacter spp.* In wild-type (WT) mice, bacteria were compartmentalized between the luminal content and the mucosal surface of the cecum, a separation not found in knockout mice. This

indicates that pIgR and sIgA are crucial for maintaining epithelial stability and a clear compartmentalization between luminal and mucosal-associated bacteria (Reikvam et al., 2012).

The compartmentalization between mucosal and luminal bacteria is reflected in IgA coating. When comparing IgA-coated microorganisms with those that are not coated, the IgA-bound fraction is more similar to the mucosal community than to the fecal community. This suggests that IgA primarily acts on organisms closely associated with host tissues, which are easily recognized by APCs (Kubinak et al., 2015). Supporting this, IgA preferentially targets organisms known for their close interaction with mucosal tissue, such as segmented filamentous bacteria (SFB) (Palm et al., 2014), flagellated members belonging to *Enterobacteriaceae* (Cullender et al., 2013), and mucus-degrading bacteria like *Akkermansia muciniphila* (Ansaldo et al., 2019). The preferential coating of these mucosal-associated bacteria is due to the localization of IgA, which, thanks to its glycosylated secretory component, is predominantly anchored to the intestinal mucosal coating (Fernandez et al., 2003). Therefore, differences in the physical properties of intestinal mucus can influence local IgA concentrations (Kubinak et al., 2015). In addition to proximity to the epithelium, compartmentalization along the gastrointestinal tract affects the coating of bacteria with IgA. IgA primarily targets commensal bacteria residing in the small intestine, while in the colon, IgA<sup>+</sup> bacteria are mostly those originating from the small intestine. Thus, the specificity of IgA is not so much due to the increased immunogenicity of the bacteria present but rather their anatomical position: it is the location in the intestinal tract that determines whether a particular taxon triggers an IgA response. This anatomical regulation of the IgA response is likely due to the priming of IgA<sup>+</sup> plasma cells primarily located in secondary lymphoid tissues of the small intestine, such as PPs and ILFs (Bunker et al., 2015).

PPs and ILFs are the primary sites in the lamina propria where B cells are activated, either in a T-dependent or T-independent manner. Indeed, intestinal PCs can produce mucosal IgA through T-dependent (TD) and T-independent (TI) mechanisms (Fagarasan et al., 2001) (Fig. 4).

In the T-dependent (TD) mechanism, DCs capture microbial antigens transferred by M cells and induce differentiation of CD4<sup>+</sup> T cells into follicular helper T cells (T<sub>fh</sub>). At the germinal center level of PP, CD40 ligand (CD40L) and interleukin IL-21 secreted by T<sub>fh</sub> cells induce AID enzyme expression in B cells and promote IgA CSR. This TD pathway results in the generation of specific, high-affinity IgA responses. Bacteria such as SFB and *Mucispirillum*, which tightly bind to the intestinal epithelium, induce a TD IgA response.

However, as shown in murine models lacking functional T cells (*Tcrb*<sup>-/-d</sup><sup>-/-</sup>) or lacking GCs and Tfh (*Bcl/6* $\Delta$ T), the T-independent (TI) response can stimulate B cell activation against commensals, albeit with lower production of IgA<sup>+</sup> plasma cells (Bunker et al., 2015, 2017). TI IgA production involves direct interactions between B cells and ligands derived from epithelial or DCs, like BAFF and APRIL, and is modulated by factors such as retinoic acid (RA) and TGF- $\beta$ , which are involved in intestinal targeting and IgA CSR. In this case, IgA is polyreactive, able to recognize and neutralize a broader range of bacteria but with low affinity and is considered an "innate" form of IgA because it is produced in the absence of T cells (Honda & Littman, 2016).

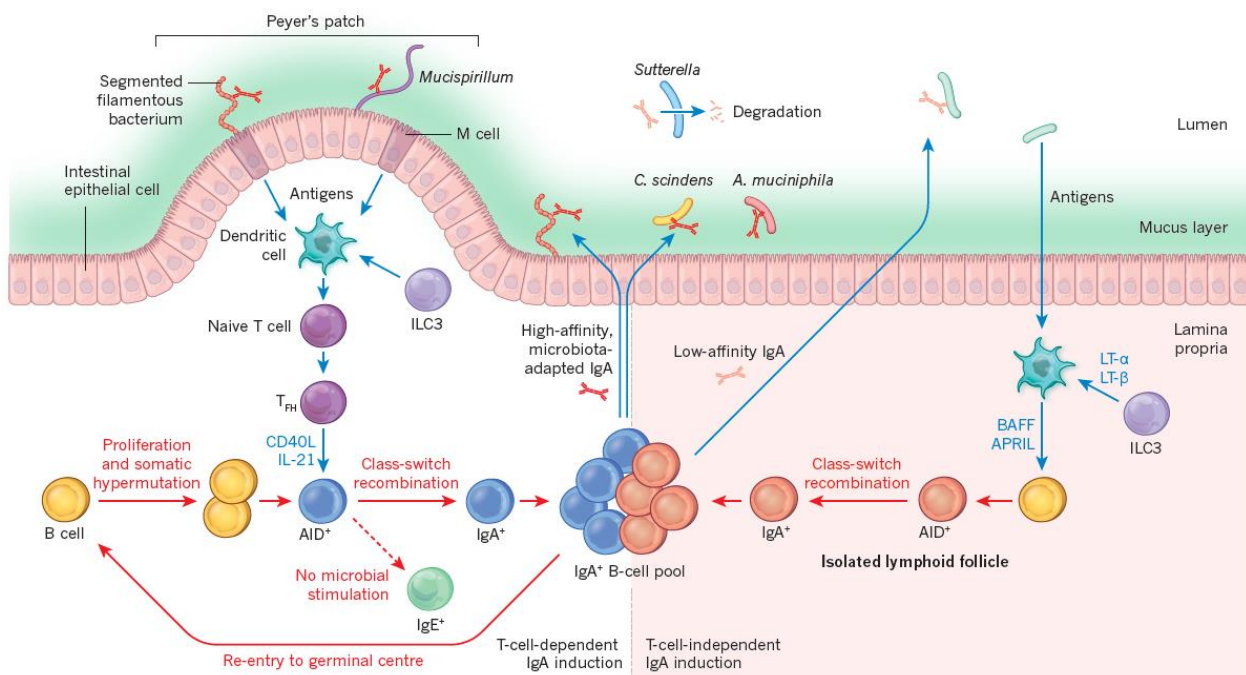


**Figure 4** T-dependent and T-independent induction of IgA in mucosal tissues (Honda & Littman, 2016)

Once activated, B cells differentiate into PCs that secrete antibodies and into memory B cells that ensure a durable and specific immune response. B cell clones expressing IgA induced by this pathway persist long after microbiota changes, even in the absence of the bacterial species that initially activated their production. This stability can be maintained through the diversification of memory B cells, which, by recirculating between lymphoid tissues within and outside the intestine, continue to produce IgA while undergoing SHM processes that improve the specificity and affinity of the antibodies produced. The frequency of somatic mutations increases with age, a phenomenon that may reflect both continuous exposure to new antigens and the need to constantly refine the immune response. Indeed, it is hypothesized that adaptations in the intestinal IgA repertoire largely depend on the diversification of already-existing B cell clones rather than the generation of naive B cells. This

approach appears to favor a stable symbiotic interaction between the host and the microbiota. sIgA exerts selective pressure on intestinal bacteria: diversifying memory B cell clones, rather than continuously recruiting new clones, may reduce the risk of introducing specificities that could threaten the stability of this relationship. Thus, a balance is established between stability and change within the IgA repertoire, enabling B cell clones to adapt and respond effectively to new immunological challenges without compromising host-microbiota symbiosis (Lindner et al., 2015).

In addition to intestinal sIgA, IgM and certain subclasses of IgG are also associated with the intestinal microbiota in a state of homeostasis (X. Li et al., 2022). Secretory IgM helps maintain a diverse community of commensals within the mucus layer, working synergistically with sIgA (Magri et al., 2017). Secretory IgG is present in negligible amounts in the intestinal lumen, and its function remains an active area of research. However, it seems that its protective role is primarily exerted systemically, complementing the activity of sIgA, which operates within the intestinal lumen (Fadlallah et al., 2019).

### **I.2.2.2 Cellular immunity**

#### ***T-helper 17 (Th17) cells***

Th17 cells establish a complex and dichotomous relationship with the gut microbiota. While traditionally linked to inflammatory responses and pathological conditions, Th17 cells also play an essential role in maintaining intestinal immune homeostasis (Honda & Littman, 2016; Q. Zhao & Elson, 2018). Under steady-state conditions, the gut microbiota significantly contributes to the constitutive development of Th17 cells in the lamina propria. Commensals presence is essential for generating and differentiating Th17 cells. Indeed, germ-free (GF) mouse models showed significantly lower levels of Th17 cells than Specific Pathogen Free (SPF) mice (Atarashi et al., 2008; Ivanov et al., 2008). In the absence of microbiota, limited Th17 differentiation may be partially compensated by the recognition of microbial products (Li et al., 2024). For instance, microbially derived ATP, by activating a unique subset of lamina propria cells, the CD70<sup>high</sup>/CD11c<sup>low</sup> cells, triggers a series of intracellular signaling pathways that lead to the expression of key cytokines essential for the induction of Th17 cells, such as IL-6 and TGF- $\beta$  (Atarashi et al., 2008). Conversely, certain bacterial metabolites inhibit Th17 cell generation. For example, 3-oxolithocholic acid (3-oxoLCA), a secondary bile acid derived from microbial metabolism, acts by blocking the transcription factor ROR $\gamma$ t, which is essential for Th17 differentiation (Hang et al., 2019).

Overall, the gut microbiome regulates Th17 levels in the lamina propria. However, key species predominating the mucosal immune system have been identified. The segmented filamentous bacterium (SFB) serves as a prototypical example, effectively modulating immune responses, including Th17 differentiation under homeostatic conditions (Ansaldo et al., 2021).

SFB is a spore-forming, anaerobic, Gram-positive bacterium that colonizes the murine terminal ileum, adhering closely to epithelial cells and inducing cytoskeletal reorganization at the contact site. This localization appears critical for Th17 differentiation (Ivanov & Honda, 2012; Sczesnak et al., 2011). Likewise, epithelial adhesive bacteria, such as *Citrobacter rodentium*, *Escherichia coli* O157 and a mixture of 20 epithelium-adherent bacterial strains isolated from the human gut, induce intestinal Th17 cells in mice (Atarashi et al., 2015). Th17 polarization relies more on CX3CR1+ monocyte-derived cells rather than traditional antigen-presenting dendritic cells. Specifically, CD103 and CD11b-expressing dendritic cells, upon activation of Notch2 and IRF4 transcription factors, appear involved in Th17 differentiation (Lewis et al., 2011; Panea et al., 2015; Schlitzer et al., 2013). SFB contact induces epithelial cells to produce serum amyloid A proteins (SAA1/2), which amplify Th17 signaling through multiple pathways. SAA1/2 locally promotes IL-17a release and creates a feed-forward loop by interacting with CX3CR1+ cells to induce IL-23 release, further stimulating IL-22 production by type 3 innate lymphoid cells (ILC3s). While IL-22 does not directly induce Th17 cells, it fosters a microenvironment conducive to their differentiation, promoting additional SAA1/2 release from epithelial cells (Sano et al., 2015) (Fig. 5).

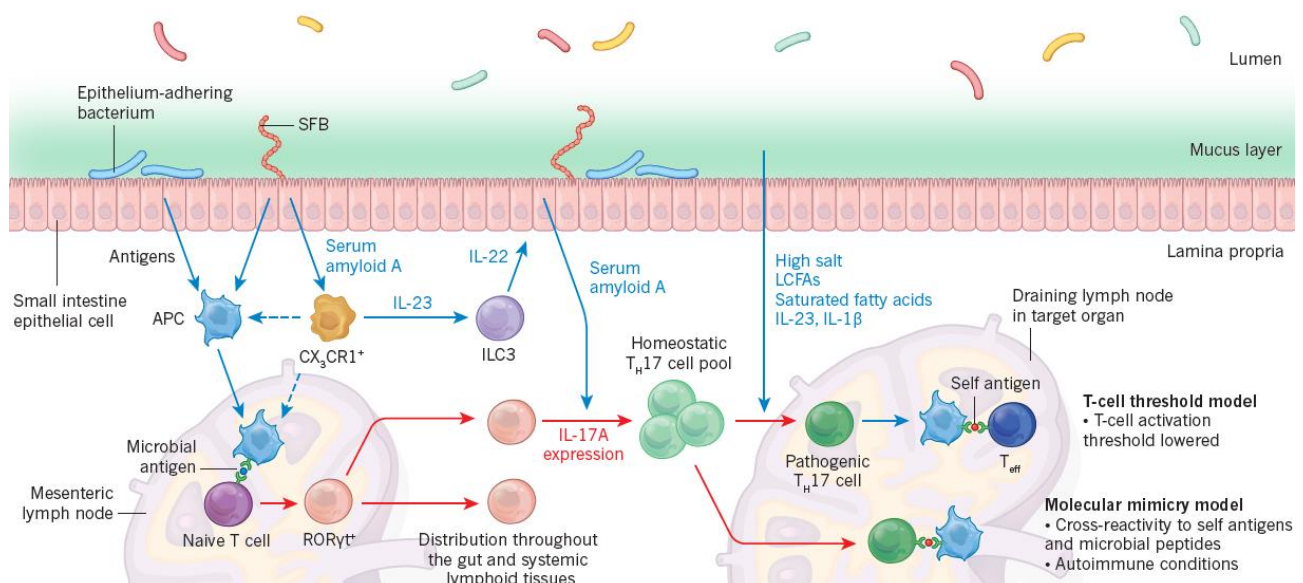


**Figure 5** Microbiota-mediated induction of Th17 cells (Honda & Littman, 2016)

According with Tan et al., *Bifidobacterium adolescentis* is similarly linked to Th17 induction, highlighting that Th17 regulation occurs through distinct pathways converging on similar objectives (Tan et al., 2016).

SFB colonization confers greater protection against pathogenic bacteria like *C. rodentium*, mediated partially by Th17 cytokines, especially IL-22, which supports antimicrobial peptide production, such as RegIII $\gamma$ , limiting pathogen growth (Ivanov et al., 2009). However, SFB-specific Th17 cells can also bring to autoimmune diseases like arthritis and encephalomyelitis (Y. K. Lee et al., 2011; H.-J. Wu et al., 2010). These pathogenic Th17 (pTh17) cells may migrate to the draining lymph nodes of target organs, promoting autoimmunity either through cross-reactivity between microbial peptides and host antigens (molecular mimicry model) or by lowering the activation threshold for self-reactive T cells, such as effector T cells (T-cell threshold model) (Honda & Littman, 2016) (Fig. 5). Thus, Th17 cells play a protective role in maintaining intestinal homeostasis while also having a central role in autoimmune and inflammatory conditions. Traditionally seen as pathogenic due to their association with various inflammatory disorders, Th17 cells can promote pro-inflammatory cytokines like IFN- $\gamma$  and GM-CSF upon stimulation with IL-23 and IL-1 $\beta$ , exacerbating inflammatory and autoimmune diseases (El-Behi et al., 2011; Harbour et al., 2015; Hirota et al., 2011). Preclinical models have shown that an increase in colonic pTh17 populations may drive tumorigenesis. Transferring colorectal cancer (CRC) patient microbiota to GF mice significantly increased Th17 cells compared to controls, promoting carcinogenesis (Wong et al., 2017).

Despite their potential pathogenicity, Th17 cells also fulfil a crucial homeostatic function in the intestine. In SPF animals, Th17 cells are among the most abundant CD4<sup>+</sup> T cells in the healthy intestinal lamina propria, exhibiting reduced expression of pro-inflammatory genes like *Tnf*, *Ifn $\gamma$* , and *IL-23 $\alpha$* , suggesting they do not actively participate in inflammatory processes. Instead, they express high levels of anti-inflammatory genes like *Ctla4*, *Icos*, and *IL-22*, which aid in regulating immune responses and protecting against inflammation and infection (Q. Zhao & Elson, 2018). This homeostatic role is further confirmed by their ability to promote IgA production in the GALT, converting themselves to Tfh cells under homeostatic conditions and interacting with B cells to induce IgA release (Hirota et al., 2013).

### ***Regulatory T-cells (Treg)***

The maintenance of tissue homeostasis in response to continuous immune stimulation by the microbiota necessitates both constitutive immunoregulatory responses, such as epithelial barrier

integrity and mucus secretion, and inducible responses that actively modulate interactions between the immune system and microbiota. In this context, regulatory T cells (Tregs) play a crucial role in preserving the delicate immune balance, maintaining both peripheral and mucosal homeostasis throughout the host's lifetime. Disruption of this balance in the gut can impair oral tolerance, leading to the development of aberrant immune responses (Dominguez-Villar & Hafler, 2018; Mucida et al., 2005).

The conventional education of T cells occurs through thymic selection, a mechanism in which immature T cells with high autoreactivity are either eliminated or converted into regulatory T cells, thus preventing autoimmunity. However, this concept has expanded to include “peripheral education” within the gut, mediated by commensal microbial antigens, a process essential for sustaining intestinal immune tolerance. In the gut, two types of Tregs exist: thymic-derived Tregs (tTregs) and peripheral-derived Tregs (pTregs), each with distinct TCR repertoires. Thanks to their collaboration, tolerance to commensal and environmental antigens can be maintained (Haribhai et al., 2011; Lathrop et al., 2011; Thornton et al., 2019). Intestinal Tregs can be categorized into three subtypes (Fig. 6):

- **Foxp3+RORyt+ cells:** These cells are characterized by the transcription factor c-MAF, CTLA-4 expression, and IL-10 release, allowing them to suppress aberrant activation of myeloid cells,  $\gamma\delta$  T cells, and Th17 cells (Huber et al., 2011; S.-G. Park et al., 2010). RORyt+ inactivation using Cre-Lox recombination induces a Th2-mediated inflammatory condition and promotes Th17 expansion (Ohnmacht et al., 2015; Sefik et al., 2015).
- **Foxp3+GATA3+ cells:** These thymic-derived cells are independent of the gut microbiota and express both the transcription factor GATA3 and the IL-33 receptor ST2. Their induction under inflammatory conditions is mediated by IL-33, which is released by epithelial cells. GATA3+ promotes the expression of Foxp3 and the ST2 receptor, amplifying Treg populations through a feed-forward mechanism (Schiering et al., 2014).
- **Foxp3+RORyt-Nrp1- cells:** Peripherally derived cells induced by dietary antigens; these cells are independent of the gut microbiota but disappear in antigen-free diets (K. S. Kim et al., 2016).

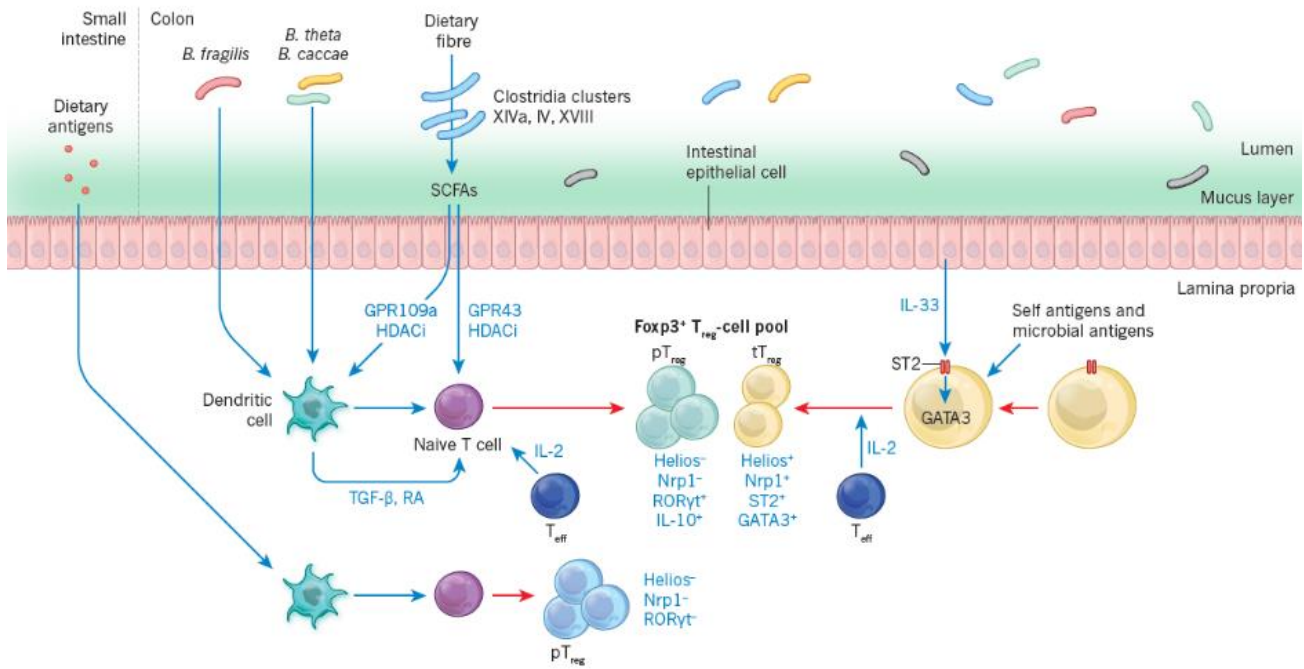


**Figure 6** Influence of gut microbiome on subsets of regulatory T cells (Tregs) (Honda & Littman, 2016)

The induction of Tregs modulated by the gut microbiome can occur via immunomodulatory cytokines or through microbial metabolites and products. One pathway for Treg induction involves the release of TGF- $\beta$  and retinoic acid (RA) by CD103<sup>+</sup> dendritic cells. This mechanism can support the induction of ROR $\gamma$ t<sup>+</sup> Tregs by Clostridia species, notably those from clusters IV, XIVa, and XVIII, which are known to modulate the immune environment within the intestine (Atarashi et al., 2011; Coombes et al., 2007; Ohnmacht et al., 2015). Oral administration of a consortium of 46 murine and 17 human Clostridia strains in mice induces a robust Treg response in the colon. Interestingly, when administered individually, these strains caused a much more moderate effect than the entire consortium (Atarashi et al., 2011, 2013; Ohnmacht et al., 2015). Induction could also occur through the release of SCFAs that are able to both inhibit histone deacetylase (HDAC) and promote the expression of the GPR109a and GPR43 receptor on dendritic cells and naive T-cells, respectively (Furusawa et al., 2013; Smith et al., 2013). In vitro, SCFAs upregulate GPR15, mediating Treg homing to the colon (S. V. Kim et al., 2013).

Similarly, certain Bacteroides species, like *B. fragilis*, induce ROR $\gamma$ t<sup>+</sup> Treg differentiation via direct and indirect mechanisms involving polysaccharide A (PSA). PSA can induce IL-10 release directly from Tregs or indirectly through PSA-containing outer membrane vesicles (OMVs) that engage TLR2 on lamina propria DCs. This process stimulates DCs to produce IL-10, which further promotes IL-10 production by Tregs (Sefik et al., 2015; Shen et al., 2012).

In conditions of immune homeostasis, the Treg/Th17 axis is critical, with Tregs maintaining tolerance toward dietary and commensal antigens and Th17 cells mounting responses to infections. Proper balance between these cells is essential to avoid both excessive inflammation and impaired immune response. The Th17/Treg axis is characterized by dynamic plasticity, allowing Tregs and Th17 cells to interconvert depending on environmental cues and immune conditions (Xu et al., 2018).

The regulatory role of Foxp3<sup>+</sup> Tregs also influences B cells and IgA release. Depletion of Tregs, for example through anti-CD25 antibodies, significantly reduces IgA B cells in the intestinal tissue, indicating that Tregs provide survival and support signals necessary for IgA production against commensal bacterial antigens (Cong et al., 2009).

### ***T follicular helper (Tfh) cells***

T follicular helper (Tfh) cells represent another subset of helper T cells essential in the immune crosstalk with the gut microbiota, promoting the maintenance of immune homeostasis. These cells are crucial for GC formation and maintenance, where they support B cell activation and immunoglobulin affinity maturation (Crotty, 2014). GF mouse models demonstrate that the absence of a microbiota reduces Tfh cell populations within GCs, underscoring the influence of commensals on Tfh cells (Kubinak et al., 2015).

Notably, SFB promote Tfh cell differentiation within PPs and their systemic dissemination. If unregulated, this process can lead to pathological conditions. Indeed, SFB limit interleukin-2 (IL-2) availability to CD4<sup>+</sup> T cells within PP. IL-2 typically supports Treg and Th17 differentiation: by limiting IL-2 access, SFB hinder these pathways and instead favor Tfh differentiation through increased Bcl-6 expression, a transcription factor pivotal for Tfh cell development (Teng et al., 2016).

The role of Tfh cells under homeostatic conditions is further illustrated by Ansaldo et al., who report adaptive immune responses induced by *Akkermansia muciniphila* colonization under homeostatic conditions. In murine models, colonization by *A. muciniphila* elicited a response dominated by Tfh cells over other T cell subsets. This suggests that specific commensals may induce Tfh cells, thereby modulating mucosal immunity, enhancing antibody production, and contributing to homeostasis (Ansaldo et al., 2019).

Microbial metabolites can also affect Tfh cell induction. For instance, microbe-derived ATP interacts with P2X7 ionotropic receptors, limiting Tfh cell expansion and consequently negatively affecting IgA production (Proietti et al., 2019).

It is also essential to recognize that Tfh cell and commensal interaction is bidirectional. Tfh cells can, in turn, influence gut microbiota composition. For example, in Bcl-6-deficient murine models, the absence of Tfh cells reduced microbial diversity, leading to an increased Firmicutes/Bacteroidetes ratio (Kawamoto et al., 2014).

### ***CD8+ T Cells***

IFN $\gamma$ -producing CD8+ T cells are significantly reduced in GF mice or those treated with ABX compared to SPF mice. Tanoue et al. demonstrated that the colonization of GF mice with a defined consortium of human commensal bacterial strains can restore the deficit of IFN $\gamma$ -producing CD8+ T cells in the colon. This finding indicates that commensal bacteria play a fundamental role in inducing CD8+ T cell activation and proliferation in the intestinal tract, thus contributing to a more robust immune response (Tanoue et al., 2019).

Furthermore, CD8+ cells can convert unidirectionally into CD4+ cells, a mechanism essential for promoting immune homeostasis. CD8+ T cells not only mediate immune responses against infections but, through their conversion to CD4+ T cells, actively contribute to the regulation and maintenance of intestinal homeostasis. Although this process is independent of commensals, the presence of cognate bacteria can facilitate the expansion of converted CD4+ T cells, increasing their numbers and contributing to a more robust and balanced immune response. This cellular plasticity is therefore crucial for sustaining a stable equilibrium between immune tolerance and responsiveness (Lui et al., 2015).

### ***CD4+ T cells***

Although the understanding of the specific role of the commensal microbiota in the differentiation of Th1 and Th2 cells remains limited, some studies have highlighted a tendency for the microbiota, under homeostatic conditions, to induce Th1 responses at the expense of Th2 responses (Q. Zhao & Elson, 2018). Several studies have demonstrated that certain probiotic strains of *Lactobacillus* are positively correlated with Th1 cells, leading to the release of TNF $\alpha$  and IFN $\gamma$ , while the release of IL-4 is reduced (Matsusaki et al., 2016; Takeda et al., 2011). In agreement with this, Mazmanian et al. reported that in GF mice, the immune response was skewed toward Th2, with a reduced Th1 response. Colonization of GF mice with *Bacteroides fragilis* induced the activation of Th1 cells following the recognition and presentation of polysaccharide A (PSA) via MHCII, correcting the imbalance between Th1 and Th2 cells (Mazmanian et al., 2005). Microbial metabolites, such as SCFAs, also promote Th1-mediated responses: they not only enhance IL-10 production in Th1 cells

through activation of the GPR43 receptor but also stimulate the expansion of the transcription factor T-bet, which is crucial for Th1 cell function (J. Park et al., 2016).

### **1.3. Gut microbiota impact on anticancer therapy outcomes**

Drug resistance and toxicity represent two of the major challenges in cancer treatment, often limiting the effectiveness of oncological therapies and compromising patients' quality of life. Recent studies have revealed that the gut microbiome not only plays a role in cancer development and progression but can also significantly influence the outcome of anticancer treatments (Blake et al., 2024; Fernandes et al., 2022; Ting et al., 2022; Zhao et al., 2023).

This involvement has become evident through the use of germ-free (GF) animal models, as well as models in which the gut microbiota has been depleted using broad-spectrum ABX. In these models, the efficacy of chemotherapeutic drugs and immune checkpoint inhibitors is reduced, suggesting a key role of the microbiota in modulating the response to anticancer therapy (Zitvogel et al., 2018). Supporting these preclinical experimental findings, several independent retrospective analyses in human cohorts with metastatic cancers of the lung, kidney, and bladder have identified the detrimental impact associated with certain classes of ABX administered at the onset of mAb treatment targeting PD1/PDL-1 (Daillère et al., 2016). The most compelling evidence for the importance of the gut microbiota in the efficacy of anticancer treatments arises from studies based on the metagenomic sequencing of baseline stool samples from patients. Advances in sequencing technologies, such as 16S ribosomal RNA amplicon sequencing and shotgun DNA sequencing, now allow patients to be stratified according to their metagenomic profiles. By analyzing pre-treatment fecal samples, specific commensal bacteria have been identified as more abundant in patients who respond to therapy compared to non-responders. Furthermore, some studies have demonstrated that lower alpha diversity or reduced bacterial richness in fecal composition correlates with a detrimental prognosis and a decreased patient survival (Alves et al., 2024; Thomas et al., 2023).

Additional evidence supporting the causal role of the microbiome in the effectiveness of antitumor therapies comes from studies on fecal microbiota transplantation (FMT), where FMT refers to the administration of biological material from donors to recipients. FMT of the intestinal microbiota from patients can humanize the gut microbiota of recipient mice, creating a human microbiota "avatar." It has been observed that GF mice or antibiotic-treated mice, after receiving fecal samples from non-responder patients, developed a response profile similar to that of the donors, characterized by reduced therapeutic efficacy, leading to a non-responder "avatar" mouse status. Conversely, the

transfer of fecal samples through FMT from responder patients or oral gavage with commensal microbial species associated with a favorable treatment response converted GF mice into responder "avatar" mice and was also able to restore therapeutic efficacy of non-responder "avatar" mice. These findings highlight the microbiota's capacity to modulate antitumor therapy responsiveness, offering new prospects for improving therapeutic strategies through microbiota manipulation (Zitvogel et al., 2018).

To fully harness the clinical potential of the relationship between the microbiota and anticancer therapies, it is crucial to understand the mechanisms underlying this interaction, such as microbial metabolism and immunomodulation of the TME (Zitvogel et al., 2016).

The gut microbiome can actively participate in drug metabolism, influencing drug bioavailability and consequently impacting both therapeutic efficacy and the side effect profile (Roy & Trinchieri, 2017). A prominent example of this interaction is seen with the chemotherapeutic agent irinotecan, whose gastrointestinal toxicity is attributed to microbial  $\beta$ -glucuronidase. This enzyme reactivates the drug by converting it back into an active form, which then causes damage to the intestinal mucosa. This example illustrates how microbial composition influences not only chemotherapy effectiveness but also its tolerability (Fujita & Sparreboom, 2010; Wallace et al., 2015).

Beyond metabolic effects, one of the key mechanisms through which the microbiota impacts therapeutic outcomes and side effects is by immunomodulating the TME. This effect has been observed in both chemotherapy and immunotherapy contexts. It is now well recognized that the clinical activity of various chemotherapies involves stimulation of antitumor immunity by either inducing the release of immunostimulatory molecules from tumor cells or by off-target effects on immune cell populations. Thus, the gut microbiota can act as a sensitizing agent, enhancing the efficacy of chemo-immunotherapy. We can conclude that the long-term effects of antitumor drugs are not solely attributable to their direct action on tumor cells but require an immune component. In this context, the microbiome appears to play a crucial role by modulating both the immune response and the TME (Galluzzi et al., 2020; Zitvogel et al., 2016).

The subtle mechanisms that explain the contribution of the microbiome to therapy-induced antitumor immune responses may differ for each treatment modality. In general, these mechanisms can be distinguished into two main categories based on the site of activation and stimulation of innate or adaptive immune cells by commensal bacteria. (1) On one hand, the activation of anti-commensal immune responses occurs at the level of the intestinal mucosa (Zitvogel et al., 2024). (2) On the other hand, bacterial translocation or translocation of bacterial products across the

intestinal epithelium results in an immunomodulatory response directly activated within the TME (Blake et al., 2024).

In both modalities, the microbiome's adjuvanticity mechanism emerges as central to modulating the intestinal antitumor immune response. However, a third mechanism, known as cross-reactivity, has been proposed as a potential additional contribution. While highly intriguing, the number of reports illustrating this latter mechanism and its influence on therapy remains limited and will be discussed in detail in the following chapter, where it will be compared to adjuvanticity mechanisms.

### **1.3.1 Tumor microenvironment immunomodulation through anti-commensal immune activation in the intestinal mucosa**

Contrary to the notion that the immune system is strictly compartmentalized among distinct organs (Kroemer et al., 1993), more recent studies have demonstrated that a variety of immune cells can circulate between the intestine and extraintestinal sites and vice versa. The concept of a "common mucosal immune system" supports the hypothesis that bacteria-specific immune cells, once activated in intestinal mesenteric lymph nodes, can circulate and migrate to extraintestinal sites where they can exert immunomodulatory actions (Galván-Peña et al., 2024). Consequently, the intestinal microbiota ecosystem can regulate not only intestinal immune homeostasis but also the inflammatory and/or immune tone of secondary lymphoid organs, contributing to shaping the TME (Daillère et al., 2016).

This section will explore the mechanisms of adjuvanticity and antigenicity, providing examples of therapeutic approaches that exploit microbiota-mediated immunomodulation to enhance cancer treatments.

#### **1.3.1.1 Adjuvanticity**

Adjuvanticity represents a strategy through which commensal microbes contribute to modulating the immune tone of the TME, thereby indirectly influencing the efficacy of oncological therapies by regulating inflammation and antitumor immune responses. TME immunomodulation by gut microbiota involves both innate and adaptive immune system cells (Zitvogel et al., 2016).

An emblematic example of microbiota-mediated innate and adaptive immunomodulation supporting chemotherapy is oxaliplatin. Although DNA-bound drug levels in GF mice were comparable to those in control mice with an intact microbiota, the absence of microbiota was

associated with reduced treatment efficacy and decreased expression of pro-inflammatory genes, particularly those encoding NADPH oxidase 2 (NOX2). NOX2, expressed on tumor-infiltrating macrophages and neutrophils, is responsible for producing reactive oxygen species (ROS) that contribute to oxaliplatin's genotoxic action. The interaction of commensals with TLR4 receptors, via MyD88-dependent pathways, appears to stimulate NOX2 expression; the absence of commensals thus leads to reduced ROS release by phagocytic cells and a consequent compromised treatment efficacy (Iida et al., 2013). In addition to modulating innate immune responses through phagocytic cells, the microbiota supports oxaliplatin's antitumor action by promoting adaptive immune responses through T-cell polarization into Tfh cells within the TME. Specifically, oxaliplatin-induced apoptosis of ileal epithelial cells in colorectal cancer treatment triggers the release of tumor antigens that can be recognized by the immune system. Activated DCs present these antigens and promote the differentiation of T cells into Tfh cells, which can subsequently stimulate B cells to produce antibodies and contribute to immunosurveillance against tumor cells (Roberti et al., 2020).

Roberti et al. study demonstrates that oxaliplatin modulates the intestinal microbial composition, favoring immunogenic bacteria such as *Bacteroides fragilis* and members of the *Erysipelotrichaceae* family. These immunogenic bacteria induce activation of DCs, which can migrate to tumor-draining lymph nodes (TdLNs), where they promote a T-cell response polarized toward Tfh cells at the expense of Th17 cells. DCs activated by immunogenic bacteria not only enhance tumor antigen presentation but also create an immunogenic environment that fosters a more robust and lasting immune response against cancer. The synergy between oxaliplatin-induced apoptosis and the presence of immunogenic commensals fosters an antitumor environment, enhancing the immune response (Roberti et al., 2020).

The ability of immunogenic bacteria to enhance the action of DCs in antitumor antigen presentation also emerges in the context of anti-PD-L1 immunotherapy, as reported by Sivan et al. The involvement of the microbiota in the outcome of the antitumor response was confirmed through subcutaneous melanoma mouse models and co-housing experiments. Notably, the presence of *Bifidobacterium longum* was associated with an accumulation of tumor-infiltrating CD8<sup>+</sup> T cells and increased IFN $\gamma$  release, attributable to more efficient priming by bacterium-activated DCs (Sivan et al., 2015). The role of commensals in boosting CD8<sup>+</sup> T cells infiltrating the tumor to support therapy has also been confirmed in neoadjuvant chemotherapy (NACI) for resectable esophageal squamous cell carcinoma (ESCC). *Streptococcus* enrichment in responders was positively correlated with the

infiltration of GrzB+ and CD8+ T cells into tumor tissues accompanied by a lower rate of Treg (H. Wu et al., 2023).

The gut microbiota's immunomodulatory action supporting antitumor treatment can also involve CD4+ T cells. For example, Di Modica et al. observed in broad-spectrum antibiotic-treated murine models and avatar mice that the efficacy of trastuzumab in HER2+ breast cancer is reduced. Indeed, the ABX causes the creation of an immunosuppressive TME by reducing DCs activation in lymph nodes and diminishing the recruitment of CD4+ T cells and NK cells at the tumor level. Consistently, a reduction in IL-12p70 levels—a cytokine critical for trastuzumab efficacy—was detected in plasma (Di Modica et al., 2021).

The involvement of CD4+ T cells has also been confirmed in treatments with anti-PD-1 and anti-CTLA-4. In the case of anti-PD-1, oral administration of *Akkermansia muciniphila* in non-responder avatar mice restored treatment efficacy, promoting tumor recruitment of CCR9+CXCR3+CD4+ T lymphocytes in an IL-12-dependent manner (Routy et al., 2018). In the case of anti-CTLA-4 (Ipilimumab), gut microbiota depletion in GF or antibiotic-treated sarcoma mouse models resulted in a less effective drug. Beyond impaired tumor progression control, a reduction in activation of splenic CD4+ effectors as well as tumor-infiltrating lymphocytes (TILS) was observed. The antitumor response in microbiota-depleted mice was restored through recolonization with *Bacteroides fragilis* and *Bacteroides thetaiotaomicron*. Interestingly, Ipilimumab induced changes in the intestinal microbiota composition, favoring precisely the colonization of immunogenic species belonging to the orders *Bacteroidales* and *Burkholderiales*, such as *B. fragilis*, *B. thetaiotaomicron*, and *B. cepacia*, which thus appear to directly contribute to its therapeutic efficacy. In particular, *B. fragilis*, in addition to promoting the maturation of intratumoral DCs, resulted in the accumulation of Th1 memory lymphocytes in TdLNs: its antitumor action can be traced back to these memory cells. Indeed, the injection of these *B. fragilis*-specific memory Th1 cells into GF mice partially restored Ipilimumab's antitumor efficacy. Beyond an adjuvant effect, it is hypothesized that antigenic cross-reactivity allows Th1 cells activated by *B. fragilis* to reach the tumor environment and recognize tumor epitopes resembling commensal antigens (Vétizou et al., 2015).

### **1.3.1.2 Antigenicity**

Antigenicity is a strategy whereby commensal immune cells induce a direct immune response against tumors, mediated by mechanisms of molecular mimicry and cross-reactivity. In this process,

anti-commensal immune cells recognize not only microbial antigen epitopes but also tumor-associated antigen (TAA) epitopes (Zitvogel & Kroemer, 2022).

In the previous chapter, with the study of Vétizou et al. the concept of cross-reactivity was induced, whereby anti-commensal memory T cells are capable of recognizing non-microbial tumor antigens (Vétizou et al., 2015). This hypothesis is based on the idea that the commensal microbiota, as the largest source of non-self antigens to which the host is exposed, might contain antigens sufficiently similar to tumor antigens to activate cross-reactive T cells. This phenomenon, known as molecular mimicry, implies that microbial and tumor antigens share structurally similar epitopes, enabling the activation of bacteria-specific T cells against tumors as well. In the oncological context, however, it is essential to distinguish these cross-reactive T cells from other T cell populations. These include T cells that respond to oncogenic viruses and “bystander” tumor-infiltrating T cells, which recognize bacterial antigens due to prior infections and are only occasionally present in the TME. Currently, there are only a few reports documenting cross-reactive T cells that recognize both tumor antigens and non-pathogenic intestinal commensals (Alves et al., 2024b; Blake et al., 2024a; Zitvogel & Kroemer, 2022).

For instance, in the study by Bessel et al., a B16-F10 melanoma mouse model was utilized, modified to express a neoantigenic peptide (SIYRYYGL) capable of binding the MHC Class-I Kb complex. In this context, a peptide derived from the intestinal commensal *Bifidobacterium breve* was identified, differing from the tumor neoantigen by only a single amino acid. This enabled the microbial peptide SVYRYYGL to elicit a robust immune response mediated by cross-reactive CD8<sup>+</sup> T cells against the tumor, leading to a reduction in melanoma growth. Naturally, the efficacy of this response depended on the expression of the neoantigenic epitope by the tumor (Bessell et al., 2020) (Fig.7).

Another recent study shows that in the prophage of a specific strain of *Enterococcus hirae*, the tail length tape measure protein (TMP) may contain peptides capable of eliciting immune responses against unmutated tumor antigens in both murine and human models. Specifically, the microbial peptide TSLARFANI, which binds to MHC Class-I Kb, has been shown to induce a cross-reactive CD8<sup>+</sup>-mediated immune response against MCA205 fibrosarcomas by recognizing a similar peptide present in the oncogenic protein PSMB4. Similarly, another microbial peptide from TMP has been shown to activate immune responses against a non-mutated tumor antigen within the GPD1-L protein, associated with adenocarcinomas and renal cancer. Interestingly, the presence of *Enterococcus* expressing TMP was correlated with favorable prognoses in patients treated with PD-1 inhibitors (Fluckiger et al., 2020) (Fig.7).

These findings strongly suggest the clinical relevance of cross-reactivity between commensal and tumor antigens, highlighting that immune responses induced by intestinal microbiota may play a significant role in cancer immune surveillance.

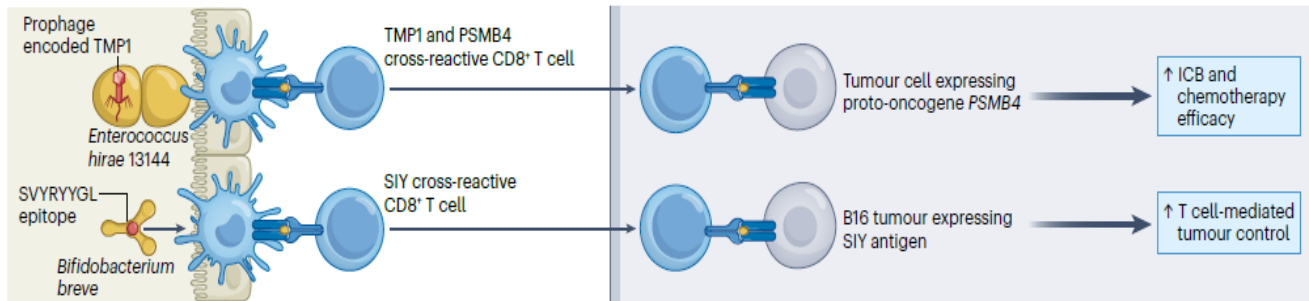


**Figure 7** Cross reactivity antigens (Adapted from Blake et al., 2024)

### 1.3.2 Tumor microenvironment immunomodulation mediated by bacteria and bacterial products translocated through the intestinal epithelium

#### *Bacterial Translocation*

The mucosal barrier creates a "demilitarized" environment that aims to prevent bacteria from crossing it and triggering systemic immune responses. Intestinal permeability has been associated with diseases such as chronic inflammatory conditions, including IBD and Crohn's disease, and appears to be linked to carcinogenesis (Belkaid & Hand, 2014; Yonekura et al., 2022). Actually, the translocation of pathobionts like *Fusobacterium nucleatum* into tumor sites, particularly in colorectal cancer, contributes to the formation of an immunosuppressive environment that undermines the effectiveness of immunotherapeutic treatments. *F. nucleatum* reduces the antitumor activity of CD8<sup>+</sup> and NK cells through two main mechanisms (Fig. 8). On one hand, it produces succinic acid, which inhibits the cGAS-interferon- $\beta$  pathway, thereby reducing the presence of CD8<sup>+</sup> cells in the TME (Jiang et al., 2023). On the other hand, the *F. nucleatum* surface protein FAP2 facilitates immune evasion by binding to the inhibitory receptor TIGIT, whose activation suppresses T and NK cell activity (Gur et al., 2015). Furthermore, a study by Yonekura et al. revealed that the tumor itself can impair the integrity of the epithelial barrier. This occurs through the ileal mucosal atrophy and constriction of the villous microcirculation, with a predominance of sympathetic over cholinergic signaling. Tumorigenesis causes a sudden and excessive release of REGIII $\gamma$  by ileal cells temporarily making the epithelial barrier permeable, resulting in overt and long-lasting dysbiosis characterized by an increase in Gram-positive *Clostridium* species (Yonekura et al., 2022).

However, there are examples of bacterial translocation that promote the efficacy of antitumor therapy (Fig.8). Chemotherapy with cyclophosphamide (CTX), for instance, compromises the integrity of the intestinal epithelium, facilitating the translocation of Gram-positive bacteria into secondary lymphoid organs. Specifically, bacteria such as *Lactobacillus johnsonii* and *Enterococcus hirae* were detected in varying amounts in the lymph nodes of mice treated with CTX, alongside observed alterations in the composition of the commensal microbiota (Viaud et al., 2013). To evaluate the role of the microbiota in CTX responsiveness, Daillère et al. employed murine sarcoma models treated with ABX, demonstrating that the absence of commensals impaired CTX efficacy (Daillère et al., 2016). However, subsequent oral administration of *E. hirae* selectively restored the antitumor effects of CTX. Indeed, *E. hirae* induced the proliferation of  $\gamma\delta$  T cells and CD8<sup>+</sup> T cells in the intestinal mucosa, as well as induced hyperplasia of mucin-producing calyciform cells and lysozyme-containing Paneth cells. These changes suggest an enhancement of the local immune response. Following colonization, splenocyte analysis revealed that *E. hirae* induced pTh17 and Th1 responses, while in the TME, it reduced the number of tolerogenic Tregs. The increased CD8<sup>+</sup>/Treg ratio induced by *E. hirae* consequently increased the immune tone within the TME. Thus, the translocation of “oncomicrobiotics,” such as *E. hirae*, can orchestrate the anticancer therapeutic effects of chemotherapy (Daillère et al., 2016).

Immunotherapeutic treatments have also been shown to promote bacterial translocation to TdLNs, a phenomenon that enhances the efficacy of immune checkpoint blockade (ICB). In murine melanoma models treated with anti-CTLA4 and anti-PD1 antibodies, a translocation process of live bacteria—including *Enterococcus faecalis*, *Escherichia coli*, and *Lactobacillus johnsonii*—mediated by DCs was observed. These DCs transported live bacteria from mesenteric to tumor-draining lymph nodes and even to the tumor itself, resulting in enhanced antitumor responses mediated by CD8<sup>+</sup> T cells. This bacterial translocation was facilitated by “lymph node remodeling” induced by ICB, a process characterized by venular enlargement that supported DC migration. Although inflammation, with the resulting increase in intestinal permeability, could promote translocation, it was not a prerequisite for its occurrence; instead, the expression of CCR7 on DCs, crucial for homing to tumor-draining lymph nodes, was essential for this process (Choi et al., 2023).

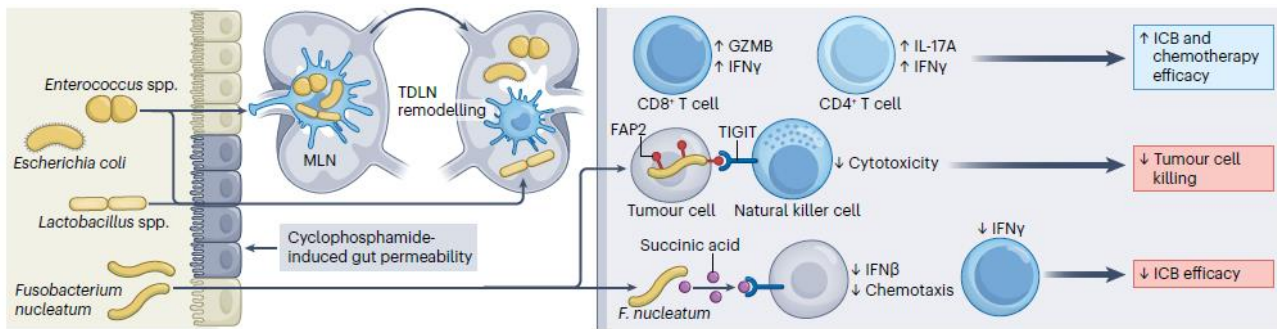


**Figure 8** Immunomodulation Mediated by Bacterial Translocation in Tumor-Draining Lymph Nodes or Tumors (Adapted from Blake et al., 2024)

### Immunomodulatory Bacterial Metabolites

The microbial metabolism has the potential to generate molecules capable of modulating the immune tone of the TME and, consequently, influencing therapeutic response (Blake et al., 2024) (Fig. 9).

A notable example is the production of inosine by *Bifidobacterium pseudolongum*, which enhances the efficacy of anti-PD-L1 and anti-CTLA-4 treatments in various tumor types, including CRC, bladder cancer, and melanoma. Indeed, inosine supports antitumor immunosurveillance by interacting with the adenosine A2A receptor (A2AR) expressed on T lymphocytes: the release of IL12Rβ2 and IFN-γ promotes the differentiation of Th1 cells and their recruitment to the TME (Mager et al., 2020). Furthermore, inosine has been shown to enhance the immunogenicity of tumor cells by facilitating the recognition of TAAs by tumor-specific T lymphocytes in addition to serving as a carbon source for CD8+ T cells (Allard et al., 2020; Ma et al., 2020).

Similarly, tryptophan metabolites released by *Lactobacillus reuteri* support antitumor immunosurveillance in anti-PD-L1 treatments. Daily administration of this bacterium improves tumor growth control in B16-F0 melanoma through the secretion of indole-3-aldehyde (I3A), which, by interacting with the aryl hydrocarbon receptor (AhR), increases intratumoral CD8+ T-cell activity (Bender et al., 2023). Conversely, in pancreatic ductal adenocarcinoma (PDAC), the interaction of tryptophan metabolites with AhR, expressed on macrophages, reduces the efficacy of ICB therapy (Hezaveh et al., 2022). These findings suggest that the effects of tryptophan metabolites depend on both the tumor context and type.

Tryptophan metabolites have also demonstrated the ability to reduce the immunotoxicity of oncological therapies. For instance, administration of indole-3-carboxaldehyde (3-IAld) in murine models of ICB-induced colitis protected mice from intestinal damage by activating the AhR/IL-22

pathway and promoting host colonization by butyrate-producing bacteria, a short-chain fatty acid (SCFA) derived from dietary fiber metabolism. This study suggests that 3-IAld regulates butyrate levels to optimize its benefits, maintaining protective effects on the epithelium without compromising the antitumor efficacy of anti-CTLA-4 antibodies (Renga et al., 2022).

Butyrate is one of the most extensively studied SCFAs in the context of antitumor treatment as well as in the management of immune-related adverse events (irAEs). This metabolite has traditionally been associated with favorable outcomes in immunotherapy. Among the mechanisms by which butyrate promotes antitumor immunosurveillance, one of these is based on the enhancement of CD8<sup>+</sup> T cell cytotoxic activity by inhibiting IL-12-dependent signaling mediated by DNA-binding inhibitor 2 (ID2) (He et al., 2021). Regarding irAE mitigation, butyrate-producing bacteria have been shown to reduce cardiotoxicity associated with anti-PD-1/PD-L1 treatment (Y. Chen et al., 2022) and to decrease the incidence of graft-versus-host disease (GVHD) (Fujiwara et al., 2018). However, studies highlight a duality in butyrate's action: on one hand, it can modulate the induction and severity of colitis through Treg cell activation (Fujiwara et al., 2018); on the other hand, this same activation could induce an immunosuppressive response against the tumor (Smith et al., 2013). Additionally, high concentrations of SCFAs such as butyrate and propionate have been associated with reduced efficacy of CTLA-4 blockade (Coutzac et al., 2020).

In conclusion, although the impact of microbial metabolites on the TME and antitumor treatments is evident, their often-dichotomous effects, dependent on the tumor context and type, underscore the need for further studies to fully understand the complex mechanisms involved and to develop targeted therapeutic approaches.

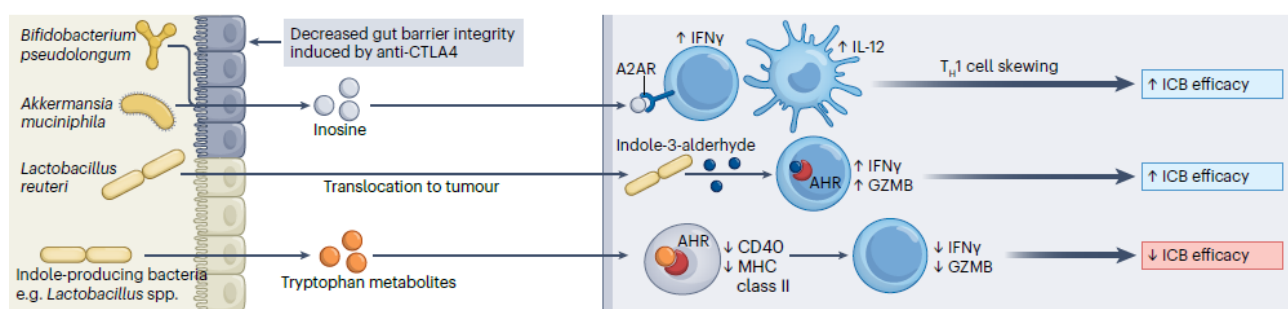


**Figure 9** Immunomodulatory bacterial metabolites (Adapted from Blake et al., 2024)

## **I.4. Gut microbiota-related biomarkers**

Resistance to oncological therapies is one of the major challenges in oncology, making optimisation of therapy selection for each individual patient crucial. In light of the influence of microbiota on cancer therapy outcomes, microbiota-based prognostic or predictive biomarkers emerge as promising tools to accurately select patients most suitable for specific oncological treatments. Furthermore, these biomarkers could play a key role in effectively integrating novel microbiota modulation strategies (microbiota-centred interventions) into clinical practice.

In this chapter, we identify some of the major gut microbiota-associated biomarkers that could potentially be translated into simple diagnostic tools to guide routine treatment decisions in immuno-oncology. These biomarkers open new perspectives for personalised and precision-based medicine centred on the microbiota.

### **I.4.1 Fecal metagenomics**

The role of the gut microbiota in influencing therapy outcomes has been confirmed through metagenomic profiling of intestinal microbiota performed on faecal samples from cancer patients. Next-generation sequencing (NGS) approaches have enabled the identification of microbial signatures associated with disease progression, supporting the hypothesis that cancer patients treated with antitumor therapies can be categorized as responders or non-responders based on their gut microbiota composition (Daillère et al., 2020).

The predictive biomarker value of gut microbiota has been proposed in various cancer patient cohorts. Many of these cohorts were included in a comprehensive meta-analysis that taxonomically profiled the fecal microbiota of 1,879 adults with eight cancer types (colon, kidney, breast, lung, ovarian, prostate, melanoma, and chronic leukemia), comparing them to a control group of 5,341 healthy individuals. Statistically significant differences in microbiota taxonomic profiles clearly distinguished cancer patients from healthy subjects, confirming dysbiosis as a hallmark syndrome of cancer and leading to the identification of Gut OncoMicrobiome Signatures (GOMS) (Thomas et al., 2023). Gram-positive bacteria such as *Enterocloster*, *Clostridium* spp., and *Eisenbergiella* spp. were predominant in six of the eight cancer types analyzed. Conversely, bacterial taxa belonging to the families *Ruminococcaceae*, *Prevotellaceae*, and *Lachnospiraceae*, as well as species from the genera *Faecalibacterium*, *Akkermansia*, and *Bifidobacterium*, were overrepresented in individuals without cancer. Interestingly, taxa dominant in healthy individuals were associated with better outcomes in antitumor therapy, particularly immunotherapy. Thus, GOMS hold dual value: as prognostic

biomarkers—identifying clinical events such as disease progression or mortality independently of therapy—and as predictive biomarkers, providing insights into the likelihood of benefiting from specific therapeutic interventions (Thomas et al., 2023).

For instance, *Faecalibacterium prausnitzii*, within the context of melanoma treatment using the anti-CTLA-4 antibody ipilimumab, has been associated with longer progression free survival (PFS) and lower levels of circulating Tregs (Chaput et al., 2017; Frankel et al., 2017). Its antitumor immunomodulatory action and role in maintaining intestinal homeostasis were further confirmed following treatment with DCs-based immunotherapy. The presence of *F. prausnitzii* correlates with an increase in *CD4+ ICOS+* T cells and serum levels of sCD25 (Alves Costa Silva et al., 2024).

A significant example of the prognostic and predictive value of intestinal microbial signatures is represented by *Erysipelotrichaceae ramosum*, an opportunistic pathogen associated with CRC. A recent study highlighted that *E. ramosum* is present in significantly higher amounts in fecal and intestinal mucosal samples of CRC patients compared to those of healthy individuals or patients with adenomatous polyps. This finding demonstrates how imbalances in commensal bacteria may converge towards tumour pathogenesis and lays the foundation for the identification of a biomarker for CRC screening. Indeed, quantification of this bacterium in stool samples via qPCR can predict CRC with a specificity of 72.7% and a sensitivity of 64.7% (Iadsee et al., 2023). Moreover, as discussed in the previous chapter, the *Erysipelotrichaceae* family has been shown to enhance oxaliplatin-based chemotherapy in CRC by improving TME immune tone. While large-scale validation studies are needed, the quantification of *E. ramosum* using qPCR could serve not only as a prognostic biomarker but also as a predictive tool to optimize oncological therapies (Roberti et al., 2020).

It is essential, however, to emphasize that despite the potential of GOMS in clinical practice, several confounding factors contribute to variability, including DNA extraction and sequencing methodologies, cohort sizes, age, sex, geography, diet, lifestyle, comorbidities, and concomitant medications. The effectiveness of GOMS may vary depending on the cancer type, disease stage, or specific therapies used. Therefore, broader and more rigorous validation of GOMS across diverse cohorts and prospective clinical trials is required to confirm their utility as biomarkers (Thomas et al., 2023).

For instance, the need to validate the predictive value of *Akkermansia muciniphila* has highlighted its prognostic and predictive superiority over PD-L1 in NSCLC immunotherapy. Retrospective cohorts of RCC and NSCLC patients identified *A. muciniphila* as being associated with long-term benefits following anti-PD-1 treatment (Derosa et al., 2020; Routy et al., 2018). Studies using avatar mice

confirmed its predictive role by demonstrating its capacity to restore the efficacy of antitumor drugs after ABX treatment. A prospective validation study by Derosa et al. in an NSCLC patient cohort (n=338) showed that the relative abundance of *A. muciniphila* influenced prognosis more than its simple presence or absence (Derosa et al., 2022). A trichotomous stratification of patients into Akk–, Akk low, and Akk high (absence, relative abundance <4.8%, and >4.8%) proved to be a more accurate independent prognostic factor than a binary Akk– versus Akk+ division. While the presence of *A. muciniphila* correlated with improved treatment response and survival, its overrepresentation resulted in negative clinical outcomes comparable to the absence of the bacterium. Normal levels of *A. muciniphila* (Akk low) were associated with better intestinal fitness and a highly diversified gut microbiota dominated by *Lachnospiraceae* and *Oscillospiraceae* families. Conversely, the taxonomic composition of fecal samples from patients with either absent or overrepresented *A. muciniphila* showed a tolerogenic microbiota dominated by *Clostridium* species at the expense of healthy commensals (Derosa et al., 2022). Pathobiontic species of the *Clostridium* genus, enriched by ABX use and metastatic cancer states, were further associated with the loss of intestinal epithelial barrier integrity and tumorigenesis-induced dysbiosis (Yonekura et al., 2022).

These results not only suggest a predictive biomarker of treatment response but also indicate that the relative abundance of *A. muciniphila* is itself associated with specific microbiota compositions. Although understanding microbial interactions within communities remains limited, identifying guilds, functional groups of diverse but cooperative bacteria, could be a promising approach for developing diagnostic tools to assess intestinal dysbiosis in routine oncological management (Derosa et al., 2021).

Metagenomic analysis of fecal samples from 245 NSCLC patients constructed a co-abundance network that defined microbial sub-consortia, termed species-species interaction groups (SIGs). Two SIGs guided the response to anti-PD-1 treatment: SIG1 comprised 37 bacteria associated with poor responses, while SIG2 included 45 species linked to favorable outcomes. The number of species from each SIG in individual fecal samples enabled the calculation of a SIG1/SIG2 ratio. Combined with the trichotomous abundance analysis of *A. muciniphila*, this ratio defined the topological score (TOPOSCORE) (Derosa et al., 2024). This predictive biomarker of immunotherapy response and dysbiosis was validated in a second NSCLC cohort (n=254) and a third cohort of RCC and urothelial carcinoma (UC) patients (n=216). The TOPOSCORE was later condensed to 21 microbial genome species (MGS), validated across NSCLC, CRC, and melanoma cohorts, and adapted into a user-friendly qPCR-based test with a 48-hour turnaround, suitable for routine use. Despite some biases—

such as the transition from metagenomic analysis to qPCR, the selection of cancer histological types, treatments, or the need for serial sampling to confirm biomarker stability—these holistic approaches, which avoid simply categorizing bacteria as positive or negative, open new opportunities for developing predictive microbiota-based scores to better guide precision medicine (Derosa et al., 2024).

In conclusion, while fecal metagenomic biomarkers hold substantial predictive and prognostic potential, further validation is needed to integrate them effectively into clinical practice.

#### **1.4.2 Gut-vascular barrier (GVB) related biomarkers**

The gastrointestinal vascular barrier (GVB) constitutes the innermost defensive layer of the intestinal mucosa. Located beneath the lamina propria, under homeostatic conditions, it prevents the systemic dissemination of commensal bacteria and their toxins, thereby averting systemic immune reactions. At the same time, it maintains selective permeability to allow, for example, the diffusion of nutrients from the intestinal lumen (Di Sabatino et al., 2023).

The GVB's semipermeable structure arises from a fenestrated endothelium coated with plasmalemmal vesicle-associated protein-1 (PV-1) (Di Sabatino et al., 2023). The expression of PV-1 correlates with increased vascular permeability, facilitating the translocation of bacteria such as *E. coli* to the liver and contributing to the formation of premetastatic niches in CRC. Furthermore, bacterial factors like Virf1 can modulate PV-1 expression, suggesting its potential role as a biomarker for CRC recurrence (Bertocchi et al., 2021).

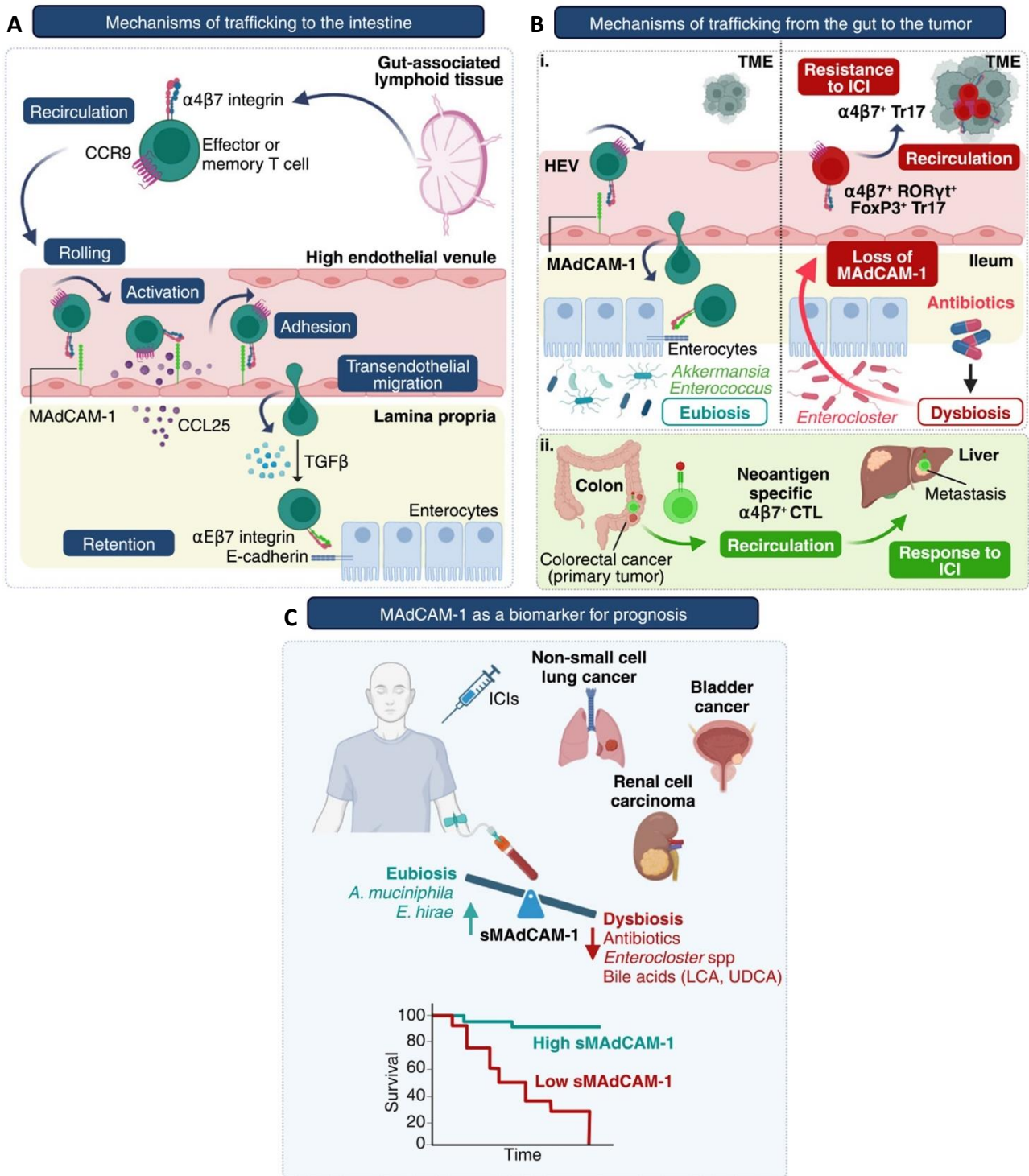
The GVB's semipermeability is also critical for immune cell migration, such as CD103<sup>+</sup> dendritic cells, that need to migrate to mesenteric lymph nodes to sustain immune homeostasis (Di Sabatino et al., 2023). Several mechanisms mediate the trafficking of leucocytes to and from the gut. One of the main mechanisms of intestinal *homing* involves mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1), which is specifically expressed on high endothelial venules (HEVs) of the intestinal tract, in mLNs and in PPs. MAdCAM-1 interacts with the  $\alpha 4\beta 7$  integrin expressed by lymphocytes migrating to the intestine. This interaction ensures leukocyte adhesion to HEVs and subsequent transendothelial migration, ensuring their retention within the intestinal environment. Intestinal homing is further supported by the release of endothelial chemokines such as CCL28 and CCL25, which interact with GPR15 in the colon and CCR9 in the small intestine, respectively. Additionally, exposure to TGF- $\beta$ 1 induces CD103<sup>+</sup> expression, which, through its interaction with E-cadherin, ensures the anchoring of T cells to the intestinal epithelium. Disruption of this retention mechanism

can lead to uncontrolled leukocyte trafficking (Berlin et al., 1993; Kunkel & Butcher, 2002; Nguyen et al., 2015; Schenkel & Masopust, 2014) (Fig. 10A).

The study by Fidelle et al. highlights how MAdCAM-1 downregulation caused by ABX leads in an uncontrolled migration of a specific subset of  $\alpha 4\beta 7$ -expressing ROR $\gamma$ t<sup>+</sup> FoxP3<sup>+</sup> Tr17 cells to distal sites, including tumor-draining lymph nodes (TdLNs) and the TME (Fidelle et al., 2023) (Fig. 10B). This accumulation of enterotropic Tr17 cells within tumors creates a pro-tumoral immunosuppressive environment that compromises the efficacy of ICB treatments in murine models. Similar immunosuppressive effects, characterized by Tr17 accumulation in tumors and tdLNs, were observed following genetic or antibody-mediated neutralization of MAdCAM-1 or the  $\alpha 4\beta 7$  integrin (Fidelle et al., 2023). MAdCAM-1 downregulation after ABX use could be explained by the induction of gut dysbiosis to the extent that downregulation is associated with the increase of a predominant genus under dysbiosis, the genus *Enterocloster* (Derosa et al., 2020; Routy et al., 2018). Additionally, microbial metabolism-derived secondary bile acids, such as lithocholic acid, contribute to MAdCAM-1 downregulation (Fidelle et al., 2023).

The inhibition of immunosurveillance caused by dysbiosis can be counteracted by blocking IL-17 or by inducing ectopic expression of MAdCAM-1 in the liver. This intervention causes the retention of enterotropic T-cells in the liver, while demonstrating their dysbiosis-induced migration. Immunosurveillance can also be restored by fecal transplantation of a eubiotic microbiota devoid of *Enterocloster* predominance or by administering *A. muciniphila* (Fidelle et al., 2023).

Low MAdCAM-1 concentrations have the potential to serve as indicators of dysbiosis and predictive biomarkers of response. A breakthrough in its clinical application came with the discovery that the soluble form of the MAdCAM-1 protein (sMAdCAM-1), detectable in plasma via ELISA, serves as a proxy for its ileal counterpart (Fig. 10C). Studies in NSCLC, renal carcinoma, and bladder carcinoma cohorts have confirmed that low sMAdCAM-1 levels are independent indicators of dysbiosis and predictors of poorer overall and progression-free survival in patients treated with anti-PD-1/PD-L1 ICBs. Combining sMAdCAM-1 with other circulating biomarkers indicative of intestinal permeability or lymphocyte immunophenotyping could enhance prognostic capabilities, paving the way for more effective composite biomarkers in oncological management (Fidelle et al., 2023).



### 1.4.3 Commensal-Specific Cellular and Humoral Responses

Several mechanisms mediate leukocyte trafficking to and from the intestine. Commensal bacteria-reactive lymphocytes, in addition to being present in intestinal tissues, have also been detected circulating in the host under homeostatic conditions. For example, a study conducted on healthy volunteers revealed that CD4<sup>+</sup> T cells reactive to commensal bacteria were present at a frequency of 40–500 cells per 10<sup>6</sup> circulating CD4<sup>+</sup> T cells. These cells exhibited a distinct repertoire of homing receptors and a unique cytokine secretion profile, differentiating them from those reactive to non-enteric microorganisms and highlighting their role in maintaining homeostasis (Hegazy et al., 2017). These cells, physiologically present, gain critical importance in oncology, as evidenced by clinical and preclinical studies. Numerous lines of evidence suggest the clinical relevance of these microbial antigen-specific memory T lymphocytes in cancer patients.

The clinical relevance of commensal bacteria-specific memory cells was first highlighted in the study by Vétizou et al.. Memory Th1 cells specific for *Bacteroides fragilis* partially restored the efficacy of anti-CTLA-4 treatment in GF mice through a mechanism of cross-reactivity (Vétizou et al., 2015; see Chapter 1.3.1.2), thereby influencing therapeutic outcomes. Subsequently, Routy et al. demonstrated the predictive value of anti-commensal memory responses in a clinical setting (Routy et al., 2018). Specifically, Routy and colleagues analyzed the baseline fecal microbiota composition of RCC and NSCLC patients, comparing metagenomic data between responders and non-responders to anti-PD-1 treatment. With the aim of investigate the relationship between microbial content and systemic immune tone, they examined the peripheral blood memory T-cell response elicited against microbiota after starting immunotherapy. The analysis of the CD4<sup>+</sup> and CD8<sup>+</sup> T-cell recall response was performed by coculture of autologous monocytes pre-incubated with different pasteurised commensal bacteria and autologous T-cells. The extent of the memory response was assessed by quantifying cytokines secreted following bacterial stimulation, representative of T-cell activation. The study identified memory T-cell immune responses that proved to be associated with clinical outcomes of anti-PD-1 treatment. Specifically, IFN $\gamma$  secretion revealed that CD4<sup>+</sup> and CD8<sup>+</sup> T-cell memory responses specific for *Akkermansia muciniphila*, as well as CD8<sup>+</sup> T-cell responses against *Enterococcus hirae*, correlated with prolonged PFS in immunotherapy-treated patients. Among the 10 commensals tested, only specific responses to *Akkermansia muciniphila* and *Enterococcus hirae*, two species characterizing the gut microbiota of responders, showed significant correlation with clinical outcomes (Routy et al., 2018).

As reported by Daillère et al., the immunogenic profile of *E. hirae* was also confirmed to be protective in NSCLC patients treated with CTX (Daillère et al., 2016). Similar to Routy et al., the correlation between memory response magnitude and clinical outcome was investigated (Routy et al., 2018). Following bacterial stimulation of autologous monocyte co-cultured with CD4+ T-cell, IFN $\gamma$  secretion by memory cells revealed that activation of Th1 cells specific for *E. hirae* and *Blautia intestinihominis* correlated with longer PFS. Interestingly, the efficacy of CTX relied precisely on these two bacterial species, which were able to modulate the TME by reducing Treg activity and stimulating CD8+ T-cell antitumor action. Conversely, recall responses of Th1 cells against other bacteria were not clinically relevant (Daillère et al., 2016). IFN $\gamma$ + Th1 memory responses against immunogenic bacteria present in the gut microbiota of responders were associated with favorable outcomes (Zitvogel et al., 2024). The correlation between commensal bacteria-specific memory T-cell responses and clinical outcomes highlights their potential predictive biomarker value. However, further studies are needed to validate their predictive potential and, crucially, to clarify the causal and mechanistic aspects underlying this association.

The analysis of commensal bacteria-specific memory responses was also conducted by Goubet et al., further emphasizing their predictive biomarker value (Goubet et al., 2022). This study aimed to elucidate mechanisms underlying immunotherapeutic responses to pembrolizumab in muscle-invasive bladder cancer (MIBC) patients, considering the presence of intratumoral bacteria. Indeed, urine is not a sterile environment and commensal bacteria are present in the urinary tract even of healthy individuals. The study focused on a specific strain, uropathogenic *Escherichia coli* (UPEC), capable of long-term invasion of bladder epithelial cells. Memory recall response analysis revealed that pre-existing immune responses specific toward pathogenic *E. coli*, particularly those of Tfh lymphocytes producing CXCL13, correlated with disease-free progression at 24 months. These findings underscore the role of *E. coli*-specific Tfh cells not only as predictive biomarkers of response but also as potential mediators of protective effects. In responders, circulating central memory Tfh cells tend to accumulate inside tumors, where they differentiate into effector memory Tfh cells. Their activation was associated with the secretion of CXCL13 and CCL19, chemokines characteristic of tertiary lymphoid structures (TLS). These structures facilitate in situ B-cell maturation and differentiation into IgG-secreting plasma cells, which support CD8+ T-cell antitumor activity (Goubet et al., 2022).

In this context, the study by Overacre-Delgoffe further highlighted that Tfh cells specific for the commensal bacterium *Helicobacter hepaticus* (Hhep) supported TLS maturation and antitumor

activity against CRC. This effect was independent of CD8+ T cells but depended on tumor-infiltrating CD4+ and NK cells and antibody-producing B-cell activation (Overacre-Delgoffe et al., 2021).

Goubet's study also demonstrated that high levels of serum IgG specific to UPEC, but not to other urinary commensals, were associated with favorable clinical responses (2-year recurrence-free survival [RFS]). This finding was confirmed across three independent cohorts of stage III-IV MIBC patients treated with ICB. Thus, in addition to memory T-cell responses, pre-existing humoral responses may also serve as predictive biomarkers of response. Only patients with high pre-existing serum IgG levels against *E. coli* were able to exert protective effects and properly sustain antitumor immunotherapy (Goubet et al., 2022).

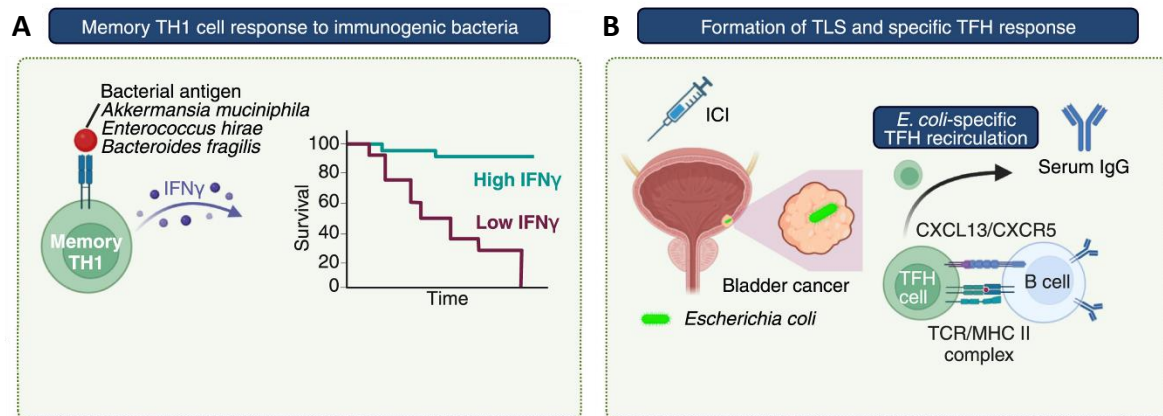


**Figure 11** Anticommensal-specific immune responses as predictive biomarkers (A) Anticommensal-specific circulating Th1 cells (B) Anticommensal-specific circulating Tfh promote TLS formation and anti-commensals humoral response (Adapted from Zitvogel et al., 2024)

## I.5. Microbiota-centred interventions (MCIs)

The microbiome's involvement in tumor prognosis, as well as its role in influencing the outcomes of antitumor therapies, establishes it not only as a biomarker with prognostic and predictive value but also as a direct target for antitumor intervention. The microbiota itself can become an object of therapeutic manipulation aimed at enhancing and supporting antitumor treatments. These emerging strategies for microbiota modulation have been defined as microbiota-centred interventions (MCIs) and will be discussed in detail in this chapter.

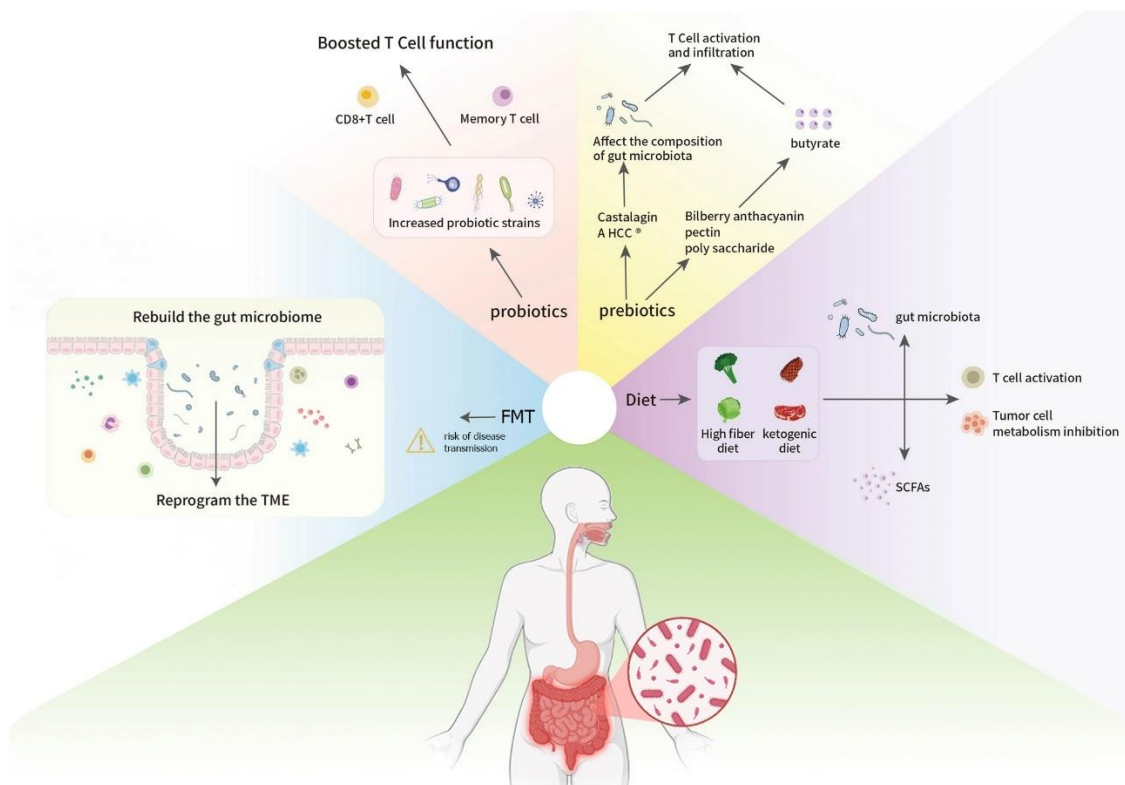


**Figure 12** Overview of microbiota-centered interventions (MCIs): (from left to right) Fecal microbiota transplantation (FMT), probiotics and prebiotics, dietary regimens (adapted from Li et al., 2024)

### I.5.1 Fecal microbiota transplantation

Fecal microbiota transplantation (FMT) is a well-established direct clinical approach for modulating the gut microbiota, involving the transfer of fecal microbial content from a healthy donor (or a cured patient) to the intestine of a recipient (Derosa et al., 2021). This procedure can be performed via oral administration of lyophilized or frozen capsules or through direct procedures like colonoscopy or gastroscopy (Kang et al., 2024). The FDA has approved FMT for treating recurrent *Clostridium difficile* infections (CDI), regulating two biological products specifically indicated for preventing CDI

recurrence after ABX treatment for recurrent infections (FDA, 2023). The primary objective of FMT is to restore the recipient's microbial homeostasis, and it is being investigated for various conditions, including inflammatory bowel disease, irritable bowel syndrome, obesity, stroke, heart failure, and oncological disease (Derosa et al., 2021). Indeed, several ongoing clinical trials are exploring the utility of FMT for oncological purposes, primarily in combination with ICB to overcome primary or acquired resistance to immunotherapy. For instance, two phase I studies demonstrated that FMT could overcome ICB resistance in patients with metastatic melanoma (Baruch, Youngster, et al., 2021; Davar et al., 2021). In the study by Baruch et al., ten patients with anti-PD-1-refractory metastatic melanoma received allogeneic FMT provided by two donor patients who had shown complete responses (CR) to anti-PD-1 therapy. Although FMT induced changes in gut microbiota composition in all patients, these changes varied depending on the donor and this was reflected in the response to ICB treatment. Among the five patients who received fecal material from the first donor, three exhibited complete (CR) or partial responses (PR) with microbiota characterised by a greater relative abundance of potentially favorable bacteria for immunotherapy, such as *Ruminococcus spp.*. Conversely, none of the patients who received microbiota from the other donor responded to anti-PD-1, and their resulting gut microbiota showed an overrepresentation of *Coprococcus spp.* and members of the *Clostridiaceae* family, typically associated with dysbiosis (Baruch, Youngster, et al., 2021). The same trend was observed in 6 out of the 15 patients with refractory melanoma enrolled in the study by Davar et al., who received FMT and regained responsiveness to pembrolizumab. The microbiota of the responders was enriched with taxa associated with favorable ICB outcomes, such as *Ruminococcaceae*, *Bifidobacteriaceae*, *Lachnospiraceae*, and *Erysiplotrichaceae* (Davar et al., 2021). Enriching the host's microbiota with intrinsically immunogenic species may thus circumvent primary resistance and restore ICB responsiveness (Derosa et al., 2021). In both studies, FMT not only modified the gut microbiota but also reshaped the TME. Recipients of immunostimulatory microbiota in the Baruch study, as well as responders in the Davar study, exhibited increased CD8<sup>+</sup> T-cell tumor infiltration, enhanced APC activity, and a reduced frequency of IL-8-expressing myeloid cells. Additionally, serological profiles revealed significant differences between responders and non-responders, with responders displaying lower levels of inflammatory markers such as CXCL8, IL-18, and CCL2, alongside higher concentrations of cytokines and chemokines indicative of a favorable immune response, such as Flt3L and CX3CL1 (Baruch, Youngster, et al., 2021; Davar et al., 2021). These studies provide proof-of-concept evidence of FMT's potential to influence immunotherapy responses in cancer patients

through gut microbiota modulation, reprogramming of immune and inflammatory tones, and TME remodeling. To provide further evidence of the impact of FMT, further clinical trials are underway to explore the combined use of FMT and immunotherapy in melanoma patients as well as in those with other cancer types including RCC, NSCLC, and CRC (Kang et al., 2024).

FMT-mediated reprogramming of the intestinal microbial composition increases microbiota  $\alpha$ -diversity and promotes a balanced distribution of health-associated bacteria. This offers a potential solution to address both broad-spectrum antibiotic-induced dysbiosis and ICB-induced colitis (Derosa et al., 2021; Wang et al., 2023). Notably, combining FMT with immunotherapy has been associated with a reduced incidence of high-grade irAEs. This suggests that FMT may be useful for both enhancing the efficacy of cancer treatment and reducing related immunotoxicity (Blake et al., 2024).

It is, however, crucial to emphasize that despite these promising results, FMT remains a high-risk intervention: rigorous screening of donor samples is essential to prevent the transfer of pathogenic or antibiotic-resistant bacteria (DeFilipp et al., 2019). Therefore, it is imperative to establish guidelines for a rational donor selection based on the specific context of FMT use, as well as to define standardization of the procedure. Such safety restrictions, however, necessitate a large pool of pre-screened donors. In this regard, large stool banks with multiple preselected healthy donors are emerging, which could help manage the considerable variability in donor microbiota. Furthermore, many unresolved questions remain regarding the definition of a "healthy" donor. Beyond the absence of known pathogens, which is clearly a non-negotiable exclusion criterion, other fundamental aspects require clarification. Additionally, the issue of recipient selection is critical, as not all patients benefit from FMT. Thus, in addition to safety screenings, effective donor-recipient matching strategies are essential (Baruch, Gaglani, et al., 2021; Duvallet et al., 2019; Porcari et al., 2023).

In summary, several clinical, regulatory, and scientific challenges must be addressed for this approach to become an approved, safe treatment capable of delivering significant benefits to patients (Fillon, 2021).

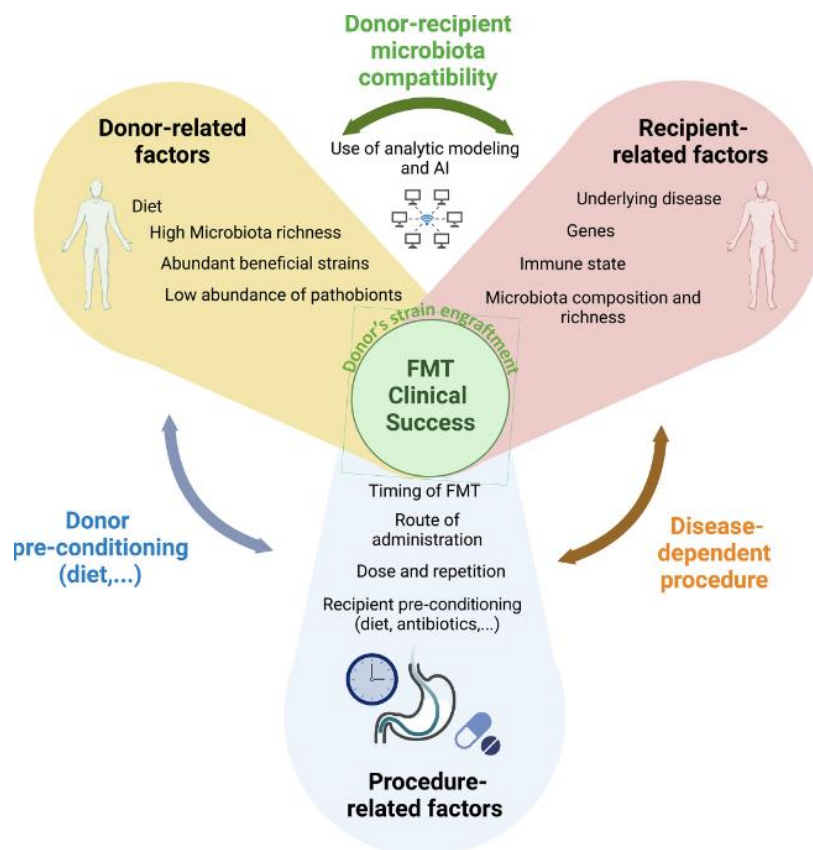


**Figure 11** Factors that influence FMT success (Porcari et al., 2023)

### 1.5.2 Prebiotics and probiotics

Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). Through processes such as lyophilization (or sporulation) and subsequent encapsulation, probiotics can be easily administered orally. Given the social acceptance challenges, reproducibility issues, and consistency concerns associated with FMT, as well as its potential risks, probiotics represent a microbiota modulation strategy that could offer more feasible solutions for widespread clinical use (Blake et al., 2024; Zitvogel & Kroemer, 2024). The primary goal of probiotic administration is to induce lasting ecological changes in the gut microbiota and affect the host immune system (Kang et al., 2024).

As proof of concept, a phase I randomized clinical trial showed that administering the commercial probiotic *Clostridium butyricum* 588 (CB588) significantly extended PFS in a cohort of patients with metastatic RCC undergoing ICB treatment, despite no significant changes in gut microbial composition being observed (Dizman et al., 2022; Ebrahimi et al., 2023). Similarly, retrospective studies in a cohort of patients with metastatic NSCLC demonstrated that the probiotic strain *C. butyricum* MIYAIRI 588 restored the clinical benefit of ICB by modulating the microbiota to favor

*Bifidobacteria* in patients who had taken ABX 60 days prior to immunotherapy or were receiving proton pump inhibitors (PPI) (Tomita et al., 2020, 2022). This finding is particularly relevant, as a recent meta-analysis of 12,000 patients treated with ICB demonstrated that ABX use, especially before treatment, increases mortality risk (Derosa et al., 2021). Similarly, PPIs have been associated with a negative impact on immunotherapy efficacy in a study of 1,012 oncological patients (Cortellini et al., 2020). Thus, besides enhancing the benefits of ICBs, probiotics could also mitigate the deleterious effect on immunotherapy caused by some comedications.

Another promising probiotic candidate is *Akkermansia muciniphila*, associated with favorable clinical outcomes in patients with NSCLC and RCC (Derosa et al., 2022; Routy et al., 2018). Dubbed the "next-generation probiotic star" (Shin et al., 2019), *A. muciniphila* highlights the importance of determining the optimal dose for probiotic administration. As discussed in Ch. 1.4.1, trichotomising the relative abundance of *A. muciniphila* has allowed for better stratification of treatment outcomes in patients with NSCLC and has the potential to identify patients who might benefit most from its administration as well as those who, already having a high abundance (>4.8%), might further compromise their response to therapy (Derosa et al., 2022).

A qPCR-based diagnostic test is being used to identify patients lacking *A. muciniphila* who are suitable for inclusion in an ongoing phase I trial (NCT05865730) where the live bacterial product *A. muciniphila*-based (Oncobax AK) is administered to restore ICB efficacy in NSCLC and RCC patients. In addition to monoclonal live biotherapeutics, microbial consortia are emerging as promising options in oncology. In murine models of CRC, oral administration of a consortium of four *Clostridiales* strains (CC4) induced tumor infiltration and activation of CD8<sup>+</sup> T cells, prevented and even successfully treated the oncological pathology, even overcoming the efficacy of anti-PD-1 therapy. This provides a robust preclinical basis for the clinical use of microbial consortia (Montalban-Arques et al., 2021). Along these lines, a consortium of 30 bacteria, the Microbial Ecosystem Therapeutic 4 (MET4), designed as an alternative to FMT, has been shown to be safe and capable of altering both the intestinal microbiota and serum metabolome in patients with advanced solid tumors, paving the way for a phase II study in head and neck cancers (Spreafico et al., 2023). However, despite their potential, probiotics are not without limitations. They may fail to colonize effectively due to rejection by the native microbiota or may alter the functionality of the recipient microbiota (Derosa et al., 2021). As with FMT, it remains crucial to establish accurate parameters for selecting patients most likely to benefit from probiotic-based treatments.

In addition to probiotics, the administration of prebiotics has also emerged as a potential strategy to modulate the composition of the gut microbiota. Prebiotics are defined as substrates that are selectively utilized by host microorganisms to confer a health benefit. This property allows prebiotics to promote the growth of beneficial microbial species by providing targeted nutrition (Blake et al., 2024; Gibson et al., 2017). Furthermore, prebiotics have been shown to enhance immunomodulatory effects, strengthen the intestinal barrier, and improve various metabolic functions (Monteagudo-Mera et al., 2019). In the oncological context, for instance, certain prebiotics have demonstrated the ability to enhance the efficacy of cancer immunotherapy, although such evidence is currently limited to preclinical studies. For example, the oral administration of castalagin has been shown to enrich the gut microbiota with beneficial bacteria, such as *Alistipes* and *Ruminococcaceae*, which are associated with effective immunotherapeutic responses. These taxa not only increase in abundance but also appear to interact directly with the prebiotic, which fosters their proliferation and amplifies their immunostimulatory benefits. Specifically, castalagin has demonstrated an ability to enhance the CD8+/FOXP3+CD4+ ratio within the TME, thereby contributing to a more robust antitumor response. Notably, this prebiotic supported the efficacy of anti-PD-L1 therapy in patients who had undergone FMT (Messaoudene et al., 2022).

Since the effectiveness of prebiotics depends on the presence of beneficial taxa already existing in the host microbiota, the combined use of prebiotics and probiotics, also known as synbiotics, could provide a more effective approach than using either in isolation. Synbiotics take advantage of the synergistic action of probiotics, which introduce specific taxa, and prebiotics, which provide the necessary nutrients to support their proliferation and activity (Panigrahi et al., 2017).

### **I.5.3 Dietary interventions**

Diet is one of the key factors able to influence the composition of the gut microbiota. Considering the significant impact of the microbiome on cancer therapy outcomes, dietary intervention has gained attention as a potential strategy to enhance the efficacy of antitumor treatments through modulation of the gut microbiota (Kang et al., 2024). However, direct evidence that diet-induced microbial modulation affects cancer and its therapies remains limited. Recently, in an attempt to elucidate the microbiota-diet-cancer axis, Spencer et al. study demonstrated that a high-fiber diet significantly improved PFS in melanoma patients undergoing neoadjuvant therapy combined with ICB. This response was attributed to increased expression of genes related to both T-cell activation and IFN $\gamma$  release (Spencer et al., 2021).

Another dietary strategy extensively studied in oncology is the ketogenic diet (KD), characterized by high-fat, moderate-protein, and low-carbohydrate intake, leading to the accumulation of ketone bodies, particularly 3-hydroxybutyrate (3HB). KD induces alterations in gut microbiota composition and reduces pro-inflammatory Th17 cells in the small intestine and adipose tissue (Ang et al., 2020). Preclinical studies have shown that KD or oral administration of 3HB can exert immunostimulatory effects against aggressive tumors, restoring the efficacy of ICB treatments when they fail to reduce tumor growth. Mechanistically, 3HB promotes the expansion of CXCR3<sup>+</sup> T cells and inhibits IFN $\gamma$ -mediated PD-L1 induction in myeloid cells (Ferrere et al., 2021).

Understanding the immunostimulatory role of KD and 3HB has paved the way for new clinical trials investigating dietary strategies integrated into standard oncology treatment protocols. It is also interesting to note that the increase in 3HB levels also occurred following fasting strategies (Kroemer et al., 2018), which have been particularly explored in oncology. Long-term caloric restriction in non-human primates has been shown to reduce cancer incidence and mortality (Colman et al., 2009), while short-term fasting or alternate-day fasting in mice has been shown to slow tumor progression (C. Lee et al., 2012). The fasting-mimicking diet (FMD), which has gained particular interest, not only proved well-tolerated by patients but also demonstrated the ability to reshape systemic and intratumoral immunity by reducing immunosuppressive myeloid cells and Tregs in peripheral blood while enhancing Th1/cytotoxic responses in the TME (Caffa et al., 2020; Vernieri et al., 2016).

Our research group is currently involved in the BREAKFAST I study (NCT04248998) that aim to investigate the gut microbiota's response to fasting cycles and its potential role in antitumor activity. Thus, dietary regulation through gut microbiota modulation could represent a promising clinical strategy to enhance cancer treatment efficacy. However, a major challenge with these microbial manipulation strategies is the need to ask patients to adopt long-term changes in their lifestyle and dietary habits (Derosa et al., 2021). It is crucial to consider that the response of the gut microbiota to dietary changes is rapid but reversible, suggesting a transient effect of dietary regulation, that therefore requires maintenance strategies for long-term effects (Carmody & Turnbaugh, 2014).



## **II. RESEARCH CONTEXT AND OBJECTIVES**



Gut microbiome has been recognized as one of the hallmarks of cancer. It can contribute to tumorigenesis and tumor progression while significantly influencing the outcomes of oncological treatments. In light of these findings, the gut microbiota is increasingly emerging as a promising tool to address the challenges of modern oncology. This project aimed to investigate the gut microbiota-cancer relationship, with the ultimate goal of paving the way for new therapeutic strategies to improve outcomes in neoplastic diseases. Our objective was achieved through two levels of analysis, detailed as follows.

- One of the predominant challenges in oncology is the development of therapy resistance. Identifying predictive biomarkers is crucial to accurately select the most suitable patients for specific oncological treatments, and the intestinal microbiota has proven to address this issue. Metagenomic analysis of fecal samples from oncological patients has confirmed the influence of the intestinal microbiota on therapeutic outcomes and led to the definition of intestinal microbial signatures with prognostic and predictive power. However, not all cohorts and treatment strategies have been explored. In this regard, the first part of the thesis aimed to profile and compare the composition of the intestinal microbiota in patients with metastatic HR+/HER2- breast cancer. The patients were distinguished between responders and non-responders based on the outcome of treatment with CDK4/6 inhibitors combined with endocrine therapy. The identification of a distinct microbial signature between responders and non-responders may address the need for predictive biomarkers and further enhance the understanding of the relationship between the intestinal microbiota and breast cancer, the state of the art of which was investigated through the writing of a review. (See Results section: Article#1; See Appendix section: Article#3; See Appendix section: Abstract#1)

- The intestinal microbiome, with its central role in maintaining immune homeostasis and modulating both local and systemic immune systems, exerts a crucial influence on the tumor microenvironment (TME) and, consequently, on the outcomes of oncological treatments. This underscores the need to clarify the role of microbiota-mediated immune responses in optimizing therapeutic strategies. The second part of this project thus focused on studying pre-existing immune memory, both cellular and humoral, directed against specific commensal bacteria, which were either positively or negatively associated with the outcome of immunotherapy treatment. The aim was to understand how these recall immune

responses could impact the effectiveness of immunotherapy. The analysis, conducted across several patient cohorts, could thus contribute to understanding the microbiota-immune system-cancer relationship and lay the foundation for new intervention strategies based on microbiota modulation.

(See Results section: Article#2; See Appendix section: Abstract#2; See appendix section: Merit Award; See appendix section: Abstract#3)



### **III. MATERIALS AND METHODS**



## CLINICAL TRIAL, REGULATORY APPROVALS AND SAMPLING

### For Article 1 (See chapter IV.1.2 in RESULTS section)

Exploratory prospective cross-sectional observational study (IRB n. 440/11/10/2019) involving patients affected by HR+/HER2-negative MBC, treated at the Breast Unit of the Cremona Hospital in Italy between March 2019 and March 2021 and fulfilling with the following inclusion criteria:

- Tumors had to be endocrine-sensitive according to ESMO definition (Gennari et al., 2021), though progression on 1<sup>st</sup>-line endocrine monotherapy lasting >6 months was allowed;
- No personal history of inflammatory bowel disease (IBD), intestinal resection for any cause and irritable bowel syndrome;
- Absence of visceral crisis (Gennari et al., 2021);
- Candidate to receive a CDK4/6-inhibitor-based regimen as *per* standard clinical practice;
- Willingness to provide a stool sample before starting anticancer treatment.

Patients were not allowed to have received antibiotics in the previous 2 weeks and previous anticancer treatment with CDK4/6-inhibitors, as well as having experienced diarrhea in the previous 2 months. Out of N=52 patients with HR+/HER2- MBC assessed for eligibility, N=14 patients were included and analyzed.

All patients were treated with either palbociclib, ribociclib or abemaciclib with an AI or fulvestrant, according to standard treatment schedules. A mandatory stool sample was collected at baseline before treatment started from each patient. Clinico-radiological reassessments were performed every 2-3 months according to local clinical practice and tumor responses were evaluated according to RECIST 1.1 criteria (Eisenhauer et al., 2009).

**Sampling collection.** Before starting treatment, a blood sample was collected, as well as a stool sample, which was collected using eNAT Copan Kit at baseline before treatment. Approximately 5g of the fresh stool was stored at +4°C until processing and then kept at -70°C until analysis. Blood samples were processed as elsewhere described to obtain blood cells counts (Schettini et al., 2020).

**Clinical endpoints.** Patients were divided into responders (R) and non-responders (NR) to CDK4/6-inhibitors if disease progression happened after or before 10 months, respectively. This timepoint was selected since 9.5 months was the lowest median PFS obtained with a CDK4/6-inhibitor-based regimen in pivotal trials.

## **For Article 2 (See chapter IV.2.2 in RESULTS section)**

**Oncobiotics.** NCT04567446 is a multicentre study involving 14 participating centres in France and Canada, aimed at evaluating the microbiome's impact on treatment outcomes in patients diagnosed with RCC, UC, and advanced NSCLC. This report focuses solely on patients receiving ICB therapies at Gustave Roussy, either as monotherapy or in combination with other treatments, such as chemotherapy or tyrosine-kinase inhibitors. Blood and stool samples were collected at various time points between June 2021 and March 2024, beginning at or shortly after treatment initiation. All experiments and analyses were conducted at Gustave Roussy.

The study followed standard care protocols until disease progression, unacceptable side effects, the completion of two years of ICB treatment, or the treating physician's discretion. Eligibility criteria and baseline data, including recent medications, are detailed in the trial protocol and recorded.<sup>8</sup>

**Sampling collection and time frames.** Faecal and blood samples were prospectively collected at specific time points: T0, up to and including 2 days post-first ICB infusion (post-treatment); T1, 3–42 days post-treatment; T2, 43–99 days post-treatment; T3, 100–190 days post-treatment; T4, 191–896 days post-treatment; and T5, ≥897 days post-treatment. To enhance statistical power, the time points were grouped into broader “periods”: T0 and T1 were merged as T<sub>0-1</sub>; T2 remained separate as T<sub>2</sub>; and T3, T4, and T5 were combined as T<sub>3-4-5</sub>. For analysis, only the sample collected closest to the first treatment infusion was considered, and unless otherwise specified, only results from the T<sub>0-1</sub> period were analysed.

Blood samples were collected in heparinized tubes. Participants self-collected stool samples at home following the IHMS\_SOP 004 protocol. The samples were immediately stored at -4°C after collection. Upon arrival at the recruitment centre's biobank, within 4 to 24 hours, the samples were stored at -80°C. All samples were processed in accordance with the International Human Microbiome Standards (IHMS) guidelines (SOP 03 V1).

**AtezoTRIBE.** The NCT03721653 phase 2 study enrolled 218 patients with unresectable stage IV CRC. Patients were randomised in a 2:1 ratio to receive a combination of FOLFOXIRI and bevacizumab, with or without atezolizumab (anti-PD-L1). All patients were treatment-naïve at the time of the first stool collection. Baseline samples were obtained from 184 patients, with stool collected from 172 patients and plasma from 135 patients. Of these, 135 patients provided both stool and plasma samples. All samples were collected in accordance with the ONCOBIOTICS cohort guidelines (Antoniotti et al., 2022).

**MIMic.** NCT03772899 is a multicentre, single-arm phase I trial involving treatment-naïve patients diagnosed with advanced cutaneous melanoma, receiving first-line anti-PD-1 therapy (Routy et al., 2023). Patients were administered a PEG laxative 24 hours prior to receiving FMT from screened donors via frozen capsules. Stool and blood samples were collected at multiple time points: pre-FMT (S1), one-week post-FMT (S2, coinciding with the first anti-PD-1 infusion), and one-month post-FMT (S3, coinciding with the second anti-PD-1 infusion). In this report, the S1 samples were evaluated as “pre-FMT,” while the S2 and S3 samples were evaluated in combination as “post-treatment.”

**Clinical endpoints:** In all clinical trials, progression-free survival (PFS) was defined as time from ICB start to disease progression or death. Overall survival (OS) was defined as the time from ICB start to death. Patients were censored at follow-up if no events occurred.

## **METAGENOMICS ANALYSES OF PATIENT STOOLS**

### **For Article 1 (See chapter IV.1.2 in RESULTS section)**

**DNA extraction, 16S rRNA gene amplification and sequencing.** Total bacterial DNA was extracted from 50 mg of fecal material (wet weight) using the FastDNA™ SPIN Kit for Soil (MP Biomedicals, Eschwege, Germany), following the manufacturer’s instructions. The genomic DNA was then quantified using the Qubit HS dsDNA fluorescence assay (Life Technologies, Carlsbad, CA, USA), while the quality of the extracted DNA was assessed using agarose gel electrophoresis. DNA amplification was performed using primers 343F and 802R, which allowed the amplification of the V3–V4 regions of the bacterial 16S rRNA gene. Amplification was carried out as described elsewhere (Patrone et al., 2018). Sequencing was performed at Fasteris SA (Geneva, Switzerland) using Illumina’s MiSeq v3 platform with 2×300 bp mode.

**Bioinformatic and statistical analysis for stool samples.** Raw FASTQ files were analyzed with DADA2 pipeline v.1.14 for quality check and filtering (sequencing errors, denoising, chimerae detection) on a Workstation Fujitsu Celsius R940 (Fujitsu, Tokyo, Japan). Filtering parameters were as follows: truncLen=0, minLen=100, maxN=0, maxEE=2, truncQ=11, trimLeft=15. All the other parameters in the DADA2 pipeline for paired-end were left as default. Raw reads (5692773 in total, on average 37700 per sample) were filtered (1921911 in total, on average 12727 per sample) and 2503 Amplicon Sequence Variants (ASV) were found. Sample coverage was computed and resulted to be on average higher than 99% for all samples, thus meaning a suitable normalization procedure for

subsequent analyses. Bioinformatic and statistical analyses on recognized ASV were performed with Python v.3.8.2. Each ASV sequence underwent a nucleotide Blast using the National Center for Biotechnology Information (NCBI) Blast software (ncbi-blast-2.3.0) and the latest NCBI 16 S Microbial Database (<https://ftp.ncbi.nlm.nih.gov/blast/db/>). After blasting, the 2503 ASVs were merged into 441 species (thus excluding sub-species or strain differences), and a matrix of their relative abundances and prevalence was built. Only bacterial species having a prevalence equal or higher than 20% were considered, thus 118 species were considered for subsequent statistical analyses.

Data matrices were first transformed with pseudocount and centered-log-ratio (CLR), then normalized and standardized using QuantileTransformer and StandardScaler methods from Sci-Kit learn package v0.20.3. Normalization using the `output_distribution = 'normal'` option transforms each variable to a Gaussian-like shaped distribution, whilst the standardization results in each normalized variable having a mean of zero and variance of one. These two steps of normalization followed by standardization ensure the proper comparison of variables with different dynamic ranges, such as bacterial relative abundances, or cytokines levels. For microbiota analysis, measurements of  $\alpha$ -diversity (within sample diversity) such as Richness and Shannon index, were calculated at species level using the SciKit-learn package v.0.4.1. Exploratory analysis of  $\beta$ -diversity (between sample diversity) was calculated using the Bray-Curtis measure of dissimilarity and represented in Principal Coordinate Analyses (PCoA), along with methods to compare groups of multivariate sample units (analysis of similarities - ANOSIM, permutational multivariate analysis of variance - PERMANOVA) to assess significance in data points clustering. ANOSIM and PERMANOVA were automatically calculated after 999 permutations, as implemented in SciKit-learn package v0.4.1. In order to visualize a non-supervised clusterization as PCoA, we implemented with custom scripts (Python v3.8.2) a Hierarchical Clustering Analysis (HCA) with Bray-Curtis metrics and complete linkage method. We implemented Partial Least Square Discriminant Analysis (PLS-DA) and the subsequent Variable Importance Plot (VIP) as a supervised analysis wherein the VIP values (order of magnitude) were used to identify the most discriminant bacterial species among the cohorts. Mann–Whitney U test and Kruskal–Wallis tests were employed to assess significance for pairwise or multiple comparisons, respectively. For statistical significance, p values without false discovery rate (FDR) were used.

**Co-abundance network analysis.** Pearson matrices for network analysis (metric = Bray-Curtis, method = complete linkage) were generated on normalized and standardized data with in-house

scripts (Python v3.8.2) and visualized with Gephi v.0.9.2 (Derosa et al., 2020; Iebba et al., 2018; Riganelli et al., 2020). Microbial species having a prevalence  $\geq 2.5\%$  were considered to generate the nodes within the final network, while a significant (after Benjamini-Hochberg FDR at 10%, Q-value  $\leq 0.001$ ) Pearson correlation coefficient was employed to obtain edges (Li et al., 2008). Network analysis was performed with Gephi v.0.9.2, taking care of an optimized visual representation as proposed by current guidelines (Berry & Widder, 2014; Faust et al., 2012; Faust & Raes, 2012; Lozupone et al., 2012), using Fruchterman Reingold then ForceAtlas2 algorithms (Jacomy et al., 2014). Only connected nodes were retained in the final network, using the Gephi K-core filter ( $n=1$ ). Nodes were colored according to the group (NR or R) in which species harbored the highest mean relative abundance, after transformation, normalization and standardization. The betweenness centrality, measuring how often a node appears on the shortest paths between pairs of nodes in a network, was computed with Gephi v.0.9.2. Intra-network communities (Species Interacting Groups, SIGs) were retrieved using the Blondel community detection algorithm (Blondel et al., 2008) by means of randomized composition and edge weights, with a resolution equal to 1. Each microbial species could be assigned to a different community with a community detection number (CDN).

#### **For Article 2 (See chapter IV.2.2 in RESULTS section)**

**DNA extraction, shotgun sequencing and metagenomic analysis.** Bacteria DNA was extracted from stool samples following the IHMS SOP 07 V2 H protocol. Sequencing was performed using an Ion Proton sequencer (Thermo Fisher) at a single site (MetaGenoPolis). The sequencing reads were processed with Alien Trimmer (version 0.4.0) to remove persistent sequencing adapters and trim low-quality nucleotides from the 3' end, applying a quality score cutoff of 20 and a length cutoff of 45 bp. The cleaned reads were then filtered to remove human DNA and potential food contaminants by comparison with the human genome (GRCh38-p13), using a 95% identity score threshold. Metagenomic analysis of \*.fastq files was performed following previously published guidelines on taxonomic profiling using MetaPhlAn 4.0 (Blanco-Míguez et al., 2023; Derosa et al., 2024).

#### **BACTERIAL STRAINS AND CULTURE CONDITIONS**

All bacterial strains used in this study were clinical isolates selected for their relevance to cancer patients. *Eggerthella lenta*, *Enterocloster bolteae*, *Enterocloster clostridioformis*, *Hungatella hathewayi*, *Klebsiella pneumoniae*, *Veillonella atypica*, and *Veillonella parvula* were isolated from ileal or primary tumour biopsies and ileal mucosa biopsies (Gustave Roussy). *Escherichia coli* Q1696

was isolated from the urine of a non-cancer patient (Goubet et al., 2022), while *Fusobacterium nucleatum subsp. animalis* P6428 was isolated from the saliva of an HIV-positive patient (both at IHU, Marseille). *Akkermansia massiliensis* P2261 was provided by EverImmune.16 Bacteria were cultured on sheep blood agar plates (COS, bioMérieux) at 37°C with 5% CO<sub>2</sub> under aerobic or anaerobic conditions for 24–72 hours. Bacterial suspensions were then prepared by transferring one or more colonies into 0.85% NaCl solution to attain a concentration of 3 McFarland units – equivalent to 10<sup>9</sup> CFU/mL. Pasteurisation was carried out by incubating the suspensions in a pre-heated water bath at 70°C for 30 minutes and transferring directly to -80°C. *Faecalibacterium prausnitzii*, provided by Bioaster, was already pasteurised upon delivery. The pasteurised bacteria were stored in aliquots at -80°C until further use.

## EVALUATION OF HUMORAL IMMUNITY

**Human serum dilution.** Serum was collected by centrifuging whole blood samples in serum-separating tubes and subsequently stored at -80°C until analysis. IgG and IgA concentrations were measured using an Optilite™ turbidimetric analyser (The Binding Site®, Birmingham, UK), in accordance with the manufacturer's instructions. To normalise antibody concentrations, serum samples were thawed at 4°C and filtered through a 60 µm filtration plate (Millipore, Cat. no. MANMN6010) using a swing-bucket centrifuge at 1,500 rpm for 30 seconds at 15°C. The filtrates were then adjusted to final concentrations of 200 µg/mL for IgG and 100 µg/mL for IgA using PBS-1X (GIBCO, Cat. no. 10010-015) supplemented with 2% BSA (SIGMA, Cat. no. A7030) as the diluent in a low protein binding plate. The plates containing the diluted serum samples were sealed and stored at -80°C until further use.

**Bacterial Flow Cytometry Assay and Analysis.** All buffers were sterilised by filtration through 0.22 µm membranes before use. Thawed bacterial aliquots were pooled and washed twice in cold PBS-1X by centrifugation at 5,000 x g for 10 minutes at 4°C. The bacteria were then labelled by incubating them at 4°C in darkness for 30-60 minutes with eFluor450 (Thermo Fisher, Cat. no. 65-0842) or eFluor670 (Thermo Fisher, Cat. no. 65-0840-85) cell proliferation dyes at a final concentration of 20 µM. After incubation, the bacteria were washed twice with PBS-1X-2% BSA, then fixed with 1x BD CellFIX (BD Biosciences, Cat. no. 340181) at 4°C for 10 minutes. After three washes, the bacteria were resuspended in cold PBS-1X-2% BSA and enumerated by flow cytometry (Beckman Coulter CytoFLEX benchtop flow cytometer) using microspheres from a bacteria counting kit (Thermo Fisher,

Cat. no. B7277). The stained and fixed bacteria were stored protected from light at 4°C until use or for up to two weeks.

**Detection of anti-bacteria antibodies from serum samples.** Bacterial-surface-specific antibodies were assessed as previously described.<sup>75,76</sup> In brief,  $1 \times 10^6$  stained and fixed bacteria suspended in PBS-1X-2% BSA were added to Nunc™ 96-well polypropylene V-bottom plates and co-incubated with patient serum, normalised to 20 µg/mL of the relevant antibody, or one of the following: Ig-normalised patient sera; purified immunoglobulins (Thermo Fisher, Cat. no. 31154 [IgG] or Cat. no. 31148 [IgA]); pooled human plasma (DUTSCHER, Cat. no. S4180-100); intravenous immunoglobulin (IVIg, INTRATECT); or purified irrelevant antibodies as isotype controls, specifically anti-SARS-CoV2 S1 IgA (Proteogenix, Cat. no. PTXCOV-A575-100) or Ultra-LEAF™ purified human IgG1 isotype control recombinant antibody (Clone: QA16A12, BioLegend, Cat. no. 403502) at the same concentration (20 µg/mL). The isotype controls, plasma, IVIg, and purified antibody were all plated in triplicate.

Following a 30-minute incubation at 4°C, the plates were washed with 1X PBS (18 minutes at 3,200 x g) before adding secondary FITC-conjugated anti-human IgG (Jackson ImmunoResearch, Cat. No. 109-096-070) or anti-human IgA (Jackson ImmunoResearch, Cat. No. 109-096-011), as required, at a final dilution of 1:200. The plates were then incubated for an additional 30–60 minutes at 4°C in darkness. Following secondary antibody incubation, the plates were washed and the pellets were resuspended in cold PBS-1X-2% BSA before data acquisition with a Beckman Coulter Life Sciences CytoFLEX benchtop flow cytometer. Data were analysed using FlowJo v10.8.1 software.

Pseudo-quantification of antibody binding levels against each bacterium was based on the median fluorescence intensity (MFI) of secondary antibody, which is directly proportional to the level of bacterial-surface-specific antibodies present in patient sera. Results were expressed in stain index (SI) using the following equation. IgG and IgA anti-bacteria binding levels were categorised as high or low based on the median SI of a considered patient cohort for a given strain, with an SI above the cohort median defined as "high."

## EVALUATION OF CELL-MEDIATED IMMUNITY

**Whole blood T-test.** Stock solutions of pasteurised bacteria were pre-diluted in RPMI-1640 supplemented with GlutaMAX™ (Thermo Fisher, Cat. no. 61870-010) and then further diluted in NIL reagent from the VIDAS® STIMM™ BASIC RUO kit (bioMérieux, Cat. no. 424615). Bacteria pools were created by combining equal amounts of each constituent bacterium. For stimulation assays, 300 µL of fresh whole blood was co-incubated directly with 300 µL of pasteurised bacteria solution

(containing  $3 \times 10^5$  bacteria) or controls in 48-well flat-bottom plates. NIL reagent served as the negative control, and the mitogen (MIT) reagent from the VIDAS® STIMM™ BASIC RUO kit was used as the positive control. The samples were incubated for 22 hours at 37°C with 5% CO<sub>2</sub> injection. Supernatants were collected and stored at -20°C until further analysis.

**DC/T cell assay.** CD14<sup>+</sup> monocytes were sorted from thawed peripheral blood mononuclear cells (PBMCs) using a MACS column and CD14 MicroBeads (Miltenyi, Cat. no. 130-050-201) according to the manufacturer's instructions. The sorted monocytes were cultured for 72 hours at 37°C with 5% CO<sub>2</sub> injection in RPMI-1640 supplemented with GlutaMAX™, 10% heat-inactivated human AB serum (Jacques Boy Institute of Biotechnology, Cat. no. 201021334), 1% sodium pyruvate (Gibco, Cat. no. 11360-039), 1% penicillin/streptomycin solution (Gibco, Cat. no. 15140122), 1% HEPES (Gibco, Cat. no. 15630056), 1000 IU/mL GM-CSF (Miltenyi, Cat. no. 130-093-862), and 250 IU/mL IFN- $\gamma$ 2b (GenScript, Cat. No. Z00353). After incubation, mature DCs were recovered, washed twice (10 min; 1,100 rpm) with PBS-1X, and counted using trypan blue exclusion. Subsequently, DCs were added to a 96-well plate with  $5 \times 10^3$  cells per well in RPMI-1640 supplemented with GlutaMAX™, 10% human AB serum, 1% sodium pyruvate, 1% penicillin/streptomycin solution, and 1% HEPES. The DCs were then pulsed with  $0.5 \times 10^6$  pasteurised bacteria by co-incubating for 2 hours at 37°C with 5% CO<sub>2</sub> injection. T cells were isolated from a separate vial of PBMCs from the same patient using a MACS column and a pan T cell isolation kit (Miltenyi, Cat. no. 130-096-535) according to the manufacturer's instructions. After counting, the T cells were added to the pulsed DCs at a 10:1 ratio and incubated for 48 hours at 37°C with 5% CO<sub>2</sub> injection. T cells were also added to wells containing anti-CD3/anti-CD28 dynabeads (Thermo Fisher, Cat. no. 11131D) as a positive control at a cell-to-bead ratio of 5:1, or to wells lacking DCs and wells with non-pulsed DCs as negative controls. Wells containing only pulsed DCs served as additional negative controls. After incubation, the supernatants were collected and stored at -20°C until further analysis.

**Cytokine Quantification and Analysis.** The concentrations of IL17 and CXCL13 in supernatants collected from both the whole blood T-test and DC/T cell assay were determined using commercial enzyme-linked immunosorbent assay (ELISA) kits, following the manufacturer's instructions. IL17 was quantified using the ELISA MAX™ Deluxe Set Human IL-17A (BioLegend, Cat. no. 433914), while CXCL13 levels were measured with the Human CXCL13/BLC/BCA-1 DuoSet ELISA kit (Bio-Techne, Cat. no. DY801). IFN $\gamma$  levels were assessed using two different methods: the VIDAS® 30 semi-automated platform with the IFN $\gamma$  RUO protocol (bioMérieux, Cat. no. 423304) for supernatants from the whole blood T-cell assay, and the ELISA MAX™ Deluxe Set Human IFN $\gamma$  (BioLegend, Cat. no.

430116) for supernatants from the DC/T cell assay. A four-parameter logistic regression model was applied to generate standard curves for each ELISA plate and to estimate cytokine concentrations in µg/mL for each sample. Note that the VIDAS platform automatically calculated the IFN $\gamma$  cytokine concentration. For the whole blood T-test, a sample was considered positive if the calculated concentration was more than twice that of the NIL reagent. A positive result for IFN $\gamma$  measured by the VIDAS® platform additionally required a concentration greater than 15 µg/mL. In the DC/T cell assay, samples were deemed positive if the calculated concentration exceeded 1.5 times each individual negative control value. A participant's sample was classified as positive if it met the criteria in either the whole blood T-test or the DC/T cell assay.

## IMMUNO-PHENOTYPING BY SPECTRAL FLOW CYTOMETRY

Aliquots of  $5 \times 10^6$  to  $10 \times 10^6$  PBMCs, cryopreserved in foetal calf serum + DMSO solution, were thawed, washed, and resuspended in FACS Buffer (Brilliant Stain Buffer Plus BD + 5% FCS + 2mM EDTA). Dead cells were excluded using the LIVE/DEAD™ Fixable Blue Dead Cell Stain Kit (Invitrogen). Cells were stained using antibodies purchased from BD Biosciences, R&D Systems, MBL, BioLegend, Miltenyi Biotec, and Cytex. Data were acquired with a Cytex Aurora 5-laser spectral analyser and data analysis was performed using FlowJo v10.8.1.

**Manual gating strategies.** Flow cytometry analysis on PBMCs was conducted by filtering out debris, doublets, and dead cells, then selecting for live CD45<sup>+</sup> cells. New \*.fcs files – retaining only live CD45<sup>+</sup> cells – were generated with FlowJo v10.8.1 software for Uniform Manifold Approximation and Projection (UMAP) and Single-cell Cytometry Annotation Network (Scyan; <https://mics-lab.github.io/scyan>) analyses. The UMAPs were computed and displayed using the Scanpy package on live CD45<sup>+</sup> cells (Wolf et al., 2018).

Cell-type annotations were automatically performed using Scyan, a biology-driven neural-network model that uses prior knowledge about immune cell phenotypes which enables us to identified nine main cell populations: basophils, NK cells, B cells, monocytes, dendritic cells, CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, double-positive T cells, and double-negative T cells (Blampey et al., 2023). After annotation, new \*.fcs files for the nine main cell populations were generated for each patient. Next, the percentage of all subpopulations for each of the nine main populations were extracted after manual gating identification on FlowJo v10.8.1 software. The T cell populations were defined as follows: central memory (CM) T cells were identified as CCR7<sup>+</sup> CD45RA<sup>-</sup>; Treg cells as CD127<sup>low</sup> CD25<sup>high</sup> CD4<sup>+</sup>; Th17 cells as CCR6<sup>+</sup> CXCR3<sup>-</sup> CD4<sup>+</sup>; and Treg17 cells as CCR6<sup>+</sup> CXCR3<sup>-</sup> CD127<sup>low</sup> CD25<sup>high</sup> CD4<sup>+</sup>.



## **IV. RESULTS**



## **IV.1 Gut microbiota signatures influence cancer treatment outcomes**



### IV.1.1 Introduction

The influence of the gut microbiota on the outcome of oncological therapy has been confirmed through metagenomic analysis of fecal samples. Profiling the gut microbiota has allowed the identification of microbial signatures with prognostic and predictive value in oncological diseases. Through the analysis of the gut microbial composition, it is now possible to distinguish between responders and non-responders, enabling the targeted selection of patients most suited for a specific treatment. However, while these gut microbial signatures are promising, they are not yet available for every patient cohort and all therapeutic strategies.

For instance, the combination of cyclin-dependent kinase 4/6 (CDK4/6) inhibitors and endocrine therapy represents the standard first-line treatment for patients with hormone receptor-positive/HER2-negative metastatic breast cancer (MBC). Despite the impressive improvement in progression-free survival (PFS) and overall survival (OS) achieved with this combination of drug classes, not all patients respond to the treatment and benefit from it. In response to the need for biomarkers that can predict the response to this type of therapy, the first part of this doctoral project focused on the profiling of the fecal microbiota in a group of patients (n=14) with HR+/HER2- metastatic breast cancer, categorized as responders (R) and non-responders (NR) based on PFS. While the comparison between the fecal samples of the two groups did not reveal significant differences in terms of  $\alpha$ - and  $\beta$ -diversity, the metagenomic analysis showed differences at the phylum and species levels. Significant differences were highlighted at the species level: *Clostridium innocuum* and *Schaalia odontolytica* were exclusively present in the NR group, whereas *Bifidobacterium longum* and *Ruminococcus callidus* were significantly associated with the R group. The network analysis of the co-abundance of 118 species revealed, with high statistical significance, the presence of two bacterial clusters, SIG1 and SIG2, which consisted of 75% species associated with the NR group and 76% species associated with the R group, respectively. The bacterial species were further subdivided into two clusters (Cluster 1 and Cluster 2) through a cross-correlation analysis between all bacterial species and clinical/immunological characteristics. Cluster 1 was associated with parameters such as neutrophil-to-lymphocyte ratio (NLR) and the CD4+/CD8+ T cell ratio. Specifically, a positive association between *Clostridium innocuum* and NLR was highlighted, a parameter known to correlate with poor prognosis and systemic inflammation in breast cancer. This prospective study, although small and exploratory, highlights the potential value of predictive biomarkers based on the gut microbiota that could be integrated into clinical practice. It is important

to emphasize how this study highlights a relationship between the gut microbiota and the systemic immune component.

**This study was presented at ECCMID (European Congress of Clinical Microbiology and Infectious Diseases) 2023 (Appendix#2).**

**Further analysis of the gut microbiota in breast cancer was conducted in a review, which explores how the gut microbiota participates in the pathogenesis of neoplastic disease through endocrine, metabolic, and immunological interactions, influencing prognosis and treatment response. The review also provides an overview of the main methods of microbiota analysis and includes a brief section on the role of the intratumoral microbiota (Appendix#1).**

#### IV.1.2 Article I

**Title:** Faecal microbiota composition is related to response to CDK4/6-inhibitors in metastatic breast cancer: A prospective cross-sectional exploratory study

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## Original Research

# Faecal microbiota composition is related to response to CDK4/6-inhibitors in metastatic breast cancer: A prospective cross-sectional exploratory study

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## KEYWORDS

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Cyclin-dependent  
kinase (CDK)4/6-  
inhibitors;  
Metastatic breast  
cancer;

**Abstract Background:** Cyclin-dependent kinase (CDK)4/6-inhibitors with endocrine therapy represent the standard of treatment of hormone receptor-positive(HR+)/human epidermal growth factor receptor 2 (HER2)-negative metastatic breast cancer (MBC). Gut microbiota seems to predict treatment response in several tumour types, being directly implied in chemotherapy resistance and development of adverse effects. No evidence is available on gut microbiota impact on efficacy of HR+ breast cancer treatment.

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Metagenomic DNA;  
Faecal microbiota;  
16S targeted  
sequencing

**Patients and methods:** We assessed the potential association among faecal microbiota and therapeutic efficacy of CDK4/6-inhibitors on 14 MBC patients classified as responders (R) and non-responders (NR) according to progression-free survival. A stool sample was collected at baseline and V3–V4 16S targeted sequencing was employed to assess its bacterial composition. Statistical associations with R and NR were studied.

**Results:** No significant differences were observed between R and NR in terms of  $\alpha$ - $\beta$ -diversity at the phylum and species level. Machine-learning (ML) algorithms evidenced four bacterial species as a discriminant for R (*Bifidobacterium longum*, *Ruminococcus callidus*) and NR (*Clostridium innocuum*, *Schaalia odontolytica*), and an area under curve (AUC) of 0.946 after Random Forest modelling. Network analysis evidenced two major clusters of bacterial species, named Species Interacting Groups (SIG)1–2, with SIG1 harbouring 75% of NR-related bacterial species, and SIG2 regrouping 76% of R-related species ( $p < 0.001$ ). Cross-correlations among several patients' circulating immune cells or biomarkers and bacterial species' relative abundances showed associations with potential prognostic implications.

**Conclusions:** Our results provide initial insights into the gut microbiota involvement in sensitivity and/or resistance to CDK4/6-inhibitors + endocrine therapy in MBC. If confirmed in larger trials, several microbiota manipulation strategies might be hypothesised to improve response to CDK4/6-inhibitors.

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## 1. Introduction

The combination of cyclin-dependent kinase (CDK)4/6-inhibitors with endocrine therapy (ET), either an aromatase inhibitor (AI) or fulvestrant, represents one of the major advances of the last decades for the treatment of hormone receptor-positive (HR+)/human epidermal growth factor receptor 2 (HER2)-negative metastatic breast cancer (MBC). Despite the impressive progression-free survival (PFS) and overall survival (OS) improvements observed in pivotal trials in comparison to single-agent ET, not all patients benefit from the combination [1–3]. At present, no selective biomarkers of response to CDK4/6-inhibitors have been clearly identified, hence patient selection for such therapeutic strategy is only based on classical endocrine receptors and HER2 status, along with clinical conditions [1–3].

Gut microbiota, composed of more than 100,000 billion bacteria, fungi, archaea and viruses, plays an important role in many physiological functions, including nutrient absorption, immune system correct functioning, and various metabolic processes, like oestrogen metabolism, which is strongly correlated to HR+/HER2-negative breast cancer development [4–6]. Importantly, host-microbe interactions in physiological conditions help counteract invading pathogens and prevent tumorigenesis. As a consequence, imbalances in the local microbial environment (dysbiosis) could modulate the host immune responses and inflammation favoring disease pathogenesis and progression [4–6]. Nevertheless, there is emerging evidence regarding the capability of faecal microbiota to predict treatment response in several tumour types (e.g. to ipilimumab in melanoma, to 5-fluorouracil in colorectal cancer etc.) and being directly implied in chemotherapy resistance and development of side-effects

[7–10]. Still, very little is known regarding faecal microbiome impact on breast cancer treatment efficacy and only preliminary data are currently available [7,11,12].

Finally, neutrophil-to-lymphocyte ratio (NLR) is an inexpensive blood biomarker representative of the systemic immune-inflammatory status [13]. High levels of NLR have been associated with poor prognosis in malignant tumours, including breast cancer [14,15]. Interestingly, recent data suggest a potential relationship between inflammatory systemic status as detected via NLR and gut microbiome composition [16].

In this prospective observational cross-sectional study, we preliminarily explored 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 on a series of patients affected by HR+/HER2-negative MBC treated at the Breast Unit of the Cremona Hospital in Italy between March 2019 and March 2021.

## 2. Materials and methods

### 2.1. Patient selection and study procedures

We included in this exploratory prospective cross-sectional observational study patients affected by HR+/HER2-negative MBC, with endocrine-sensitive tumours [17] and candidate to receive a first/second-line with CDK4/6-inhibitor-based regimen as per standard clinical practice. Full inclusion/exclusion criteria are reported in [Supplementary materials](#).

All patients were treated with either palbociclib, ribociclib or abemaciclib with an AI or fulvestrant, according to standard treatment schedules. A mandatory

stool sample was collected at baseline before treatment started from each patient.

For the purpose of this study, patients were divided into responders (R) and non-responders (NR) to CDK4/6-inhibitors if disease progression happened after or before 10 months, respectively. This time point was selected since 9.5 months was the lowest median PFS obtained with a CDK4/6-inhibitor-based regimen in pivotal trials [3].

## 2.2. Sample collections

Before starting treatment, a blood sample was collected, as well as a stool sample, which was collected using eNAT Copan Kit at baseline before treatment. Approximately 5 g of the fresh stool was stored at +4 °C until processing and then kept at −70 °C until analysis. Blood samples were processed as elsewhere described to obtain blood cells counts [15].

## 2.3. DNA extraction, 16S rRNA gene amplification and sequencing

Total bacterial DNA was extracted from 50 mg of faecal material using the FastDNA SPIN Kit for Soil (MP Biomedicals, Eschwege, Germany). The genomic DNA was then quantified using the Qubit HS dsDNA fluorescence assay (Life Technologies, Carlsbad, California, United States of America). Amplification was carried out as described elsewhere [18], using Illumina's MiSeq v3 platform with 2 × 300 bp mode.

## 2.4. Bioinformatic analyses

Raw FASTQ files were analysed with DADA2 pipeline v.1.14 for quality check and filtering (sequencing errors, denoising, chimera detection). Raw reads were filtered and 2503 Amplicon Sequence Variants (ASV) were found. Analyses were performed with Python v.3.8.2. A matrix of bacterial species relative abundances and prevalence was built. Only bacterial species having a prevalence equal or higher than 20% were taken into account.

Data matrices were first transformed with pseudo count and centred-log-ratio (CLR), then normalised and standardised. For microbiota analysis, measurements of  $\alpha$ -diversity (within sample diversity) were calculated at species level. Exploratory analysis of  $\beta$ -diversity (between sample diversity) was performed using the Bray-Curtis measure of dissimilarity and represented in Principal Coordinate Analyses (PCoA), along with methods to compare groups of multivariate sample units (analysis of similarities [ANOSIM], permutational multivariate analysis of variance [PERMANOVA]). We implemented Partial Least Square Discriminant

Analysis (PLS-DA) and the subsequent Variable Importance Plot (VIP) as a supervised analysis wherein the VIP values (order of magnitude) were used to identify the most discriminant bacterial species among the cohorts.

Pearson matrices for network analysis were generated on normalised and standardised data with in-house scripts (Python v3.8.2). Intra-network communities (Species Interacting Groups, SIGs) were retrieved using the Blondel community detection algorithm [19].

## 2.5. Statistical analyses

The study was exploratory and the sample size was not based on a formal statistical assumption. Standard descriptive statistics were used, along with  $\chi^2$  test, Mann-Whitney *U* test and Kruskal-Wallis test, when appropriate. Survival differences between R and NR were estimated by the Kaplan–Meier method and the log-rank test. Exploratory univariate cox regression models were used to estimate PFS and OS hazard ratios (HR) with 95% confidence intervals (CI). Analyses were conducted with R version 3.6.1 for MacOSX. Significance was generally set at  $p \leq 0.05$ . More detailed bioinformatic and statistical methods are reported in [Supplementary Materials](#).

## 3. Results

Fourteen MBC patients complied with inclusion criteria and accepted entering the study ([Fig. 1](#)). The median follow-up at the time of the analysis was 32.5 months (95% CI: 31.6–not estimable [NE]). Seven (50%) patients were considered as R, while other seven were considered as NR. Median PFS and OS for R were not reached at the time of the analysis. For NR, median PFS was 6.2 months (95% CI: 3.8–NE) and median OS was 14.7 months (95% CI: 7.7–NE). R to CDK4/6-inhibitors showed a significantly improved OS compared to NR (HR: 19.81, 95% CI: 2.30–170.78;  $p = 0.007$ ) ([Supplementary Fig. 1](#)). Population characteristics and treatment details are fully reported in [Table 1](#).

Clinicopathological characteristics and circulating immune cells were not associated with PFS and OS (not shown), with the exception of NLR. Higher levels of NLR were significantly associated with worse PFS (HR: 4.13, 95% CI: 1.08–15.74;  $p = 0.038$ ), with a tendency towards a significantly worse OS (HR: 3.17, 95% CI: 0.87–11.72;  $p = 0.081$ ). Consistently, R showed significantly lower levels of NLR (2.23 vs. 2.57;  $p = 0.026$ ) compared to NR. No other significant differences were observed, except for a slightly lower body mass index (BMI) (median 21.2 vs. 23.8;  $p = 0.016$ ) in the group of R, with no significant association with both PFS ( $p = 0.912$ ) and OS ( $p = 0.769$ ).

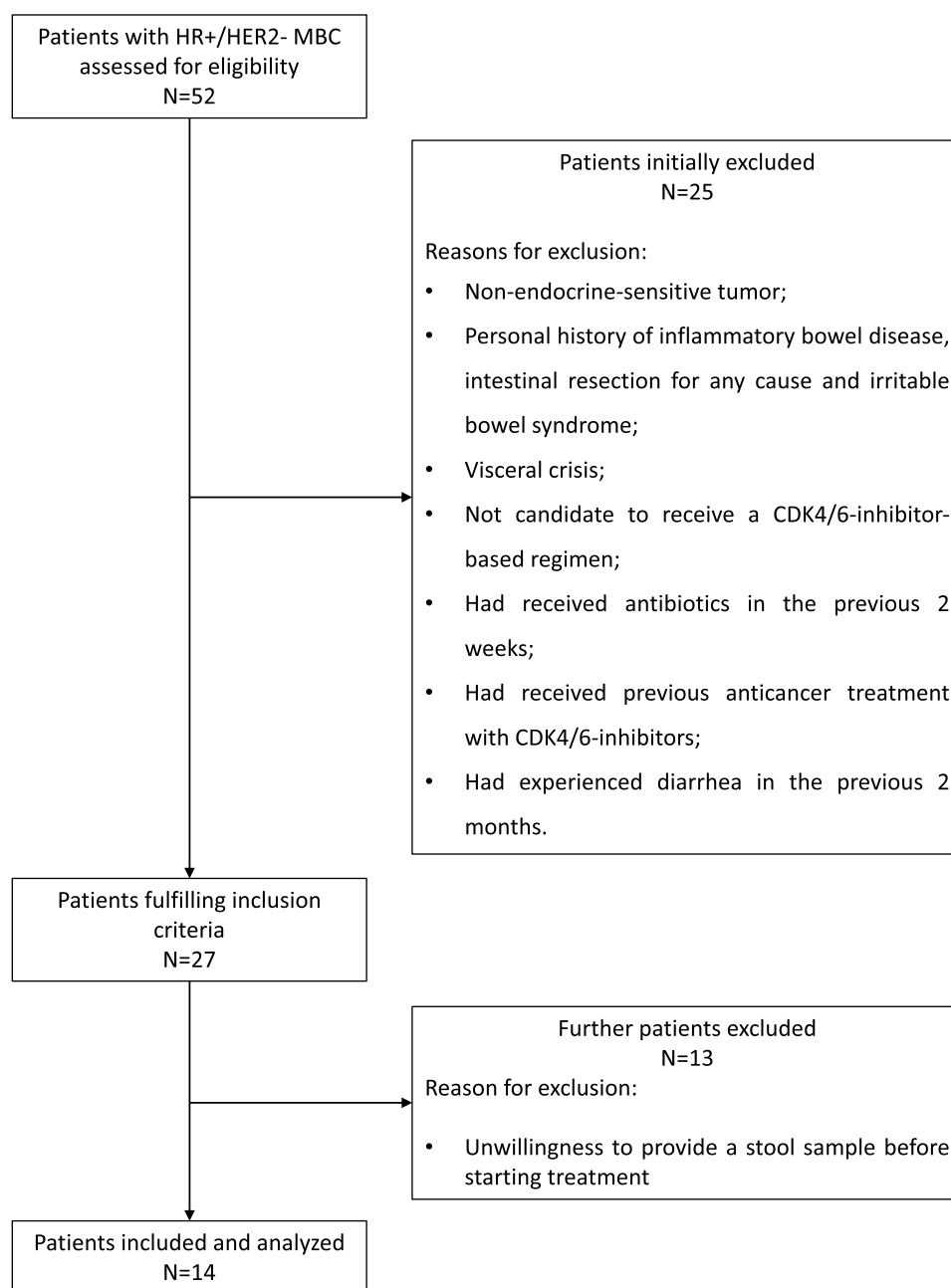


Fig. 1. STROBE flow chart for patients selection. CDK, cyclin-dependent kinase; HR+, hormone receptor positive; HER2-, human epidermal growth factor receptor 2-negative; MBC, metastatic breast cancer.

Regarding faecal microbiota composition, at phylum level was observed a higher relative abundance of *Actinobacteriota* in R patients, even if not significant (fold change = 1.8,  $p = 0.125$ ) (Table 2). Employing 118 bacterial species, after a cut-off of prevalence  $\geq 20\%$ , we found no significant differences among NR and R in terms of  $\alpha$ -diversity (richness  $p = 0.337$ , biodiversity  $p = 0.655$ , Fig. 2A) and  $\beta$ -diversity (PCoA, ANOSIM = 0.048,  $p = 0.267$ ; PERMANOVA = 1.131,  $p = 0.299$ , Fig. 2B). PLS-DA and the derived VIP showed that four bacterial species were significant in dividing NR from R, namely *Bifidobacterium longum*, *Ruminococcus callidus*, *Clostridium innocuum* and

*Schaalia odontolytica* (Fig. 2C). By using the relative abundances of these species in a random forest model, a good prediction was provided, even if limited by the sample number (area under curve [AUC] = 0.946, Specificity = 0.990, Sensitivity = 0.804) (Fig. 2D). *B. Longum* and *R. Callidus* were significantly more abundant ( $p = 0.012$  and  $p = 0.017$ , respectively) in R, with the former being also significantly more prevalent ( $p = 0.033$ ), while *C. Innocuum* and *S. Odontolytica* were present exclusively in NR (relative abundance  $p = 0.025$  both) (Fig. 2E). Other differences in relative abundance and prevalence for all other species were not significant (all  $p > 0.05$ ).

Table 1  
Population clinicopathological characteristics and circulating immune cells levels.

| Demographics                     | Responders  |       | Non-responders |       | Overall patients |       | <i>p</i> Values* |
|----------------------------------|-------------|-------|----------------|-------|------------------|-------|------------------|
|                                  | N           | %     | N              | %     | N                | %     |                  |
|                                  | 7           | 50.0  | 7              | 50.0  | 14               | 100.0 |                  |
| Age                              |             |       |                |       |                  |       |                  |
| Median                           | 70          | -     | 65             | -     | 67.5             | -     | 1.00             |
| IQR                              | 47.0–74.5   | -     | 58.5–72.0      | -     | 56.0–73.5        | -     |                  |
| BMI                              |             |       |                |       |                  |       |                  |
| Median                           | 21.2        | -     | 23.8           | -     | 23.5             | -     | 0.016            |
| IQR                              | 20.0–29.4   | -     | 23.1–28.4      | -     | 20.9–29.3        | -     |                  |
| Oestrogen receptor               |             |       |                |       |                  |       |                  |
| Positive (> 1%)                  | 7           | 100.0 | 7              | 100.0 | 14               | 100.0 | -                |
| Negative                         | 0           | 0.0   | 0              | 0.0   | 0                | 0.0   |                  |
| Progesterone receptor            |             |       |                |       |                  |       |                  |
| Positive (> 1%)                  | 4           | 57.1  | 5              | 71.4  | 9                | 64.3  | 0.577            |
| Negative                         | 3           | 42.9  | 2              | 28.6  | 5                | 35.7  |                  |
| Ki67%                            |             |       |                |       |                  |       |                  |
| ≤20                              | 4           | 57.1  | 4              | 57.1  | 8                | 57.1  | -                |
| > 20%                            | 3           | 42.9  | 3              | 42.9  | 6                | 42.9  |                  |
| HER2                             |             |       |                |       |                  |       |                  |
| 0                                | 5           | 71.4  | 7              | 100.0 | 12               | 85.7  | 0.311            |
| 1+                               | 0           | 0.0   | 0              | 0.0   | 0                | 0.0   |                  |
| 2 + FISH neg.                    | 2           | 28.6  | 0              | 0.0   | 2                | 14.3  |                  |
| Menopausal Status                |             |       |                |       |                  |       |                  |
| Pre/Perimenopausal               | 2           | 28.6  | 1              | 14.3  | 3                | 21.4  | 0.515            |
| Postmenopausal                   | 5           | 71.4  | 6              | 85.7  | 11               | 78.6  |                  |
| Treatment Line                   |             |       |                |       |                  |       |                  |
| 1st                              | 6           | 85.7  | 4              | 57.1  | 10               | 71.4  | 0.237            |
| 2nd                              | 1           | 14.3  | 3              | 42.9  | 4                | 28.6  |                  |
| Endocrine therapy                |             |       |                |       |                  |       |                  |
| Aromatase Inhibitor              | 5           | 71.4  | 5              | 71.4  | 10               | 71.4  | -                |
| Fulvestrant                      | 2           | 28.6  | 2              | 28.6  | 4                | 28.6  |                  |
| CDK4/6-inhibitor                 |             |       |                |       |                  |       |                  |
| Palbociclib                      | 2           | 28.6  | 0              | 0     | 2                | 14.3  |                  |
| Ribociclib                       | 4           | 57.1  | 4              | 57.1  | 8                | 57.1  | 0.223            |
| Abemaciclib                      | 1           | 14.3  | 3              | 42.9  | 4                | 28.6  |                  |
| ECOG                             |             |       |                |       |                  |       |                  |
| 0–1                              | 7           | 100.0 | 7              | 100.0 | 14               | 100.0 | -                |
| ≥2                               | 0           | 0.0   | 0              | 0.0   | 0                | 0.0   |                  |
| Visceral metastases              |             |       |                |       |                  |       |                  |
| Yes                              | 2           | 28.6  | 5              | 71.4  | 7                | 50.0  | 0.109            |
| No                               | 5           | 71.4  | 2              | 28.6  | 7                | 50.0  |                  |
| Number of metastases             |             |       |                |       |                  |       |                  |
| < 3                              | 6           | 85.7  | 5              | 71.4  | 11               | 78.6  | 0.515            |
| ≥3                               | 1           | 14.3  | 2              | 28.6  | 3                | 21.4  |                  |
| Best response                    |             |       |                |       |                  |       |                  |
| Complete response                | 0           | 0.0   | 0              | 0.0   | 0                | 0.0   | 0.213            |
| Partial response                 | 1           | 14.3  | 0              | 0.0   | 1                | 7.1   |                  |
| Stable disease                   | 6           | 85.7  | 5              | 71.4  | 11               | 78.6  |                  |
| Progressive disease              | 0           | 0.0   | 2              | 28.6  | 2                | 14.3  |                  |
| CD3+ CD4+ Lymphocytes (cells/UL) |             |       |                |       |                  |       |                  |
| Median                           | 633.0       | -     | 722.7          | -     | 677.8            | -     | 0.710            |
| IQR                              | 559.3–784.1 | -     | 601.1–877.6    | -     | 592.0–803.4      | -     |                  |
| CD3+ CD8+ Lymphocytes (cells/UL) |             |       |                |       |                  |       |                  |
| Median                           | 463.0       | -     | 361.0          | -     | 442.5            | -     | 0.456            |
| IQR                              | 402.0–647.0 | -     | 253.0–633.5    | -     | 295.8–688.8      | -     |                  |
| NK Lymphocytes (cells/UL)        |             |       |                |       |                  |       |                  |
| Median                           | 381.0       | -     | 258.0          | -     | 296.0            | -     | 0.805            |
| IQR                              | 157.0–470.0 | -     | 194.0–355.5    | -     | 183.2–429.0      | -     |                  |
| Tregs (cells/UL)                 |             |       |                |       |                  |       |                  |
| Median                           | 59.00       | -     | 69.0           | -     | 62.0             | -     | 0.565            |
| IQR                              | 39.0–67.0   | -     | 45.5–82.0      | -     | 42.8–75.0        | -     |                  |
| CD4/CD8 Ratio                    |             |       |                |       |                  |       |                  |
| Median                           | 1.21        | -     | 1.83           | -     | 1.43             | -     | 0.289            |
| IQR                              | 0.99–1.56   | -     | 1.24–2.66      | -     | 1.045–2.145      | -     |                  |

(continued on next page)

Table 1 (continued)

| Demographics | Responders    |      | Non-responders |      | Overall patients |       | <i>p</i> Values* |
|--------------|---------------|------|----------------|------|------------------|-------|------------------|
|              | N             | %    | N              | %    | N                | %     |                  |
|              | 7             | 50.0 | 7              | 50.0 | 14               | 100.0 |                  |
| NLR          |               |      |                |      |                  |       |                  |
| Median       | 2.23          | -    | 2.57           | -    | 2.39             | -     | 0.026            |
| IQR          | 1.52–2.27     | -    | 2.53–3.01      | -    | 1.87–2.57        | -     |                  |
| PLR          |               |      |                |      |                  |       |                  |
| Median       | 111.11        | -    | 179.88         | -    | 140.97           | -     | 0.128            |
| IQR          | 100.38–140.97 | -    | 152.79–193.89  | -    | 108.69–182.66    | -     |                  |

BMI, body mass index; HER2, human epidermal growth factor receptor 2; IQR, interquartile range; NLR, neutrophil-to-lymphocyte ratio; PLR, platelet-to-lymphocyte ratio.

Italic values are statistically significant.

\* Exploratory, unadjusted.

With the aim of finding other bacterial species within the faecal microbiota of our MBC patients that would better describe the separation of NR and R, we employed a co-abundance network analysis of all the 118 species (Fig. 3A). Network analysis evidenced two major clusters of interacting bacterial species, named SIGs, in which a SIG1 group harboured 75% of NR-related species, while a SIG2 group harboured 76% of species with higher relative abundance in R (Fig. 3A). This topological distribution was highly significant (Chi-square  $p < 0.001$ ), thus meaning that these two communities could have an opposite role in responsiveness to CDK4/6-inhibitors.

A cross-correlation analysis of all the 118 bacterial species with all of the 14 patients' immunological features and BMI was carried out. Two different clusters were formed by the correlation of the species with CD8+, CD4+, NK and Tregs lymphocytes, CD4/CD8 ratio, platelet-to-lymphocytes ratio (PLR), NLR and/or BMI (Fig. 3B). Among the four species evidenced by the VIP plot (Fig. 2C), only *C. Innocuum* showed a positive association with NLR ( $r = 0.53$ ,  $p = 0.049$ ) (Table 3), falling within the cluster1 (Fig. 3B). Interestingly, a bunch of species falling within the cluster1 were positively related to NLR, CD4/CD8 and PLR, and, at the same extent, negatively related to CD8+, CD4+ and Tregs lymphocytes. When examining the species distribution among the correlogram and the network together, we found a correspondence among SIG1 and

cluster1, and among SIG2 and cluster2, evidencing four species in common for SIG1/cluster1 and six species in common for SIG2/cluster2 (Fig. 3C).

#### 4. Discussion

In a small population of 14 patients affected by HR+/HER2- MBC treated with CDK4/6-inhibitor + ET, we preliminarily observed some differential features of baseline faecal microbiome, especially in terms of phylum and species, which might be associated to poorer or better responses.

CDK4/6-inhibitors represent one of the major therapeutic advances in breast cancer of the decade. Also in our patients' cohort, although very small, women achieving the most prominent PFS results, presented a clear advantage in OS over women experiencing poorer responses. However, since a proportion of patients does not adequately respond to treatment, biomarkers for accurate patient selection are needed to maximise benefits and spare unnecessary toxicity and high treatment costs [20,21]. In the last couple of decades it has been extensively investigated the role of the immune system in cancer development and progression [22], as well as its complex interplay with chemotherapies and several targeted anticancer agents, with prognostic and predictive implications [15,23], leading also to the development of immune-checkpoint inhibitors (ICI) for the treatment of cancer [24]. To note, there is accumulating

Table 2  
Relative abundances of Phyla.

| Phylum                   | NR                 | R                  | <i>p</i> values* |
|--------------------------|--------------------|--------------------|------------------|
|                          | Mean $\pm$ SEM (%) | Mean $\pm$ SEM (%) |                  |
| <i>Firmicutes</i>        | 61.75 $\pm$ 4.95   | 55.80 $\pm$ 1.92   | 0.701            |
| <i>Bacteroidota</i>      | 22.85 $\pm$ 3.60   | 26.61 $\pm$ 1.47   | 0.443            |
| <i>Actinobacteriota</i>  | 4.14 $\pm$ 0.80    | 11.58 $\pm$ 3.35   | 0.125            |
| <i>Proteobacteria</i>    | 5.97 $\pm$ 2.73    | 4.53 $\pm$ 2.17    | 1.000            |
| <i>Verrucomicrobiota</i> | 2.38 $\pm$ 1.69    | 1.07 $\pm$ 0.58    | 0.891            |
| <i>Desulfobacterota</i>  | 0.37 $\pm$ 0.13    | 0.38 $\pm$ 0.10    | 1.000            |
| <i>Euryarchaeota</i>     | 1.53 $\pm$ 1.02    | 0.02 $\pm$ 0.02    | 0.551            |

FDR, false discovery rate; NR, non-responders; R, responders; SEM, standard error mean.

\* Two-sided Mann-Whitney *U* test, no FDR.

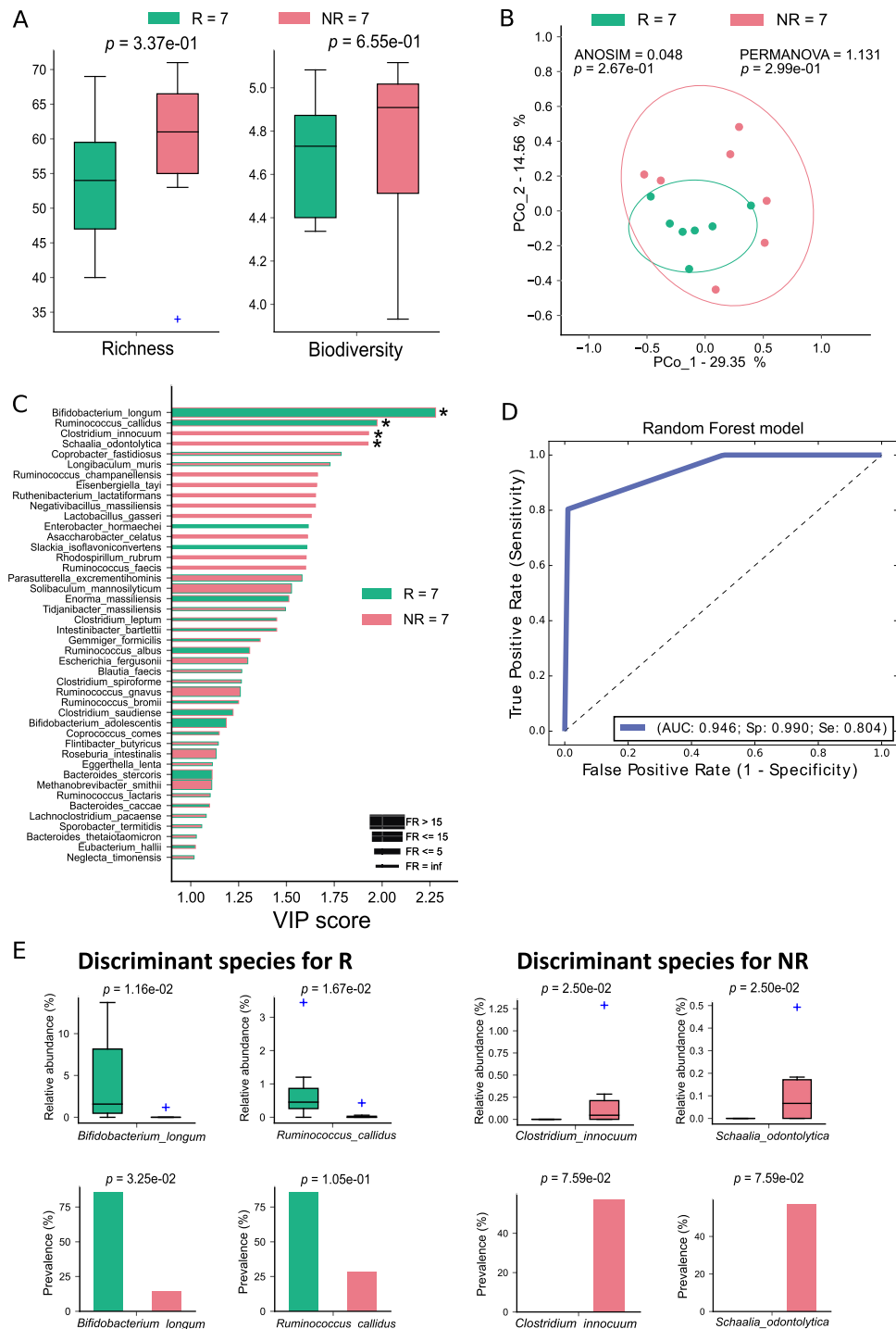


Fig. 2. Microbiota compositional shifts in responders and non-responders. Alpha- (A) and  $\beta$ -diversity (B) of responders (R, green, n = 7) and non-responders patients (NR, red, n = 7). (C) Variable Importance Plot (VIP). It shows: (i) discriminant species after Partial Least Square Discriminant Analysis (PLS-DA) in descending order of VIP score (bar length); (ii) the highest relative abundance depending on the cohort (central bar colour) and the lowest one (edge bar colour); (iii) fold ratio (FR) of the highest versus the lowest relative abundance (bar thickness), and (iv) significant difference after Mann–Whitney  $U$  test (non-false discovery rate [non-FDR],  $*p \leq 0.05$ ). Absent borders indicate mean relative abundance of zero in the compared cohort. (D) Receiver Operating Characteristic (ROC) curve with fivefold cross-validation following a Random Forest model on the four species (retrieved by the VIP plot). Area Under Curve (AUC), sensitivity (Se) and specificity (Sp) are reported. (E) Pairwise analysis of the selected four species depicts significant differences in terms of relative abundance (box plots) and prevalence (bar plots). In each sub-graph are reported the  $p$ -value (from Mann–Whitney  $U$  test) among R and NR. Blue crosses are outliers.

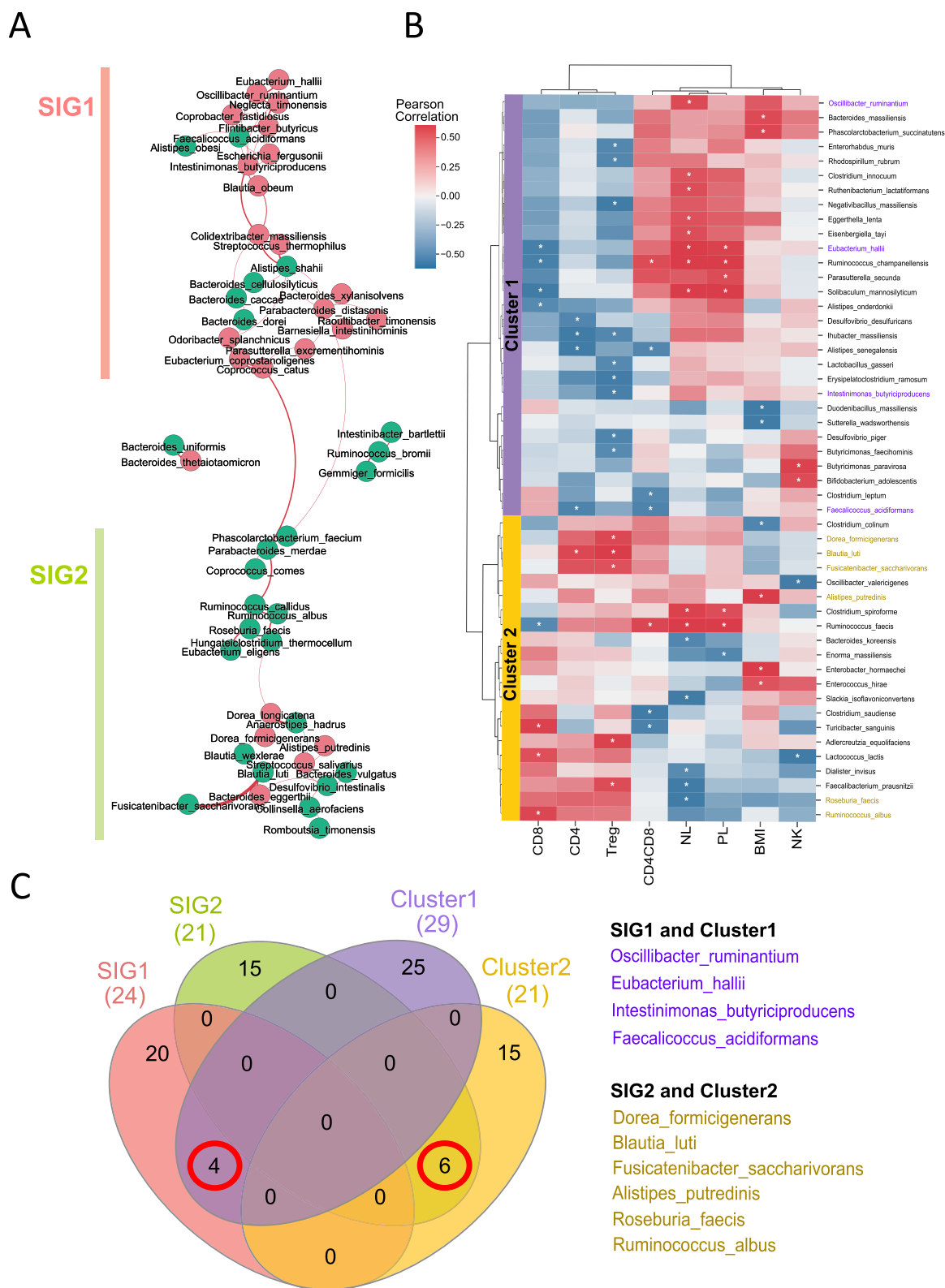


Fig. 3. Network analysis and correlation of bacterial species with immunological parameters. (A) Network analysis showing communities of bacterial species (namely, SIGs) and their positive (red Pearson coefficient) or negative (blue Pearson coefficient) relative abundances correlation. Nodes are coloured according to the cohort harbouring the higher relative abundance for a definite species, as NR (red) or R (green). Edge thickness is inversely proportional to the Pearson  $p$ -value after 10% Benjamini-Hochberg two-stages FDR, and it is coloured according to positive (red) or negative (blue) Pearson coefficient. (B) Correlogram of bacterial species and immunological

parameters shows positive (red) or negative (blue) Pearson correlation on CLR-transformed, normalised and standardised abundances. Significant correlation is marked with an asterisk inside each square: only species or parameters having at least one significant correlation were reported. Dendrograms on the x and y axes were generated following Bray–Curtis similarity, evidencing two different clusters for bacterial species (cluster1 and cluster2, shown here within white boxes). (C) Venn diagram showing species distribution among the two network SIGs and the two correlogram cluster, colours are as in panels (A) and (B). Highlighted in red the four and six species in common among SIGs and clusters. BMI, body mass index; CLR, centred-log-ratio; FDR, false discovery rate; NR, non-responders; NL, neutrophil-to-lymphocyte ratio; PL, platelet-to-lymphocyte ratio; R, responders; SIG, species-interacting group.

evidence suggesting that gut microbiota, especially bacteria, can trigger both innate and adaptive immune responses by eliciting the expression and secretion of immunomodulatory cytokines and chemokines that enter the systemic circulation and affect cancer pathogenesis, with the creation of tumour-promoting or tumour-antagonising immune microenvironments, as well as response to anticancer therapies [22,25,26]. In this perspective, the NLR indicates the dynamic relationship between innate (via neutrophil count) and adaptive (via lymphocyte count) immune responses and has been extensively investigated for the purpose of evaluating ongoing systemic inflammation during cancer development, severity stratification and prognosis of cancer disease in multiple cancer types, including breast cancer [27]. We observed in our exploratory analysis that patients experiencing more prolonged responses to CDK4/6-inhibitors-based regimens showed lower basal levels of NLR compared to poor CDK4/6-inhibitor responders. In addition, lower levels of NLR, as also observed elsewhere [27], showed an association with a better prognosis. Our results showed that some bacterial species, such as *C. Innocuum*, *Oscillibacter ruminantium* and *Eubacterium hallii*, seem to be positively related to NLR, thus probably exerting a negative effect on response to CDK4/6 inhibition. In fact, *C. Innocuum* showed higher relative abundance and prevalence in NR. On the contrary, *Roseburia fecis*, being negatively related to NLR, could have a favourable prognostic impact. These findings add to the previously mentioned postulated relationship between gut microbiota and systemic immunity [22,25,26].

Several members of the *Actinobacteria* phylum, such as *Bifidobacteria*, can be administered via probiotics [28]. Importantly, this phylum has shown to increase the efficacy of anti-PD-L1 ICI in breast and other tumours mouse models [29,30]. Intriguingly, an immunomodulatory effect for CDK4/6-inhibitors has been recently theorised after showing to promote tumour infiltration and activation of effector T cells and cytotoxic T cell-mediated clearance of tumour cells in pre-clinical studies [31,32]. In fact, a first phase I/II trial of palbociclib + letrozole and the ICI pembrolizumab already proved the combination to be well tolerated and active in the first-line setting of patients with HR+ MBC [33]. Here we found that *B. Longum* was more abundant in R, compared to NR. If *Actinobacteria* such as *Bifidobacteria* were effectively able to both improve

response to CDK4/6-inhibitors and anti-PD-L1 agents, they could be easily provided to patients via probiotics as a strategy to boost therapeutic efficacy of regimens including both or only one of the two drug classes, with the advantage of being safe and likely cost-effective.

A higher abundance of *R. Callidus* was also observed in R, as well. Although there is no specific study associating this species with breast cancer, it has been reported to be negatively associated with colorectal cancer [34].

Finally, a clear and statistically significant differential distribution of faecal bacterial species in SIGs according to response to CDK4/6-inhibitors was observed in the network analysis. Noteworthy, species associated with better responses seemed to interact more than species associated with worse responses.

Although numbers are too small to provide in-depth reliable conclusions, the tendencies observed should be further explored. Due to obvious technical limitations related to the study of gut microbiota, faeces could be considered as a potential proxy for its assessment, although reliable methodologies that adequately correlate the activity of faecal microbiota (composed of species that mostly reside in the transient luminal compartment) with the mucosa-associated microbiota (MAM) are currently missing [35].

Our study has several limitations worth nothing. First, results significance has been surely hampered by the reduced population. Nevertheless, larger studies are costly and require more education and sensibilization of both patients and operators regarding optimal collection and management of faecal samples. Furthermore, there is no current evidence regarding faecal microbiota role as a predictive factor of response to CDK4/6-inhibitors, and this study was intended to be a preliminary effort to provide translational hypothesis-generating data. Secondly, the lack of a control group that only received ET was not available, considering that, apart from specific cases, the standard of care is now represented by the combination of ET with a CDK4/6-inhibitor as upfront therapy [17]. At the same time, a recent small study in early-stage disease showed a significant difference in the mean microbial abundance between endocrine-resistant and endocrine-sensitive cases in terms of the taxonomic rank of order and family [36]. Thirdly, we had no validation cohort to make more robust the putative biomarkers retrieved by machine learning algorithms. Finally, patients could have

Table 3

Pearson correlation coefficients and respective *p*-values.

| Faecal bacterial species                   | Pearson coefficients |              |              |              |              |              |              |              |  |  | <i>p</i> values |              |              |              |              |              |              |              |  |  |
|--------------------------------------------|----------------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--|--|-----------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--|--|
|                                            | BMI                  | NLR          | PLR          | CD8+         | CD4+         | CD4+/CD8+    | NK           | Treg         |  |  | BMI             | NLR          | PLR          | CD8+         | CD4+         | CD4+/CD8+    | NK           | Treg         |  |  |
| <i>Adlercreutzia equalifaciens</i>         | 0.12                 | -0.03        | 0.20         | 0.21         | 0.28         | -0.26        | 0.08         | <b>0.59</b>  |  |  | 0.675           | 0.925        | 0.484        | 0.480        | 0.333        | 0.371        | 0.788        | <b>0.027</b> |  |  |
| <i>Alistipes onderdonkii</i>               | -0.24                | 0.35         | 0.48         | <b>-0.54</b> | -0.47        | 0.18         | 0.17         | -0.37        |  |  | 0.401           | 0.219        | 0.083        | <b>0.048</b> | 0.093        | 0.549        | 0.567        | 0.188        |  |  |
| <i>Alistipes putredinis</i>                | <b>0.72</b>          | 0.28         | -0.02        | -0.05        | 0.35         | 0.33         | 0.17         | 0.02         |  |  | <b>0.004</b>    | 0.333        | 0.948        | 0.862        | 0.221        | 0.255        | 0.552        | 0.947        |  |  |
| <i>Alistipes senegalensis</i>              | 0.10                 | 0.16         | 0.11         | -0.06        | <b>-0.61</b> | <b>-0.56</b> | 0.16         | -0.40        |  |  | 0.745           | 0.588        | 0.715        | 0.849        | 0.720        | <b>0.039</b> | 0.575        | 0.156        |  |  |
| <i>Bacteroides korensis</i>                | -0.11                | <b>-0.56</b> | -0.43        | 0.14         | 0.10         | 0.00         | 0.05         | -0.25        |  |  | 0.711           | <b>0.038</b> | 0.129        | 0.621        | 0.721        | 1.000        | 0.852        | 0.394        |  |  |
| <i>Bacteroides massiliensis</i>            | <b>0.56</b>          | 0.28         | 0.28         | -0.44        | -0.07        | 0.42         | 0.41         | -0.38        |  |  | <b>0.035</b>    | 0.330        | 0.325        | 0.119        | 0.818        | 0.136        | 0.151        | 0.179        |  |  |
| <i>Bifidobacterium adolescentis</i>        | -0.31                | -0.07        | 0.01         | -0.10        | -0.43        | -0.34        | <b>0.56</b>  | -0.10        |  |  | 0.289           | 0.822        | 0.976        | 0.725        | 0.121        | 0.232        | <b>0.038</b> | 0.733        |  |  |
| <i>Blautia luti</i>                        | -0.32                | -0.07        | 0.15         | 0.05         | <b>0.57</b>  | 0.27         | -0.11        | <b>0.61</b>  |  |  | 0.260           | 0.815        | 0.600        | 0.860        | <b>0.033</b> | 0.349        | 0.704        | <b>0.020</b> |  |  |
| <i>Butyrivibrio monas faecihominis</i>     | 0.10                 | -0.01        | -0.26        | -0.14        | -0.10        | 0.12         | 0.44         | <b>-0.54</b> |  |  | 0.731           | 0.978        | 0.373        | 0.630        | 0.745        | 0.690        | 0.119        | <b>0.047</b> |  |  |
| <i>Butyrivibrio monas paravivrosa</i>      | -0.03                | -0.09        | -0.32        | -0.12        | -0.10        | 0.10         | <b>0.59</b>  | -0.37        |  |  | 0.929           | 0.751        | 0.262        | 0.680        | 0.732        | 0.728        | <b>0.028</b> | 0.194        |  |  |
| <i>Clostridium colinum</i>                 | <b>-0.56</b>         | 0.20         | 0.25         | -0.39        | 0.20         | 0.38         | 0.22         | 0.21         |  |  | 0.645           | <b>0.049</b> | 0.076        | 0.172        | 0.486        | 0.182        | 0.453        | 0.461        |  |  |
| <i>Clostridium innocuum</i>                | 0.14                 | <b>0.53</b>  | 0.49         | -0.34        | -0.08        | 0.28         | -0.08        | -0.21        |  |  | 0.307           | 0.497        | 0.389        | 0.232        | 0.797        | 0.335        | 0.783        | 0.477        |  |  |
| <i>Clostridium leptum</i>                  | 0.05                 | -0.17        | -0.41        | 0.24         | -0.43        | <b>-0.54</b> | 0.25         | -0.04        |  |  | 0.862           | 0.570        | 0.143        | 0.418        | 0.128        | <b>0.044</b> | 0.387        | 0.894        |  |  |
| <i>Clostridium saudiense</i>               | 0.30                 | -0.22        | -0.08        | 0.44         | -0.12        | <b>-0.63</b> | -0.22        | 0.27         |  |  | 0.302           | 0.456        | 0.791        | 0.118        | 0.676        | <b>0.015</b> | 0.454        | 0.349        |  |  |
| <i>Clostridium spiroforme</i>              | 0.12                 | <b>0.59</b>  | <b>0.55</b>  | -0.19        | 0.15         | 0.09         | -0.36        | 0.21         |  |  | 0.693           | <b>0.027</b> | <b>0.041</b> | 0.520        | 0.615        | 0.757        | 0.208        | 0.475        |  |  |
| <i>Desulfovibrio desulfuricans</i>         | 0.05                 | 0.34         | 0.36         | -0.37        | <b>-0.56</b> | -0.22        | 0.14         | -0.16        |  |  | 0.861           | 0.228        | 0.204        | 0.192        | <b>0.038</b> | 0.456        | 0.631        | 0.573        |  |  |
| <i>Desulfovibrio piger</i>                 | -0.06                | -0.22        | -0.01        | -0.08        | -0.16        | 0.07         | 0.26         | <b>-0.56</b> |  |  | 0.851           | 0.451        | 0.984        | 0.776        | 0.573        | 0.811        | 0.376        | <b>0.036</b> |  |  |
| <i>Dialister invisus</i>                   | -0.19                | <b>-0.53</b> | -0.15        | 0.42         | 0.08         | 0.47         | -0.45        | 0.13         |  |  | 0.507           | <b>0.050</b> | 0.599        | 0.137        | 0.786        | 0.769        | 0.108        | <b>0.032</b> |  |  |
| <i>Dorea fornicigenans</i>                 | -0.23                | 0.26         | 0.30         | -0.16        | 0.47         | 0.42         | -0.11        | <b>0.57</b>  |  |  | 0.438           | 0.373        | 0.291        | 0.587        | 0.092        | 0.136        | 0.703        | <b>0.032</b> |  |  |
| <i>Duodenibacillus massiliensis</i>        | <b>-0.72</b>         | -0.43        | -0.10        | 0.16         | -0.09        | -0.13        | -0.19        | -0.13        |  |  | <b>0.004</b>    | 0.127        | 0.737        | 0.583        | 0.768        | 0.655        | 0.512        | 0.659        |  |  |
| <i>Egerthella lenta</i>                    | 0.44                 | <b>0.54</b>  | 0.50         | -0.44        | -0.11        | 0.39         | -0.02        | -0.47        |  |  | 0.113           | <b>0.046</b> | 0.068        | 0.118        | 0.701        | 0.165        | 0.933        | 0.091        |  |  |
| <i>Eisenbergiella taylori</i>              | 0.20                 | <b>0.56</b>  | 0.46         | -0.39        | -0.13        | 0.32         | -0.01        | -0.51        |  |  | 0.500           | <b>0.039</b> | 0.096        | 0.166        | 0.667        | 0.269        | 0.975        | 0.065        |  |  |
| <i>Enorma massiliensis</i>                 | -0.10                | -0.49        | <b>-0.55</b> | 0.38         | 0.15         | -0.04        | 0.10         | 0.14         |  |  | 0.734           | 0.073        | 0.703        | 0.185        | 0.619        | 0.886        | 0.746        | 0.632        |  |  |
| <i>Enterobacter hormaechei</i>             | <b>0.61</b>          | -0.11        | -0.12        | 0.27         | 0.07         | 0.00         | 0.03         | 0.04         |  |  | <b>0.021</b>    | 0.708        | 0.685        | 0.344        | 0.807        | 0.999        | 0.913        | 0.899        |  |  |
| <i>Enterococcus hirae</i>                  | <b>0.53</b>          | -0.17        | -0.38        | -0.01        | 0.12         | 0.09         | 0.48         | -0.03        |  |  | <b>0.050</b>    | 0.554        | 0.184        | 0.969        | 0.686        | 0.759        | 0.079        | 0.911        |  |  |
| <i>Enterorhabdus muris</i>                 | 0.09                 | 0.17         | 0.40         | -0.37        | -0.08        | 0.39         | -0.08        | <b>-0.55</b> |  |  | 0.764           | 0.551        | 0.158        | 0.189        | 0.784        | 0.168        | 0.792        | <b>0.041</b> |  |  |
| <i>Erysipelatoclostridium ramosum</i>      | 0.14                 | 0.20         | 0.25         | -0.18        | -0.53        | -0.25        | -0.03        | <b>-0.61</b> |  |  | 0.621           | 0.482        | 0.387        | 0.546        | 0.053        | 0.381        | 0.913        | <b>0.021</b> |  |  |
| <i>Eubacterium hallii</i>                  | 0.06                 | <b>0.89</b>  | <b>0.58</b>  | <b>-0.60</b> | -0.22        | 0.44         | 0.09         | -0.29        |  |  | 0.833           | <b>0.000</b> | <b>0.028</b> | <b>0.024</b> | 0.454        | 0.114        | 0.772        | 0.319        |  |  |
| <i>Faecalibacterium prausnitzii</i>        | -0.28                | <b>-0.62</b> | -0.27        | 0.32         | 0.36         | -0.05        | -0.15        | 0.56         |  |  | 0.331           | <b>0.018</b> | 0.353        | 0.266        | 0.207        | 0.853        | 0.620        | <b>0.038</b> |  |  |
| <i>Faecalicoccus acidiformans</i>          | 0.06                 | -0.06        | -0.32        | 0.24         | <b>-0.57</b> | <b>-0.57</b> | 0.14         | -0.38        |  |  | 0.831           | 0.834        | 0.260        | 0.412        | <b>0.034</b> | <b>0.034</b> | 0.637        | <b>0.186</b> |  |  |
| <i>Fuscatenibacter saccharivorans</i>      | -0.35                | -0.08        | 0.15         | -0.03        | <b>0.53</b>  | 0.34         | -0.09        | <b>0.54</b>  |  |  | 0.221           | 0.774        | 0.601        | 0.932        | <b>0.050</b> | 0.236        | 0.766        | <b>0.047</b> |  |  |
| <i>Hubacter massiliensis</i>               | 0.11                 | 0.43         | 0.43         | -0.37        | <b>-0.67</b> | -0.23        | 0.05         | <b>-0.55</b> |  |  | 0.704           | 0.121        | 0.125        | 0.191        | 0.147        | 0.951        | 0.864        | <b>0.042</b> |  |  |
| <i>Intestinimonas butyrificiproducens</i>  | 0.18                 | 0.35         | 0.08         | -0.18        | -0.41        | 0.02         | 0.09         | <b>-0.59</b> |  |  | 0.529           | 0.226        | 0.791        | 0.537        | 0.147        | 0.951        | 0.750        | <b>0.027</b> |  |  |
| <i>Lactobacillus gasseri</i>               | 0.08                 | 0.22         | 0.11         | -0.03        | -0.18        | -0.05        | -0.03        | <b>-0.54</b> |  |  | 0.790           | 0.440        | 0.713        | 0.925        | 0.529        | 0.876        | 0.930        | <b>0.045</b> |  |  |
| <i>Lactococcus lactis</i>                  | -0.23                | -0.06        | -0.13        | <b>0.54</b>  | 0.34         | -0.11        | <b>-0.62</b> | 0.42         |  |  | 0.425           | 0.847        | 0.665        | <b>0.044</b> | 0.231        | 0.704        | <b>0.018</b> | 0.139        |  |  |
| <i>Negativibacillus massiliensis</i>       | 0.11                 | 0.51         | 0.45         | -0.43        | -0.19        | 0.38         | -0.06        | <b>-0.62</b> |  |  | 0.697           | 0.060        | 0.111        | 0.123        | 0.508        | 0.185        | 0.832        | <b>0.017</b> |  |  |
| <i>Oscillibacter ruminantium</i>           | 0.51                 | <b>0.56</b>  | 0.24         | -0.32        | -0.35        | 0.16         | 0.22         | -0.35        |  |  | 0.061           | <b>0.036</b> | 0.402        | 0.262        | 0.213        | 0.579        | 0.460        | 0.220        |  |  |
| <i>Oscillibacter valericigenes</i>         | 0.11                 | 0.29         | 0.16         | 0.26         | 0.03         | 0.15         | <b>-0.61</b> | 0.08         |  |  | 0.716           | 0.322        | 0.584        | 0.373        | 0.906        | 0.618        | <b>0.021</b> | 0.776        |  |  |
| <i>Parasutterella secunda</i>              | 0.10                 | 0.50         | <b>0.56</b>  | -0.53        | -0.02        | 0.53         | -0.06        | -0.13        |  |  | 0.730           | 0.067        | <b>0.038</b> | 0.051        | 0.944        | 0.051        | 0.832        | 0.655        |  |  |
| <i>Phascolarctobacterium succinatutens</i> | <b>0.58</b>          | 0.28         | 0.33         | -0.44        | 0.03         | 0.45         | 0.37         | -0.08        |  |  | <b>0.031</b>    | 0.334        | 0.245        | 0.119        | 0.924        | 0.105        | 0.197        | 0.786        |  |  |
| <i>Rhodospirillum rubrum</i>               | 0.19                 | 0.42         | 0.13         | -0.40        | -0.05        | 0.40         | 0.06         | <b>-0.55</b> |  |  | 0.525           | 0.139        | 0.655        | 0.158        | 0.852        | 0.154        | 0.836        | <b>0.041</b> |  |  |
| <i>Roseburia faecis</i>                    | -0.45                | <b>-0.62</b> | -0.40        | 0.44         | 0.48         | -0.02        | -0.40        | 0.47         |  |  | 0.108           | <b>0.018</b> | 0.158        | 0.113        | 0.084        | 0.935        | 0.158        | 0.092        |  |  |
| <i>Ruminococcus albus</i>                  | -0.04                | -0.50        | -0.44        | <b>0.58</b>  | 0.41         | -0.04        | -0.34        | 0.40         |  |  | 0.890           | 0.066        | 0.120        | <b>0.031</b> | 0.146        | 0.901        | 0.229        | 0.154        |  |  |

(continued on next page)

Table 3 (continued)

| Faecal bacterial species               | Pearson coefficients |              |             |              |       |              |       |       |              |              | <i>p</i> values |              |       |              |       |
|----------------------------------------|----------------------|--------------|-------------|--------------|-------|--------------|-------|-------|--------------|--------------|-----------------|--------------|-------|--------------|-------|
|                                        | BMI                  | NLR          | PLR         | CD8+         | CD4+  | CD4+/CD8+    | NK    | Treg  | BMI          | NLR          | PLR             | CD8+         | CD4+  | CD4+/CD8+    | Treg  |
| <i>Ruminococcus champanellensis</i>    | 0.10                 | <b>0.66</b>  | <b>0.61</b> | <b>-0.59</b> | -0.13 | <b>0.56</b>  | -0.07 | -0.43 | 0.743        | <b>0.010</b> | <b>0.021</b>    | <b>0.025</b> | 0.648 | <b>0.038</b> | 0.122 |
| <i>Ruminococcus faecis</i>             | 0.09                 | <b>0.67</b>  | <b>0.61</b> | <b>-0.54</b> | 0.36  | <b>0.58</b>  | -0.01 | 0.37  | 0.750        | <b>0.008</b> | <b>0.021</b>    | <b>0.047</b> | 0.202 | <b>0.030</b> | 0.191 |
| <i>Ruthenibacterium lactatiformans</i> | 0.16                 | <b>0.56</b>  | 0.45        | -0.34        | -0.05 | 0.28         | 0.01  | -0.24 | 0.586        | <b>0.039</b> | 0.105           | 0.233        | 0.856 | 0.336        | 0.401 |
| <i>Slackia isoﬂavoniconvertens</i>     | 0.17                 | <b>-0.63</b> | -0.13       | 0.07         | 0.11  | 0.05         | 0.29  | 0.00  | 0.559        | <b>0.016</b> | 0.646           | 0.817        | 0.705 | 0.855        | 0.995 |
| <i>Solibaculum mammosilyticum</i>      | 0.15                 | <b>0.70</b>  | <b>0.74</b> | <b>-0.68</b> | -0.05 | 0.40         | -0.05 | -0.05 | 0.602        | <b>0.005</b> | <b>0.002</b>    | <b>0.008</b> | 0.874 | 0.155        | 0.877 |
| <i>Sutterella wadsworthensis</i>       | <b>-0.63</b>         | -0.07        | 0.03        | -0.02        | -0.13 | -0.03        | -0.15 | -0.06 | <b>0.017</b> | 0.821        | 0.917           | 0.940        | 0.646 | 0.932        | 0.851 |
| <i>Turicibacter sanguinis</i>          | 0.12                 | 0.04         | -0.10       | <b>0.59</b>  | -0.29 | <b>-0.58</b> | -0.48 | 0.07  | 0.694        | 0.903        | 0.740           | <b>0.026</b> | 0.315 | <b>0.030</b> | 0.805 |

BMI, body mass index; NLR, neutrophil-to-lymphocytes ratio; PLR, platelet-to-lymphocytes ratio. In bold, significant coefficients and respective *p*-values.

received any CDK4/6-inhibitor, thus it is hard to know if different molecules might have exerted different immunomodulatory effects and differential indirect interactions with gut microbiome. In any case, evidences in this perspective lacks and most patients in both cohorts had received ribociclib (57.1% in both cohorts).

In conclusion, although small and exploratory, and requiring validation in different cohorts, our study suggests that stool microbiota might be able to early-predict responses to CDK4/6-inhibitors. Moreover, targeted antibiotic-based depletions of specific bacteria and oral administration of probiotics like *Bifidobacteria* might improve response to CDK4/6 inhibition, in a relatively safe and cost-effective fashion. Faecal microbiota transplantation, a strategy effectively adopted in other diseases [37–39], might be also explored for the purpose.

### Ethical declaration

The study was approved by the Ethic Committee of Val Padana (IRB n. 440/11/10/2019) and was conducted following the ethic principles of the Declaration of Helsinki, in compliance with all international, national and local regulatory laws and consistently with Good Clinical Practices guidelines. All patients signed a written informed consent before entering the study.

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### Author contributions

DG conceived the study. FS and AF performed the statistical analysis. VI performed bioinformatic analysis. FS and DG interpreted study results. FS, AF, VI and DG wrote the first manuscript draft. All authors except for FS, AF and VI participated in patients' recruitment and management. All authors participated in revising and writing the final manuscript. All authors approved the final manuscript version.

### Data availability statement

All raw data (fastq.gz files) and clinical metadata, complying with FAIR principles (<https://www.go-fair.org/fair-principles/>), are available at NCBI SRA portal under PRJNA946762 Bioproject.

### Declaration of Competing Interest

FS declares travel expenses by Novartis and Gilead and personal fees for educational events by Novartis, Daiichy-Sankyo and Gilead. DG declares personal fees for educational events by Novartis, Lilly, Pfizer,

Daiichi-Sankyo, Roche; research funds from AstraZeneca, Novartis and LILT. GC declares reports advisory/consulting fees from Seagen, Roche, Novartis, Eli Lilly, Daiichi Sankyo, AstraZeneca, Pfizer, Sanofi, Pierre Fabre and Gilead, and fees for non-CME services (e.g. speakers' bureaus) from Eli Lilly, Pfizer Inc and Daiichi Sankyo. The other authors have nothing to declare.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.ejca.2023.112948](https://doi.org/10.1016/j.ejca.2023.112948).

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## **IV. 2 Pre-existing anticomensal immune response: effects and clinical implications**



## IV.2.1 Introduction

The gut microbiota significantly influences the outcome of immunotherapy: certain bacteria classified as beneficial, such as *Akkermansia muciniphila*, are associated with improved patient survival and positive treatment outcomes, while other bacteria, such as *Escherichia coli*, are known as pathobionts linked to carcinogenesis and immunoresistance. The strong connection between the intestinal microbiota and both the local and systemic immune systems, its role in maintaining immune homeostasis, and its immunomodulatory effect on the tumor microenvironment (TME) highlight the need to understand the underlying mechanisms of this complex relationship, which impacts the outcome of oncological treatment.

This second part of the thesis focuses on understanding the significance of the pre-existing immune response, both cellular and humoral, directed against specific commensal bacteria, whether beneficial or pathobionts, with the ultimate goal of defining the impact of this memory response on immunotherapy outcomes. The recall response analysis was conducted on several patient cohorts enrolled in three distinct clinical studies, all involving immunotherapy (alone or in combination with other therapeutic strategies).

In the cohort of patients with NSCLC, serum levels of IgG against *Akkermansia spp.* (Akk) were found to be correlated with intestinal dysbiosis, a lower fecal prevalence of the same bacterium, and a significantly reduced overall survival (OS). Furthermore, elevated levels of anti-Akk IgG were significantly associated with reduced levels of circulating memory T cells (CM)Th1 and CD8+ T cells. The cellular recall response also revealed that memory Th1/Th17 cell reactivity against this beneficial bacterium was linked to worse clinical outcomes and was characterized by an immunosuppressive immune tone, indicated by a significant association with the proportion of circulating CMTh17 and Tr17 cells. Overall, these results suggest that both IgG-mediated humoral responses and Th1/Th17 cellular responses against a beneficial bacterium like Akk are associated with a negative outcome in immunotherapy, as well as with the eradication of the bacterium from the gut. This observation was confirmed in a cohort of refractory melanoma patients treated with anti-PD-1 in combination with FMT, where seropositivity against Akk, along with its eradication from the intestinal microbiota, was associated with immunoresistance.

In contrast, in the cohort of patients with colorectal cancer (CRC), the elicitation of humoral memory against pathobionts such as *E. coli* was associated with significant improvements in both OS and progression-free survival (PFS). Additionally, seropositive patients were more likely to show

undetectable levels of the pathobiont in their fecal samples. It is therefore assumed that seropositivity against pathobionts, when coupled with the absence of their intestinal colonization, exerts a protective effect with favorable clinical outcomes.

The analysis of the memory response against a specific commensal bacterium, combined with metagenomic analysis of fecal samples, not only lays the foundation for identifying predictive biomarkers of response but also proposes new strategies for selecting patients most suitable for microbiota-centered interventions (MCIs).

**The component of this work related to the analysis of the pre-existing anti-commensal cellular response in cohorts of patients with lung cancer (NSCLC) and genitourinary (GU) tumors, which suggests the influence of the recall response on immunotherapy outcomes, was presented at ASCO 2024 and received the ASCO Merit Award (Appendix#3-4). The integration with the analysis of humoral anti-commensal serum responses, which showed a significant association with immunotherapy outcomes when combined with the analysis of bacterial prevalence in fecal samples, was presented at the CICON (International Cancer Immunotherapy Conference) 2024 (Appendix#5).**

## **V. CONCLUSIONS AND PERSPECTIVES**



The human organism serves as the habitat for 39 trillion symbiotic microorganisms, collectively known as the human microbiota. The vast majority, approximately 97%, are bacteria that primarily reside in the intestine, especially in the colon, where they perform essential functions for the host. These include aiding digestion and nutrient absorption, protecting against pathogens, and maintaining innate and adaptive immune homeostasis. The intestinal microbiota thus influences not only local intestinal physiology but also the systemic one (Belkaid & Naik, 2013).

Despite its stability and resilience, external perturbations can disrupt the delicate balance of its composition and function, resulting in a state detrimental to health known as "dysbiosis." Dysbiosis is recognized as a hallmark or "meta-hallmark" of various diseases, including cancer (Zitvogel & Kroemer, 2024). Increasingly robust evidence supports the microbiome's impact on cancer, leading to its inclusion among the fundamental hallmarks of oncological pathology (Hanahan, 2022). By producing bioactive metabolites and immunomodulating the tumor microenvironment (TME), the intestinal microbiome—defined as the entirety of a microbial community, including its structural components, metabolites, and interactions within its 'theatre of activity' (Berg et al., 2020)—not only plays a role in tumorigenesis and cancer progression but also reveals prognostic and predictive heterogeneity in certain cancers, influencing the outcomes of antitumor treatments (Blake et al., 2024; Fernandes et al., 2022; Ting et al., 2022; Zhao et al., 2023).

The influence of the gut microbiota on cancer treatment outcomes has been confirmed in a cohort of HR+/HER2- metastatic breast cancer patients treated with CDK4/6 inhibitors combined with endocrine therapy. Analysis of bacterial phyla and species highlighted the microbiota's role as a predictive biomarker: patients who responded to therapy showed a distinct microbial composition compared to those who showed resistance. This result was further supported by a network analysis which further identified two main bacterial clusters: SIG1, associated with non-responders, and SIG2, associated with responders. Species within each cluster exhibited similar interactions, suggesting shared ecological or functional roles in response to CDK4/6 inhibitors. While these results are promising, it is important to emphasise that the study's small sample size limits the robustness of these findings, requiring further validation.

Among four bacterial species significantly differing between the two patient groups, *Bifidobacterium longum* stood out. This bacterium has been shown to enhance anti-PD-L1 immunotherapy outcomes in murine breast cancer and melanoma models by increasing CD8+ T cell infiltration (Kim et al., 2022; Sivan et al., 2015). Furthermore, preclinical studies have also demonstrated that selective CDK4/6 inhibitors not only arrest tumor cell cycles but also promote antitumor immunity by activating

effector T cells and inducing tumor infiltration (Deng et al., 2018; Goel et al., 2017). In addition, these inhibitors exhibit synergistic potential when combined with immune checkpoint inhibitors like PD-1, a finding that has been supported by early-phase I/II clinical trials (Yuan et al., 2021). To optimise therapeutic efficacy a therapeutic strategy could involve modulating the microbiota by administering *B. longum* as a probiotic alongside CDK4/6 inhibitors, either alone or combined with immunotherapy.

A further cross-correlation analyses integrating clinical and immune data led to the categorisation of the patients into two clusters, showing a strong association between the neutrophil-to-lymphocyte ratio (NLR) and *Clostridium innocuum*. Found exclusively in non-responders, this bacterium correlates with elevated NLR—a marker associated with poor clinical outcomes, systemic inflammation, and unfavorable prognosis in breast cancer (Zahorec, 2021). This microbiota connection with a marker of immune tone underscores the close and intricate dialogue between gut microbiota and the systemic immune system.

The aim of this research was to investigate the immune mechanisms underlying the interaction between the host immune system and the gut microbiota, analyzing the impact of this relationship on the outcome of cancer therapies. Specifically, the study focused on the dynamics and clinical significance of the pre-existing immune response toward specific commensal bacteria in patients with different types of tumors treated with immunotherapy. This recall response was examined in terms of both cellular and humoral immunity, subdividing commensals between beneficial or pathobionts based on their positive or negative influence on immunotherapeutic outcomes.

Overall, the analysis revealed that pre-existing bacterium-specific immune responses, both cellular and humoral, were associated with the outcomes of immunotherapy, confirming the predictive value of these biomarkers. However, these associations were tumor-dependent and, more importantly, influenced by the prevalence of bacteria in fecal samples. For example, in a prospective cohort of NSCLC patients, it was found that an IgG or Th1/Th17 memory response triggered against beneficial bacteria such as *Akkermansia spp.* or *Faecalibacterium prausnitzii*, combined with their elimination from the gut microbiota, was associated with a poor clinical outcome and a state of gut dysbiosis. The Th1/Th17 memory response against these beneficial bacteria was also linked to an immunosuppressive profile characterized by reduced proportions of central memory (CM) CD8+ effector cells and increased Tr17 immunosuppressive cells. Overall, these findings suggested that a pre-existing memory response toward beneficial bacteria, coupled with their eradication from the

gut microbiota, had a higher likelihood of leading to unfavorable clinical outcomes, as shown in the NSCLC cohort.

In contrast, in a prospective cohort of patients with metastatic colorectal cancer (MCRC), the selective elimination of fecal pathobionts was associated with improved therapeutic success. Patients who mounted a humoral response against pathobionts such as *E. coli* and *Fusobacterium nucleatum* tended not to harbor these bacteria in fecal samples and exhibited prolonged progression-free survival (PFS) and overall survival (OS). These results suggest that in patients with MCRC, an immune system predisposed to the elimination of pathobionts could improve immunotherapeutic outcomes.

Thus, from the study emerges the hypothesis that pre-existing immune responses were detrimental when directed against beneficial bacteria but protective when targeting pathobionts. However, this conclusion was valid only when the humoral immune response was associated with the loss of bacterial colonization in the gut.

The study represents an excellent example of integrating multiple microbiota-based predictive biomarkers of response: only by combining metagenomic analysis of fecal samples with the cellular and humoral recall response specific for commensal bacteria was it possible to predict the outcome of immunotherapy treatment. Tools such as TOPOSCORE (Derosa et al., 2024) further allowed the assessment of dysbiosis status and investigation of its association with the commensal-specific humoral immune response. This study thereby identified and integrated microbiota-based predictive biomarkers of response, demonstrating their applicability and utility in clinical oncology practice. Furthermore, the integration of these biomarkers provided the rationale for microbiota-centred interventions (MCIs) such as fecal microbiota transplantation (FMT). Although this microbial manipulation strategy has been extensively validated in preclinical murine models and shown its potential in the clinical setting (Baruch et al., 2021; Davar et al., 2021), significant questions remain regarding the so-called "enterofecal compatibility code." Indeed, although donor selection is of crucial importance and characterized by numerous levels of complexity, including those related to safety or perturbation of the gut microbiota, selecting an appropriate recipient for treatment is equally challenging (Porcari et al., 2023). In clinical trials where FMT had been performed, rooting and treatment success was not observed in all patients, despite receiving fecal material from the same donor. Our study highlighted potential screening strategies through a cohort of patients with refractory melanoma treated with combined FMT and immune checkpoint blockade (ICB). This cohort first allowed us to consolidate the hypothesis that poor outcomes in cancer treatment could

result from the eradication of beneficial bacteria from the gut, coupled with the elicitation of bacterium-specific humoral memory responses. In addition, this finding also led to define potential recipient screening strategies. Specifically, the success of FMT was less likely in patients who were or became seropositive for a beneficial commensal that was either absent or eradicated from the gut microbiota. Hence, recipients should be screened for both anti-commensal immune reactivity and intestinal microbiota composition before undergoing fecal material transfer. In cases where a patient mounts a humoral immune response against a beneficial bacterium, leading to its eradication from the gut, alternative microbiota modulation strategies could be considered, such as prebiotics, postbiotics, or dietary interventions. Alternatively, targeted strategies could counter or neutralize the humoral response against beneficial anti-commensal to prevent its eradication and thus preserve its intestinal colonization. Conversely, in patients with humoral reactivity against pathobionts such as *E. coli* or *F. nucleatum*, immunization approaches could be developed to promote their eradication and improve therapeutic outcomes. For example, inducing a memory humoral immune response against these pathobionts through vaccination could represent an effective eradication strategy to improve treatment success.

However, these strategies aimed at inducing or neutralizing pre-existing humoral immune responses to eradicate or preserve bacteria in the gut rely on a causal link between humoral memory responses and bacterial eradication from the intestinal microbiota, which has not yet been investigated. If an IgG-dependent humoral response were to induce the eradication of commensal bacteria from the gut, bacterium-specific IgG should be present in the intestinal lumen, and bacteria coated with these immunoglobulins should be detectable in fecal samples. Although the presence of anti-commensal IgG in systemic circulation is well-established, their presence in the intestinal lumen is expected to be negligible in healthy individuals (Koch et al., 2016).

This migration into the gut would imply a process of IgG transcytosis across the intestinal epithelium mediated by the FcRn receptor or could result from increased intestinal permeability associated with dysbiosis. To confirm the migration of IgG into the intestinal lumen, it would be necessary to verify the presence of IgG-coated bacteria in fecal samples using flow cytometry and subsequent metagenomic analysis of the IgG-coated bacterial fraction. In such cases, careful selection of eligible patient samples for this type of analysis will be crucial: serum samples should contain bacterium-specific IgG, and their dysbiosis status should be assessed through the detection of sMAdCAM-1 (Fidelle et al., 2023). The potential involvement of FcRn-mediated transcytosis could also be

evaluated using FcRn-blocking agents. Additionally, B-cell depletion through CAR-T cells in murine models could further confirm the role of antibody-secreting cells in bacterial depletion.

One critical next step will be identifying the serum IgG subtypes reactive to commensal bacteria: while IgG1 and IgG3 activate the complement cascade, IgG2 (specifically IgG2b) and IgG4 exhibit low inflammatory potential. Such analyses will help determine whether the anti-commensal humoral response is associated with a homeostatic or inflammatory immune state. This understanding could, for example, clarify why the humoral response against beneficial bacteria in the urothelial cancer cohort was associated with eubiosis condition, the preservation of bacteria in the gut environment, and improved therapeutic outcomes.

Future research will thus focus on mechanistic investigations to support the reported correlation between humoral responses and immunotherapeutic outcomes detailed in the study.

We can therefore conclude that the profound effect of gut microbiome on responses to oncological therapies and systemic immunity, combined with our growing understanding of the underlying mechanisms, opens new opportunities to effectively manipulate the gut microbiota in order to improve therapeutic outcomes for cancer patients.



## **VI. BIBLIOGRAPHY**



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## **VII. APPENDIX**



## APPENDIX#I: Article 3

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

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## Hot Topic



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

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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

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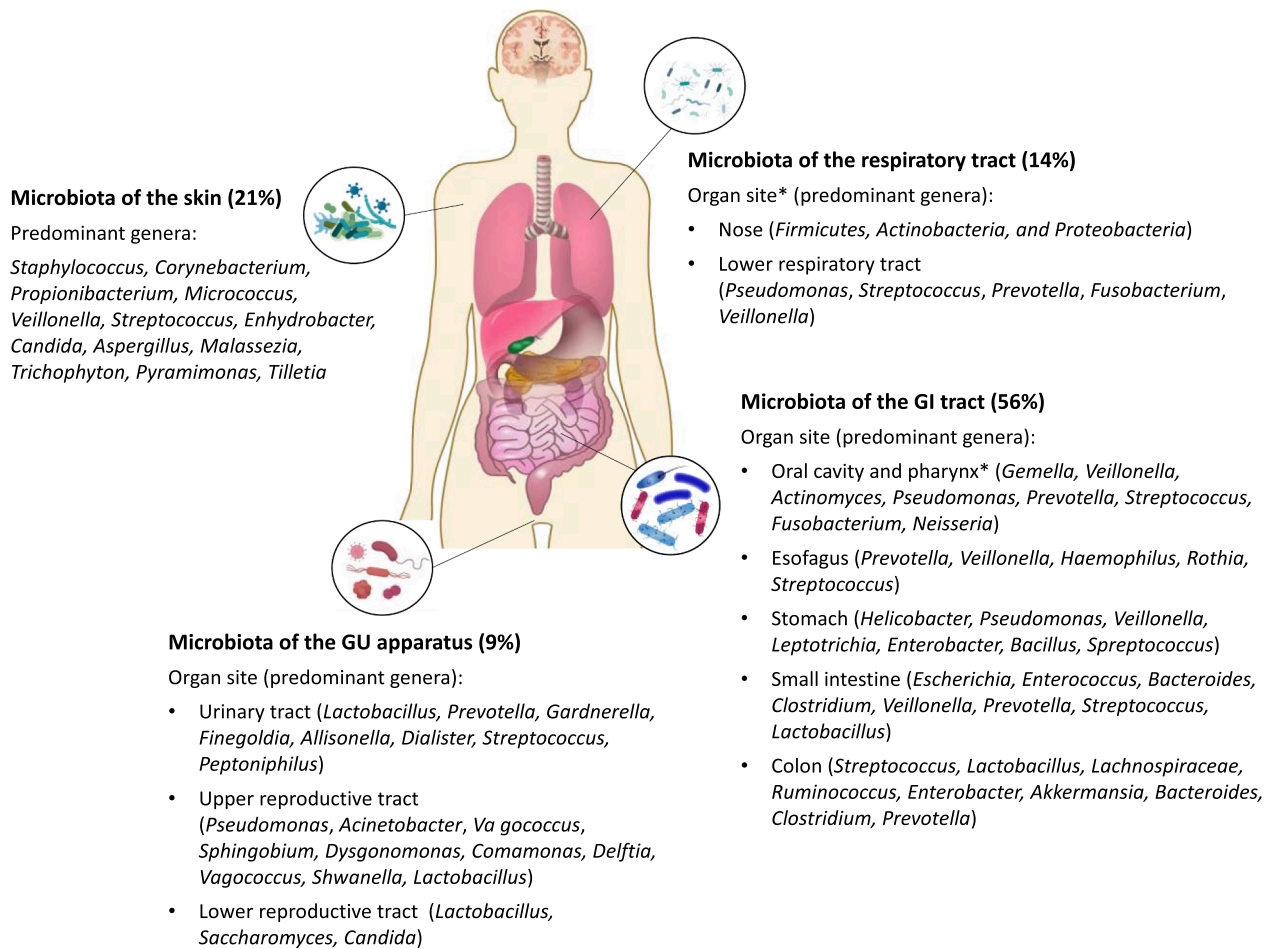


**Fig. 1. Overview of the human microbiota composition. Legend.** GU genitourinary; GI gastrointestinal. This image was created with Reactome (<https://reactome.org>) and Microsoft PowerPoint for MacOSX.

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

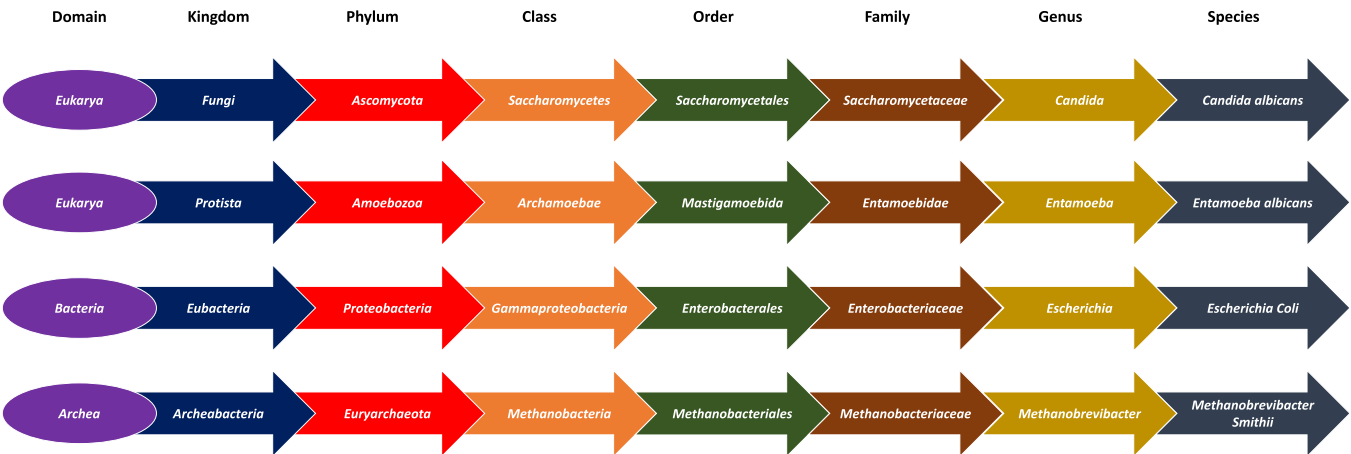


**Fig. 2. Practical example of microbial taxonomization by considering for each kingdom one of the most prevalent species at the human gut level. Legend.** Protista are more frequently pathogens rather than commensalia, while fungi are rarer in the gut microbiota than in other compartments like skin 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 *Archaea* domain, are part of the *Archaeobacteria* 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 *Archaeobacteria* 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 dysentery [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

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

| Methods                                                        | Main Limitations                                                                                                                                                                                                                                                                                                                                                                                                  |
|----------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <i>Fecal sampling</i>                                          | 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 |
| <i>Endoscopic sampling</i><br><i>Intestinal biopsy</i>         | 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.                                                                                                                                                                                      |
| <i>Luminal brush</i>                                           | Same bowel preparation effects than intestinal biopsy; invasive; inevitable contamination; costly and time-consuming                                                                                                                                                                                                                                                                                              |
| <i>Laser capture microdissection</i>                           | 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                                                                                                                                                                                     |
| <i>Sampling from aspirated intestinal fluid</i>                | Bowel preparation effects; invasive; unavoidable contamination; uncertainty of sampling sites; time-consuming; patient discomfort                                                                                                                                                                                                                                                                                 |
| <i>Sampling from surgery</i><br><i>Ileostomy</i>               | 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                                                                                          |
| <i>Other surgical procedures on the gastrointestinal tract</i> | Preoperative preparation effects on microbiome composition and distribution; significant and durable surgical-induced alterations in the composition of gut microbiota; impossible in healthy individuals                                                                                                                                                                                                         |
| <i>Ingestible sampling devices</i>                             | Potential contamination of samples with intestinal fluid from non-collected sites; costly; technical complexities                                                                                                                                                                                                                                                                                                 |

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,

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  $-20^{\circ}$  to  $-2^{\circ}\text{C}$ ) 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  $-20^{\circ}\text{C}$  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 ( $4^{\circ}\text{C}$ ), properly homogenized, processed and stored at  $-80^{\circ}\text{C}$ [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 ddH<sub>2</sub>O 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  $-80^{\circ}\text{C}$  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

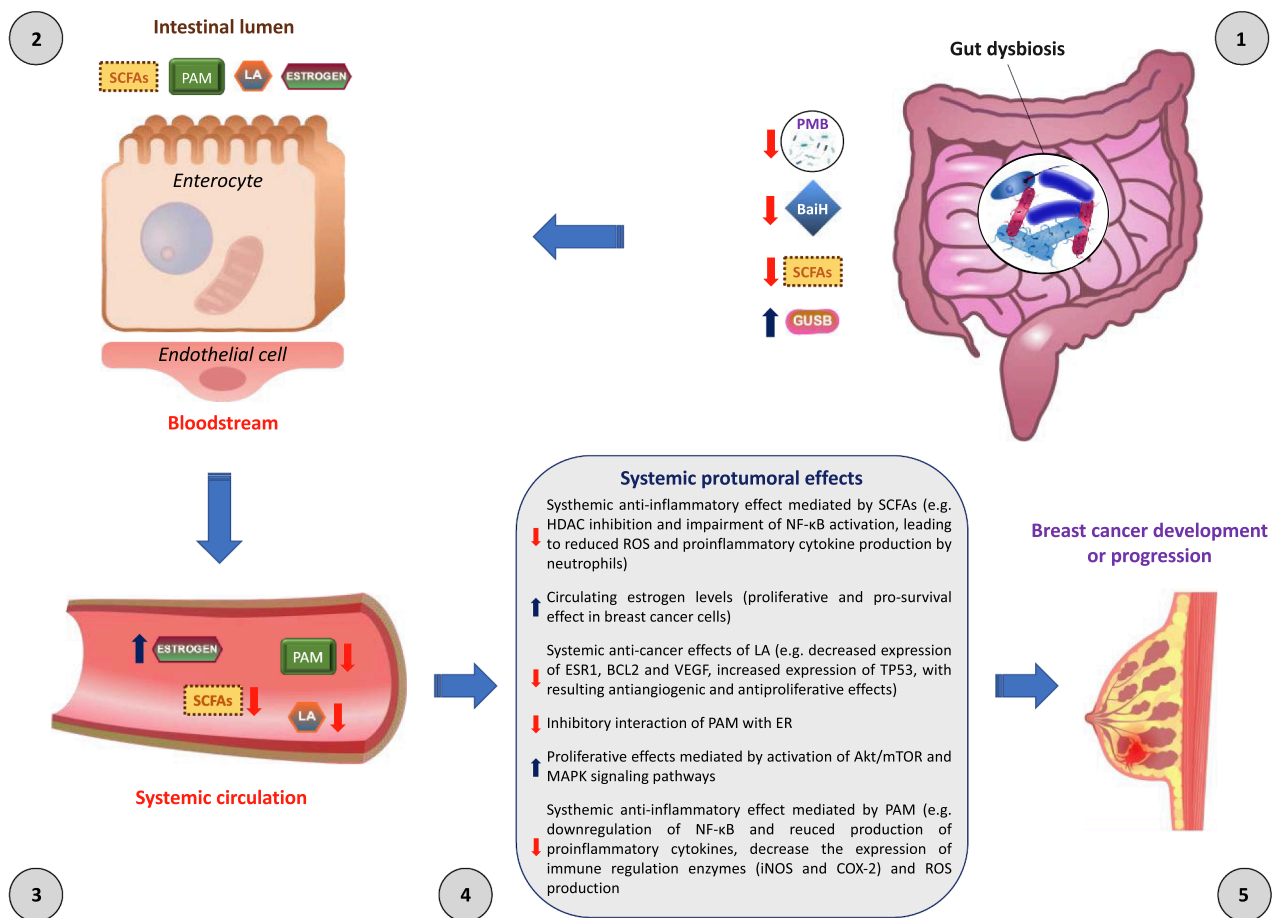


**Fig. 3. A scheme of the complex and multifaceted interaction among gut microbiota, estrogen, SCFAs and polyphenols metabolism. Legend.** 1: Dysbiosis may result in an increase in  $\beta$ -glucuronidase-producing bacteria, increasing the metabolization of glucuronized estrogens. In addition, a reduced 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  $7\alpha/\beta$ -hydroxysteroid dehydroxylase has been observed, resulting in reduced production of lithocholic 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  $\beta$ -glucuronidase; BaiH  $7\alpha/\beta$ -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  $\alpha$ - (within-sample) and  $\beta$ -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  $\alpha$ -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  $\beta$ -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  $\beta$ -glucuronidases and  $\beta$ -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  $\beta$ -glucuronidase-producing bacteria and, consequently, an increase in estrogen levels in circulation, potentially influencing the development of breast cancer [48,49]. Current evidence is characterized 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  $\alpha$ -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 *Pedococcus* 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  $\alpha$ - and  $\beta$ -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  $\alpha$ -diversity in patients compared to healthy controls, although some taxonomic differences in patients at the level of the Firmicutes phyla (higher levels of Clostridiaceae, *Feacalibacterium* and Ruminococcaceae and at lower levels of *Dorea* and Lachnospiraceae) 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  $\alpha$ -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  $\alpha$ -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 TNF $\alpha$ , 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. TNF $\alpha$ , IL-1, IL-6, IL-8, resistin and leptin, and attracts macrophages. Many of these cytokines promote the activation of the transcriptional factor NF- $\kappa$ B. Moreover, adipocytes express a functional and proinflammatory toll-like receptor (TLR) signaling system, which can promote the activation of the TLR–MD-2–NF- $\kappa$ B 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<br>Anthracycline + Taxane + Platinum                                                                                         | To assess the feasibility of gut microbiome analysis in patients undergoing NACT<br>To evaluate impact on treatment response and association with clinicopathologic factors<br>To describe longitudinal changes before and after exposure to CT | Higher $\alpha$ -diversity and richness in the pCR group at BL<br>No difference in $\alpha$ -diversity at the species level between the 2 groups at BL.<br>No significant correlation between $\alpha$ -diversity and clinical-pathological features.<br>No significant differences on $\beta$ -diversity between the two groups at BL.<br>Higher <i>B. eggerthii</i> on pCR group.<br>Stability in composition at the phyla and species levels in both groups<br>No change in median <i>Firmicutes/ Bacteroidetes</i> ratio after CT in either group.<br>No difference in $\alpha$ -diversity or $\beta$ -diversity between pCR and RD group<br>Higher species richness of <i>Bifidobacterium longum</i> in pCR group.<br>Higher richness of the <i>Bacteroides thetaiotaomicron</i> species and of the <i>Lachnospiraceae</i> genus in RD group. |
| Abuhadra, N. et al[76]  | 85          | eTNBC                      | Fecal samples | 16S genomic DNA sequencing                 | Adriamycin/<br>Cyclophosphamide                                                                                                                     | To investigate the association between gut microbiome and response to NACT                                                                                                                                                                      | No difference in $\alpha$ -diversity between the favorable and unfavorable prognosis groups.<br>$\beta$ -diversity as a stratification factor.<br>Higher $\alpha$ -diversity after CT.<br>Higher health-related species after CT.<br>$\beta$ -diversity post-CT predicted neurological side effects                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |
| Terrisse, S. et al[10]  | 76          | eBC                        | Fecal samples | Shotgun metagenomics                       | Anthracycline/Taxane/<br>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 statistical difference in $\alpha$ -diversity<br>Lower $\beta$ -diversity in metronomic group<br>Presence of <i>Slackia</i> associated with worse PFS.<br>Presence of <i>Blautia obeum</i> associated with better PFS.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |
| Guan, X et al [78]      | 31          | mBC                        | Fecal samples | DNA extraction<br>16S rRNA gene sequencing | Metronomic capecitabine (500 mg, three times daily)<br>Standard capecitabine (1,000–1,250 mg/m <sup>2</sup> , 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                                                                                                          | Higher $\alpha$ -diversity in R than in NR patients.<br><i>Bacteroidetes</i> was more abundant in NR patients.<br>Clostridiales (i.e., <i>Lachnospiraceae</i> ), <i>Bifidobacteriaceae</i> , <i>Turicibacteraceae</i> , and <i>Bacteroidales</i> (i.e., <i>Prevotellaceae</i> family) was enriched in R patients.<br>$\beta$ -diversity segregated patients according to the response to treatment                                                                                                                                                                                                                                                                                                                                                                                                                                                 |
| Di Modica, M et al [85] | 24          | HER2 + eBC                 | Fecal samples | DNA extraction<br>16S rRNA gene sequencing | Adriamycin/<br>cyclophosphamide/<br>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.                                                            | Low diversity of fecal microbiome in NR group<br><i>Bacteroides</i> genus enriched in NR group<br><i>Firmicutes</i> decreased in NRgroup<br>Greater representation of <i>Coprococcus</i> , and                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |
| Li, Y. Et al [86]       | 23          | eBC                        | Fecal samples | Metagenomic DNA sequencing                 | Anthracycline/Taxane/<br>cyclophosphamide/<br>Trastuzumab                                                                                           | To investigate the relationship between gut microbiome and responses to NACT e underlying mechanisms.                                                                                                                                           |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |

(continued on next page)

Table 2 (continued)

| Study /Reference                | Sample size | Population characteristics | Sample Source | Analysis Methods                           | Treatment Regimen           | Aim                                                                                                                                                                              | Main Findings                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |
|---------------------------------|-------------|----------------------------|---------------|--------------------------------------------|-----------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 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                                                                                                                         | <i>Ruminococcus</i> correlated with higher levels of CD4 + T cells in blood.<br><i>Coprococcus</i> , <i>Dorea</i> , and uncultured <i>Ruminococcus</i> sp. correlated with CD4 + TILs<br>Higher CD4 + and CD8 + TILs in R group<br>Shifts in the abundance were characteristic of pts receiving E but not of those receiving E+P.<br>Higher <i>Faecalibacterium</i> in the E-treated group<br>Higher levels of NLR significantly associated with worse PFS<br>No significant differences in $\alpha$ e $\beta$ -diversity between two groups<br><i>B. Longum</i> and <i>R. Callidus</i> significantly more abundant in R<br><i>C. Innocuum</i> and <i>S. Odontolytica</i> present exclusively in NR.<br>Identification of two clusters of bacterial species, SIG 1–2, SIG1 harbouring 75 % of NR-related bacterial species, and SIG2 regrouping 76 % of R.<br>Fecal metabolic profile influenced from second cycle of therapy.<br>SCFAs increased after CT<br>↑ Specific aminoacids increased in R patients |
| Schettini, F. et al[90]         | 14          | HR+/-HER2-mBC              | Fecal samples | DNA extraction<br>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 |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |
| 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                                                                  |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |

**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-epirubicin-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-2 $\alpha$  and TNF- $\alpha$ ) 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- $\kappa$ B 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 7 $\alpha$ / $\beta$ -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 an 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  $\alpha$ -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  $\alpha$  or  $\beta$  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  $\alpha$ -diversity were observed in patients with different prognosis. However,  $\beta$ -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  $\geq$ II[10]. Furthermore, adjuvant chemotherapy resulted in an increased  $\alpha$ -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 anti-cancer 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 anti-tumoral 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  $\alpha$ -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  $\beta$ -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  $\alpha$ -diversity in the NR patient group, along with a significant difference in  $\beta$ -diversity between R and NR patients [86]. Interestingly, some bacterial species were over-represented 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 pro-inflammatory 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<br>Case-control<br>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<br>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<br>Cohort<br>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<br>Cohort<br>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<br>Cohort<br>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<br>Cohort<br>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<br>EFS<br>OS<br>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<br>Phase III<br>Randomized<br>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<br>DFS in PD-L1 + ve patients                                                                                                           | Active, not recruiting |
| Neoadjuvant treatment of locally-advanced BC patients with Ribociclib and Letrozole – NEOLETRIB (NCT05163106)                                       | Interventional,<br>phase II,<br>multicentre,<br>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<br>cohort<br>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<br>Pre-treatment circulating immune population and metabolic profile                    | Recruiting             |
| Determinants of acquired endocrine resistance in mBC: A Pilot Study ENDO-RESIST (NCT04579484)                                                       | Observational<br>cohort<br>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<br>cohort<br>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 participants 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,<br>Phase II<br>Non-Randomized<br>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<br>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<br>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,<br>Creation of complex prospective clinico-biological database in metastatic breast cancer<br>specific metastatic biopsy intervention,<br>Creation of complex prospective clinico-biological database in metastatic breast cancer<br>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 |

(continued on next page)

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<br>Randomized<br>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<br>Phase I<br>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 FMT.<br>Efficacy of FMT for clinical remission/response of immune-related diarrhea/colitis. | Recruiting |
| Sacituzumab Govitecan +/- Pembrolizumab in Metastatic TNBC (NCT04468061)                                                    | Interventional<br>Randomized<br>Phase I<br>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  $\alpha/\beta$ -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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## APPENDIX#2: Abstract sent to ECCMID in 2023

### Gut microbiota components predict response to cyclin-dependent kinase (CDK)4/6-inhibitors in metastatic breast cancer (MBC): a prospective cross-sectional exploratory study

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#### Background

The CDK4/6-inhibitors with endocrine therapy represent the standard of treatment of hormone receptor-positive (HR+)/HER2-negative MBC. Gut microbiota seems to predict treatment response in several tumor types, being directly implied in chemotherapy resistance and development of side effects. Very little is known regarding gut microbiota impact on breast cancer treatment efficacy.

#### Methods

We explored the potential association among fecal microbiota and therapeutic efficacy of first line CDK4/6-inhibitors on 14 MBCs classified as responders (R) and non-responders (NR) accordingly to time of relapse. A stool sample was collected at baseline before treatment using Copan eNAT®; total DNA was extracted using the FastDNA™ SPIN Kit for Soil. Sequencing was performed using Illumina-MiSeq. Raw data sequences were elaborated with DADA2; the statistical analysis was performed using in-house Python and R scripts. Spearman's correlation analysis was performed to test the association among clinicopathological variables and gut microbiome phylum/family/genus.

#### Results

No significant compositional differences were observed among R and NR in terms of alpha and beta-diversity. At phylum level the Actinobacteria phylum was significantly higher in the R group (FDR=0.047), while the Euryarchaeota and Proteobacteria phyla were significantly higher in NR (FDR=0.003 and 0.023, respectively). *Clostridium innocuum* and *Schaalia odontolytica* were exclusively present in NR, while *Bifidobacterium longum* and *Ruminococcus callidus* were

significantly associated to R. Network analysis evidenced two major clusters of bacterial species, named Species Interacting Groups (SIGs), in which SIG1 harbored the NR-related species, while SIG2 harbored species with higher relative abundance in R, and such a topological distribution was highly significant (Chisquare=13.647,  $p=0.000221$ ). A cross-correlation analysis of bacterial species with immunological features evidenced significant correlations among SIG2 members and CD8, CD4, and Treg populations.

## **Conclusions**

The stool microbiota findings might have therapeutic implications: members of the Actinobacteria phylum, as Bifidobacteria, can be administered via probiotics, therefore, to improve response to CDK4/6-inhibitors, with the advantage of being safe and likely costeffective. These preliminary findings merit further investigation in a wider population to better understand their potential therapeutic implications.

### APPENDIX#3: Abstract sent to ASCO in 2024

Federica Gattazzo, Roxanne Birebent, Isabelle Lebhar, Marie Xiberras, Mathias Marques, Anna Reni, Caroline Flament, Adele Bonato, Lorenzo Belluomini, Hugo Chanfreau-Paris, AgatheCarrier, Benjamin Besse, Laurence Albiges, Jordi Remon, Claudia Parisi, Marie Deroussent, Franck Berthier, Carolina Alves Costa Silva, Lisa Derosa, Laurence Zitvogel

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**Background:** The gut microbiota influences the cancer immune set point and response to immune checkpoint inhibitors (ICB), participating in the differentiation and function of T cells. We aimed to investigate the potential impact of microbiota-specific circulating memory T cells in cancer immunotherapy. **Methods:** NCT04567446 provided longitudinal blood samples (T0, before starting ICB until 1.5 months; T3, between 3 and 5.5 months) from patients with lung (NSCLC) and kidney (RCC) during ICB therapy (alone or combinations) in France. Different pools of harmful (ENTERO: *Enteroclosterspp*, *Hungatella hathewayi*, VEILLEG: *Veillonella spp*, *Eggerthella lenta* and KLEBC: *Klebsiella pneumoniae*, *Escherichia coli*, *Fusobacterium nucleatum* (Fn)) or single beneficial (*Akkermansia spp.* (Akk), *Faecalibacterium prausnitzii* (Fp)) pasteurized bacteria (ppB) were used to stimulate whole blood sample (22h). The secretion of CXCL13, IL-17 (ELISA) and IFN $\gamma$  (VIDAS) was quantified at baseline and longitudinally to characterize memory T<sub>FH</sub>, T<sub>H</sub>17 and T<sub>H</sub>1 responses, respectively and analyze the effects of antibiotics. Progression-free survival (PFS), overall survival (OS) were assessed according to bacteria-specific T cell responses. **Results:** From Mar. 2023 to Jan. 2024, a total of 75 patients were screened and 39 patients enrolled in this analysis (54 assessed samples). Median age was 66yr, 72% were male, 74% had NSCLC, 26% had RCC and 78% were treated in first line. Median progression-free survival was 5.3 months (0.9-12.4); median overall-survival was 5.9 months (1.5-12.4). Firstly, we analyzed the 33 samples at baseline. 15% of patients harbored Akk-specific T<sub>FH</sub> memory responses and those patients with CXCL13 secretion superior to the median of the cohort tended to exhibit longer PFS (p=0.064) while 54% of patients harbored KLEBC T<sub>FH</sub> memory responses that were clinically irrelevant. 15% of patients harboring circulating Akk-specific T<sub>H</sub>1 memory responses had a shorter OS (p=0.055) while VEILLEG or KLEBC-specific T<sub>H</sub>1 responses detected in 24% and 42% cases were clinically irrelevant. 26/32 patients who did not show Akk-specific T<sub>H</sub>17 responses had a better OS. ATB tended to decrease bacteria-specific

CXCL13 and IFN $\gamma$  responses but increased T<sub>H</sub>17 memory T cells. While boosting the systemic T<sub>H</sub>1 TCR tonus (IFN $\gamma$  secretion by fresh blood T cells stimulated with the positive control (mitogen)), ICB decreased the most prominent reactivities against KLEBC, VEILLEG or *Akk*, suggesting that bacteria-specific T cells may traffic to tumoral or intestinal locations. **Conclusions:** Although awaiting further validation and correlations with humoral IgG/A titers, circulating memory T cells against distinct commensals may be clinically relevant to predict benefit to immunotherapy, suggesting that such bacteria may invade tumor cells or share molecular homology with cancer antigens.

APPENDIX#4: MERIT AWARD FROM ASCO IN 2024



2024

*Conquer Cancer Merit Award*

*Presented to*

*Frederica Gattazzo*

*in recognition of the high quality abstract entitled*

*"Impact of microbiota specific circulating memory T cells in response to immunotherapy."*

A handwritten signature in black ink, appearing to read "Howard A. Burris III".

*Howard A. Burris III, MD, FACP, FASCO  
Chair, Conquer Cancer*

A handwritten signature in black ink, appearing to read "Lynn M. Schuchter".

*Lynn M. Schuchter, MD, FASCO  
President, ASCO*

## APPENDIX#5: Abstract sent to CICON in 2024

### Humoral and cellular immune responses against gut commensals and cancer Immunotherapy Outcomes

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**Background:** Cancer or antibiotics-related gut dysbiosis is associated with resistance to immunotherapy. A deviation in the taxonomic composition of the gut microbiota may favor the relative outgrowth of distinct proinflammatory or immunosuppressive pathobionts such as *Fusobacterium nucleatum* spp. *animalis* or *pks+* *E. coli*. Whether the absence of memory B and T cells directed against these pathobionts could lead to immunoresistance remains an open question. This study investigated the dynamics and clinical significance of humoral and cellular immunity against select bacteria in patients (pts) diagnosed with lung (NSCLC) or genitourinary (GU) cancers (urothelial carcinoma [UC] or renal cell carcinoma [RCC]) receiving first-line anti-PD1 (ICB) therapies.

**Methods:** NCT04567446 prospectively enrolled NSCLC (n=74) and GU (n=41) cancer pts undergoing ICB at Gustave Roussy. Blood samples were collected at various time points between June 2021 and March 2024, starting at or shortly after the first treatment infusion. B and T cell memory responses were analysed against individual (*Akkermansia massiliensis* P2261 (Akk), *pks+* *E. coli* Q1696, *Faecalibacterium prausnitzii*, and *F. nucleatum* spp. *animalis* P6429) or pools (*E. coli*+*Klebsiella* spp, *Veillonella*+*Eggerthella* spp, *Enterocloster* spp, *H. hathewayi*) of bacteria. Plasma was analyzed by flow cytometry for bacteria-specific IgG and IgA antibodies. T cell-mediated responses were assessed by co-incubating fresh whole blood with pasteurized bacteria or in a DC/T cell crosstalk assay. In both cases, supernatants were evaluated for CXCL13, IL-17, and IFN $\gamma$  concentrations (follicular helper T [Tfh], Th17, Th1 cytokines, respectively) using ELISA and a Vitek ImmunoDiagnostic Assay System (VIDAS). Bacteria-specific seroreactivities and T cell responses were compared between cancer pts and healthy volunteers (HV), correlated with a score for gut dysbiosis (SIG1), and analyzed with comprehensive immune profiling by spectral flow cytometry.

**Results:** IgG titers were stable over time in NSCLC but tended to decrease during ICB in GU malignancies and after antibiotics. In NSCLC, *E. coli*-specific IgG was associated with a shorter PFS ( $p=0.022$ ). *Akk*-specific IgG correlated with SIG1 ( $p=0.009$ ) and anti-correlated with gut colonization by *Akk*. It was also associated with a dismal prognosis (OS  $p=0.045$  in multivariate analyses, PFS  $p=0.027$ ). In contrast, *Akk*-specific IgG correlated with a favorable prognosis in GU malignancies (OS  $p=0.006$ , PFS  $p=0.05$ ). The T cell assays were performed in 46 NSCLC and 24 GU pts. The whole blood assay revealed that, compared with HV, cancer pts showed defects in memory TFH response against *E. coli* and memory TH17 responses against *F. prausnitzii*, *Veillo/Egg*, and *E. coli/Klebs*. In contrast, they had exacerbated TH1 responses against *Akk*, *Veillo/Egg*, and *E. coli/Klebs*. NSCLC pts harboring *Akk*-restricted TH1 or TH17 responses had a shorter PFS ( $p=0.022$ ,  $p=0.06$  respectively), while pts harboring a TH17 response against *Enterocloster/HH* spp or *Veillo/Egg* had prolonged OS ( $p=0.036$ ,  $p=0.01$  respectively). In RCC pts, *Veillo/Egg*-specific TFH were of good prognosis (OS,  $p=0.05$ ). Using the DC/T cell assay, we corroborated that *Enterocloster/HH* spp-specific TH17 responses correlated with a favorable prognosis in NSCLC and that *F. prausnitzii*-specific TH17 correlated with shorter PFS in NSCLC ( $p=0.09$ ) and GU cancers ( $p=0.099$  for PFS,  $p=0.075$  for OS).

**Conclusions:** These findings suggest that a TH1/IgG immune profile set to kill beneficial microbes like *Akk* is associated with dysbiosis and predicts reduced benefit to ICB in NSCLC. Conversely, a TH17 immunization against harmful microbes (*Enterocloster/HH* spp or *Veillo/Egg*) may promote favorable outcomes. Importantly, these findings are context-dependent, as unlike in NSCLC, GU cancers respond better to ICB after immunization against *Akk*. This study highlights the potential interest in monitoring B and T cell responses to commensals during ICB.



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