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Final degree project

THE ONCOMICROBIOTICS AND THE INFLUENCE OF THE MICROBIOTA IN THE CARCINOGENESIS

Microbiology

Immunology

Pharmacology and therapeutics

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Abbreviations

ALD: Alcohol liver disease

APC: antigen-presenting cell

CRC: colorectal cancer

CTL: Cytotoxic T lymphocyte

CTLA: Cytotoxic-T-lymphocyte-associated

CTX: Cyclophosphamide

DAMP: danger-associated molecular pattern

DC: Dendritic cell

FMT: faecal microbial transplantation

GF: germ-free

HCC: hepatocellular cancer

ICB: immune check blocker

IEC: intestinal epithelial cells

IT: immunotherapy

LPS: lipopolysaccharide

mAb: monoclonal antibodies

MAMP: microbe-associated molecular patter

MHC: major histocompatibility

mLN: mesenteric lymph node

NAFLD: non-alcoholic fatty liver disease

NK: natural killer

PAMP: pathogen-associated molecular patter

PD: programmed death

PRR: pattern recognition receptor

SCFA: short chain fatty acid

SFB: Segmented filamentous bacteria

SPF: specific pathogen free

TCR: T cell receptor

Th: T cell helper

TIL: tumour infiltrated lymphocytes

TLR: toll-like receptor

TME: tumour microenvironment

Treg: T regulatory cell

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Abstract

The microbiome also known as “The forgotten organ” embraces the collective genome of all bacteria, archaea, fungi, protists, and viruses found in the host body surfaces and cavities. It is acknowledged that the microbiota has a role in many physiological functions such as the regulation of inflammation, immune response or haematopoiesis among others. The involvement of dysbiosis (alteration of the homeostatic microbiota) in the development of many pathologies like depression or cancer is a current topic of interest. On the other hand, carcinogenesis results as an accumulation of genetic and epigenetic alterations which are favoured by risk factors such as lifestyle, diet or dysbiosis. The latter has recently been discovered to either contribute or prevent carcinogenesis by modulating tumour or host cell microenvironment, respectively. Moreover, the microbiota has been found to influence chemotherapy, radiotherapy and immunotherapy efficacy and toxicity. Therefore, in this dissertation it is discussed the role of the microbiota as the cause, consequence or both of carcinogenesis as well as the mechanisms and the bacterial species that are involved in the efficacy or toxicity of certain anticancer drugs. It is relevant because it opens new strategies to prevent cancer or to enhance therapeutic agents against cancer which could result in positive clinical outcomes for cancer patients. One new strategy is the oncomicrobiotics, a select “cocktail of bacteria and/or bacterial products” as an adjunctive therapy to cancer with the intention of improving the immune response through optimizing the gut microbiota.

Resum

La microbiota també coneguda com “l’òrgan oblidat” és el conjunt de bacteris, arqueus, fongs, protists i virus que es troben a les superfícies corporals de l’hoste. Se sap que la microbiota desenvolupa un paper en moltes funcions fisiològiques com la regulació de la inflamació, la resposta immunològica o l’hematopoesi entre d’altres. La implicació de la disbiosi (l’alteració de la microbiota homeostàtica) en el desenvolupament de patologies com la depressió o el càncer és un tema actual d’interès. D’altra banda, la carcinogènesi és la conseqüència d’acumulacions d’alteracions genètiques i epigenètiques que es veuen potenciades per certs factors de risc tals com l’estil de vida, la dieta o la disbiosi. Recentment, aquesta última, s’ha vist que contribueix o pot prevenir la carcinogènesi modulant el microambient tumoral o el de les cèl·lules hoste respectivament. A més, s’ha vist que la microbiota influeix en l’eficàcia i la toxicitat de la quimioteràpia, la immunoteràpia i la radioteràpia. Així doncs, en aquest treball es discuteix el paper de la microbiota com a causa, conseqüència o ambdues de la carcinogènesi així com els mecanismes i les espècies bacterianes involucrades en l’eficàcia o toxicitat de certs medicaments anti-cancerígens. Aquesta discussió és rellevant ja que obre noves portes a estratègies, per prevenir el càncer o millorar els agents terapèutics contra el càncer, que podrien esdevenir en resultats clínics positius pels pacients oncològics. Un exemple d’això seria l’ús dels oncomicrobiòtics un “còctel de bacteris i/o productes bacterians” com a teràpia addicional al càncer, per a la millora de la resposta immunitària a través de l’optimització de la microbiota intestinal.

Integration of the different fields included in the thesis

The main field of this dissertation is microbiology since it revolves around the microbiota and its influence on cancer initiation and it spotlights the oncomicrobiotics, a new therapeutic field based on microbiota modulation and/or its products in order to prevent cancer or improve its treatment. Secondary fields such as immunology and pharmacology and therapeutics are equally important to fully understand and develop a comprehensive view of this work. On the one hand, the immune system has a crucial role in carcinogenesis and in shaping the microbiota, and, at the same time, it is affected by cancer and microorganisms. On the other hand, efficacy and toxicity of certain anticancer drugs depend on the gut microbiota state. Therefore, the immune system, the microbiota and cancer are three concepts that are interconnected in a bidirectional manner and they cannot be seen separately (**Figure 1**).

For all the reasons stated before, microbiology, immunology and pharmacology and therapeutics are integrated in this thesis.

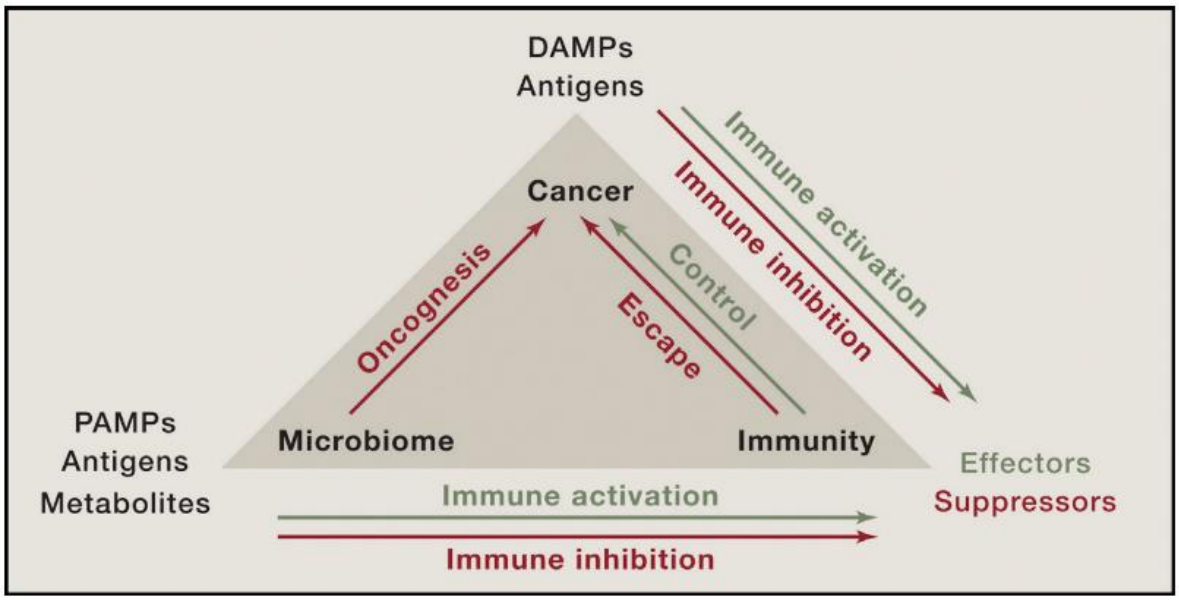

Figure 1. *How the microbiota, the immune system and cancer are interconnected. DAMP, danger-associated molecular pattern. PAMP, pathogen-associated molecular pattern* (1).

1. Introduction

1.1. What is carcinogenesis?

Carcinogenesis is the production or development of cancer through genotypic, phenotypic and epigenetic changes which upset the normal balance between cell proliferation and cell death. Most cancers arise from random somatic alterations in key genes of cell proliferation, survival and growth. These genetic changes are importantly favoured by a number of risk factors such as lifestyle, diet and inflammation, and the environment where tumours evolve provides a unique source of signalling cues that affects cancer cell growth, survival, movement and metastasis (2).

Carcinogenesis appears as a result of accumulations of genetic alterations including activation of oncogenes or deregulation of tumour suppressor genes and DNA repair systems.

Proto-oncogenes are physiologic regulators of cell proliferation and differentiation that can become oncogenes when suffering from a mutation. The latter encodes oncoproteins which have the ability of skipping the cell cycle strict regulation and thus, cell growth control is lost. Examples of oncoproteins frequently mutated in cancer are growth factors (epithelial, vascular…), growth factor receptors, signal transduction proteins, nuclear regulatory protein and cell-cycle regulators.

Tumour suppressor genes on the other hand encode proteins that are related with the inhibition of cell proliferation or growth signalling pathways, and with the liberation of proteins that promote apoptosis and DNA repair systems. Tumour suppressor genes loss their function in cancer cell whereas oncogenes are more expressed (3).

Carcinogenesis is a process that consist of several steps that could be sum up with proliferation cell (tumour), malignant transformation (cancer), local invasion (neighbouring tissues) and metastasis (invasion of distant sites of the body through blood stream/ lymphatic vessels), being the last step the more dangerous and lethal form of carcinogenesis because at that point cancer have the ability to spread all around the body and control of the disease becomes extremely difficult (3).

1.2. The human microbiota

It might be thought that microorganisms only involve infections, when in fact they do not only have a parasitic relationship with human beings but also a mutualistic and commensal one. This statement is endorsed by two different theories that are believed to explain eukaryotic cells inception. One of them is Lynn Margulis' theory of endosymbiosis which holds that the organelles distinguishing eukaryote cells such as mitochondria and

chloroplasts were acquired through intracellular symbiosis with bacteria. The other one, which is gathering strength and is also backed by Lynn Margulis holds that the first eukaryotic cell was formed as a result of the symbiosis between bacteria and archaea (4).

So, the **human microbiota** is the ecological community of microorganisms – from bacteria to archaea, fungi, protozoa or viruses– that reside the epithelial barrier surfaces of our body which are exposed to the external environment (gastrointestinal and respiratory tract, vagina, skin, etc.) and exhibits mainly commensalism and mutualism with its host. It is acquired after birth through vertical transmission and shaped throughout life by environmental exposure. The term microbiome, which is usually confused with microbiota, refers to the collective genome of those microorganisms. In order to put into perspective the relevance of this symbiotic relationship with microorganisms, it is worth knowing that commensal microorganisms (which are normally present on body surfaces covered by epithelial cells) are at least equally numerous as human cells (approximately $3.0 \cdot 10^{13}$) although some studies claim that are three times or even ten times higher than human cells. Moreover, the number of microbial genes is around 100 times higher than that of the human genes although many proteins and metabolites are shared (4, 5). Therefore, the symbiosis between human being and microorganisms is acknowledged. On the one hand, the intestine offers an ideal microenvironment for microbes to reside, e.g. nutrient rich, warm, with adjusted pH and protected. On the other hand, humans greatly benefit from microbiota present at the epithelial barrier (particularly in the gut) because they are involved in several physiological processes such as host immunity modulation –which modulate cancer initiation, progression and response to anticancer–, bone marrow haematopoiesis promotion or maturation, local and systemic metabolic functions and inflammation. In addition to that, microbiota in contact with intestinal cell regulates mucosal immune homeostasis, prevents infestation by occupying ecological niches that could be otherwise colonised by pathogen microorganisms, digest complex carbohydrates (dietary fibre), lipids and proteins, synthesizes vitamins like vitamin K and controls the overpopulation of pathobionts (described as resident microbes with pathogenic potential)(6, 7).

It is in the gut where most of the human microbiota is located forming a dynamic and complex ecosystem. Its distribution, diversity, species composition and metabolic outputs set the balance of benefit and harm for the host. The intestinal microbiota is shaped by constant peristalsis, which move fractions of microbes from low pH environment (stomach) to a higher ones (colon), and by antagonistic relationship between species such as competition of resources, secretion of antibiotic toxins by some bacteria, or infection chains between bacteriophages, bacteria and eukaryotes (8). Depending on how this work, there are two different microbial states. On the one hand, **eubiosis** is the natural equilibrium

between the microbiota and the host in order to maintain homeostasis. It is achieved by continuous crosstalk between the gut microbiota, immune cells and the mucosal barrier that happens mainly in the gut (7). On the other hand, **dysbiosis** is an alteration of the composition, distribution and/or diversity of the microbiota and is statistically associated with a pathology and contributes to the aetiology, diagnosis or treatment of the disease. A reference population for eubiosis or dysbiosis state is difficult to determine since there is an unquestionable variability between healthy individuals attributed to geography, age, gender or dietary habits (9).

Conversely, it has to be understood that eubiosis is not always a positive term as well as dysbiosis is not always a negative one (**Figure 2**). Some authors have used the expression "beneficial dysbiosis" to refer that sometimes an alteration of the microbiota can result in benefit of the host while others have stated that "eubiotic gut microbiota may limit the unwarranted side effects of various antineoplastic agents" (6).

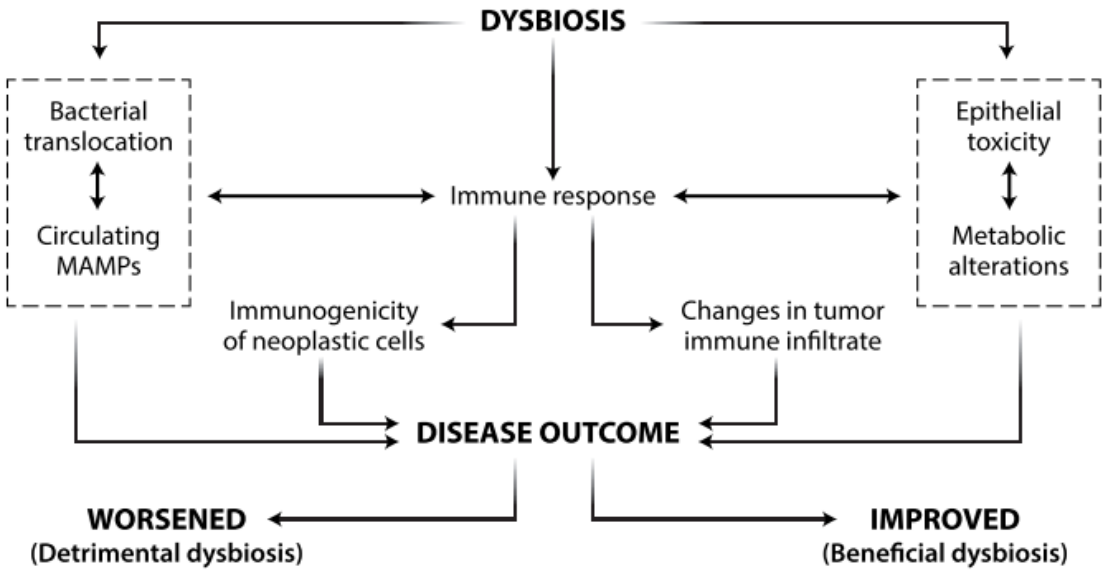

***Figure 2**. Detrimental and beneficial effects of dysbiosis in disease outcome. MAMP, microbe-associated molecular pattern* (6).

Dysbiosis that can be featured by the bloom of pathobionts, the loss of commensal (microbial killing or diminished proliferation) and the loss of diversity. Likewise, it can be caused by infection, inflammation, diet and xenobiotics, genetics, familial transmission and other factors such as circadian disruption (9).

As it will be explained in this thesis as a major field of study, dysbiosis has a role in carcinogenesis and in the efficacy and toxicity of some cancer therapies. That makes commensal microbiota manipulation as one of the most promising therapeutic tools as an approach to enhance efficacy and avoid toxicity in cancer therapy.

2. Aims

The main objective of this dissertation is to explain the relationship status between the microbiota and carcinogenesis as well as the oncomicrobiotics. In order to do so, more concrete aims, listed below, will be achieved:

- To discuss to what extend carcinogenesis is influenced by microbiota.
- To discern microbiota alteration in cancer setting and in anticancer therapies setting.
- To explain the influence of the microbiota in host immune response.
- To explain the influence of the microbiota in the efficacy and toxicity of anticancerogenic drugs.
- To outline the therapeutic benefits of bacteria in cancer setting.

3. Material and methods

In order to conduct this bibliographical research, I have widely search for the most trust-worthy and up-to date articles, reviews and books. To achieve that, I mainly used PubMed® a free resource developed by the National Centre for Biotechnology information (NCBI) which provided free access to National Library of Medicine's®(NLM) databases like Medline. Medline has citations and abstracts from different fields including medicine and preclinical science and each bibliographical reference has a Medical Subject Hiding (MeSH) which allow to search articles not only through keywords but also through subjects.

After carefully choose the most relevant articles to me, I conducted a review of the subject previously planned. It starts with a global vision of how carcinogenesis, the human microbiota and immune system are interconnected as well as an explanation of basic concepts in microbiology and immunology. It follows a gathering of different situations that are used to exemplify what is intended to be explained. Finally, it ends with possible scenarios in the therapeutic field that this subject might lead, and leaves open the option of hypothetical solutions to an efficient anticancer treatment.

All the articles that I used in this dissertation are referenced with Mendeley®. Thereby, most of the information compiled correspond to an article that can be found in the bibliography.

4. Results and discussion

4.1. The influence of the microbiota in the carcinogenesis.

The role of bacteria in carcinogenesis is complex, as both pro- and anticarcinogenic functions have been attributed to microorganisms. On the one hand, the microbiota has positive outcomes in fighting cancer located at distant sites from the gut, due to its ability to modulate local and systemic metabolism, inflammation and immunity (10). On the other hand, microbiota can induce carcinogenesis through different pathways. For example, by direct oncogenic effects of microorganisms or their products or by alterations in carcinogenic metabolites that are microbiota-mediated or by producing trophic factors, such as growth factors or by inducing proinflammatory and immunosuppressive effects that undermine anticancer immunosurveillance. In addition to that, broad-spectrum antibiotic treatment, pathogen exposure, fasting, long-lasting changes in diet and other factors, such as cold stress or perturbations of diurnal rhythms, can alter microbiome steadiness and thus, can develop carcinogenesis (8).

There are many studies that support the idea that microbiome and carcinogenesis are not isolated concepts.

To begin with, there is evidence that suggests increased cancer risk with the use of antibiotics which links the idea of dysbiosis may cause carcinogenesis. The first clinical evidence was published in 2008 by the National Public Health Institute in Finland. A nationwide cohort study that included 3,112,624 individuals, aged 30–79 years, with no history of cancer was conducted. Antibiotic data used from 1995 to 1997 was collected and later, for the next 6 years (1998-2004) those individuals were followed up in order to detect if they developed any kind of cancer to finally estimate a relative risk of cancer. Antibiotic use was associated with an increased risk of cancer despite having little knowledge of the importance of microbiota in carcinogenesis. However, the study pointed out that antibiotic use should be considered as an indicator rather than a cause of cancer because there are many other factors that can contribute to carcinogenesis and limitations in this study were recognised (11).

Moreover, it has been tested in mouse that pro-carcinogenic phenotypes (induced colitis or colon carcinogenesis) expressed by genetically mutated mice (in genes which produce, process, or respond to IL-18 that mediate mucosal protective mechanisms) can be transferred into wild-type mice by microbiota transfer (7).

Lastly, resistance or susceptibility to carcinogenesis can be noticed in different studies in germ-free mice. This nonsense fact can be explained by the dual role that microbiota may play in carcinogenesis. While it is true that it participates in epithelial cell damage, genetic

instability and mutation by secreting reactive oxygen and nitrogen species and by downregulating DNA repair genes; it is also true that it is required to repair epithelial damage in mucosal tissues (7).

Those examples clearly associate microbiota with carcinogenesis, even though there is a lack of insight into the mechanisms by which they happen. One mechanism, though, could be inflammation, that on the one hand, creates a carcinogenic environment resulting from bacteria and host cells that trigger carcinogenesis and, on the other hand, it is used as a communication channel between microbiota and cancer (**Figure 3**) (2). This communication channel occurs through peptides in a quorum sensing fashion and it is thought to contribute to metastasis (12).

Because of microbiota-carcinogenesis association has been made, new therapeutics interventions such as microbiota manipulation have been thought. Testing antibiotics, probiotics, prebiotics, etc. in preclinical models has just started (13).

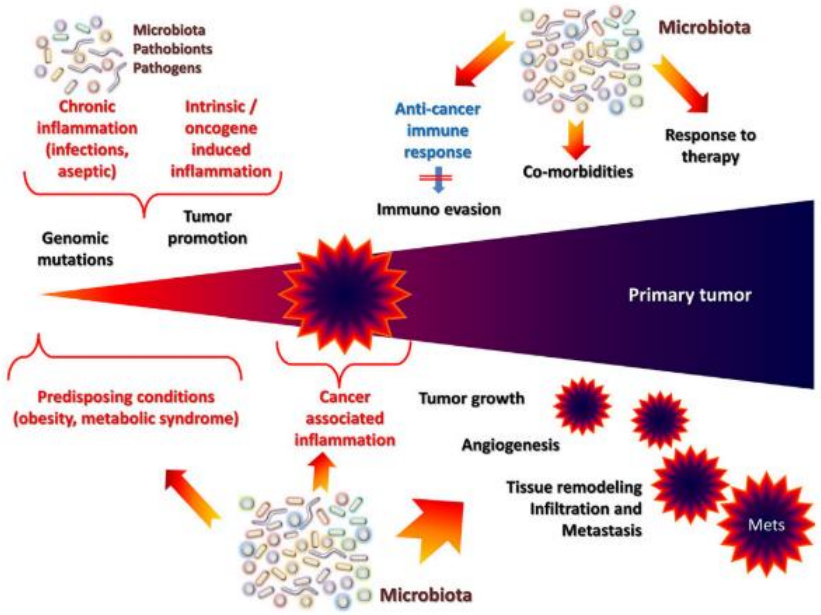

Figure 3. *The role of inflammation and the microbiota in carcinogenesis. Inflammation, known as a hallmark of cancer is influenced in many different ways by the microbiota* (4).

4.2. Immunology and cancer

Similar to the relationship between the microbiota and carcinogenesis, immunology plays a dual role in cancer. On the one hand, it is responsible of immunosurveillance, processes by which cells of the immune system look for and recognise foreign pathogens such as cancerous cells. On the other hand, inflammation –which is mediated by the immune system– is present in almost all the steps of carcinogenesis from initiation and propagation

to metastasis, being chronic inflammation highly related with cancer risk. Plus, the inflammatory microenvironment developed by tumours, that lead to immunosuppression and thus, tumour progression, is also supported by the immune system (14).

4.2.1. The basis of onco-immunology.

In an optimal immune situation, the immune system has enough efficacy and specificity to eliminate cancer cells. What come next is the description of a **seven-step cyclic-process** that sums up the way the human body generates anticancer immune response to lead to effective killing of cancer cells (**Figure 4**).

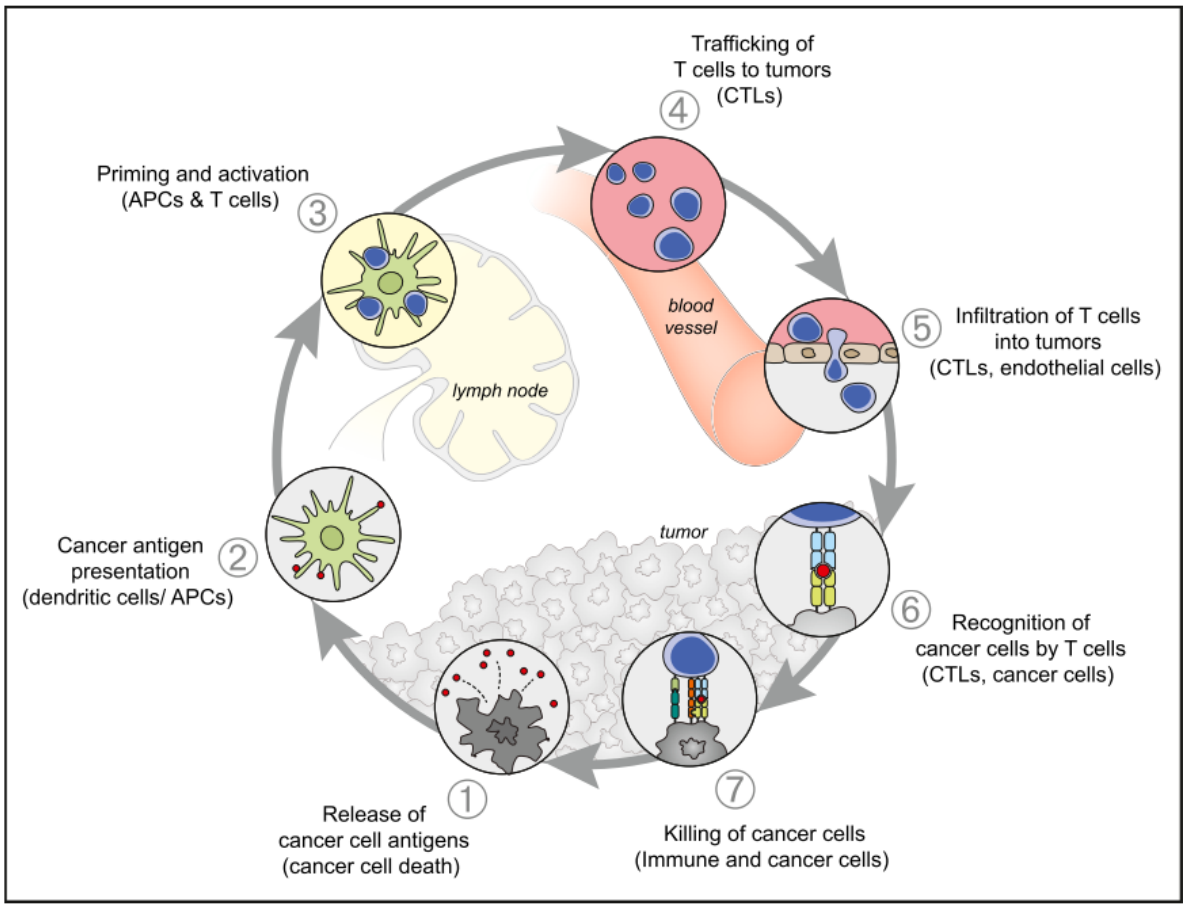

Figure 4. *The cancer-immunity cycle* (15).

Cancer cell death –caused by cytotoxic CD8 T cells, natural killers (NK) or chemotherapy among others– releases cancer antigens that can be captured by antigen-presenting cells (APC) being dendritic cells (DC) the most efficient ones to induce antigen-specific T cell response (step 1). Later, antigens are processed by DCs and displayed in major histocompatibility I and II (MHC I or II). (*MHC I is a cell surface protein that is expressed in all cells to display own cell antigens in order to develop self-tolerance. MHC II cell surface protein is only present in APCs to display rare or foreign antigens in order to activate antigen-specific T*

lymphocytes). At this moment DCs have matured and they move to the lymph nodes where they present the antigen to naïve CD4+ /CD8+ T cells (steps 2 - 3). Naïve CD8+ T cells then become cytotoxic T lymphocytes (CTL) (which eliminate cancer cells) while naïve $CD4^+$ T cells, depending on the different pattern of signals, become helper T cells (Th) (with different profiles that generally helps CTLs) or regulatory T cell (Treg) (which contribute to immune suppression and diminish CTLs activity). It is a critical step that determines the outcome of the immune response towards cancer. If the number of Treg cells outweighs the number of Th cells it will be difficult for the immune system to undermine the tumour. Finally, effector T cells (primed and activated T cells) traffic to tumour through blood vessels (step 4) and infiltrate the tumour bed through endothelial cells (step 5). Eventually, cancer cells are recognised by the receptor of effector T cells (TCR) that bind to cancer cells MHC I that display the antigen previously presented (step 6) and kill their target by secreting cytotoxic mediators such as granzymes A and B and perforin (step 7). This fact provokes the release of tumour-associated antigens that not only closes the cancer-immunity cycle but also enlarges the immune response (15, 16).

However, in cancer pathogenesis this cycle does not work as it should because tumours can develop mechanisms to scape immunity. There are three major immune escape mechanisms. The first one is the **loss of antigenicity** which is produced by a decrease or mutation in MHC I molecules. Therefore, cancer cells can no longer display the cancer-cell antigen and CTL lose their target. The result is less specific T cell and less T cell killing. The second mechanism is regarding the **loss of immunogenicity or immunosuppression**. Tumours are surrounded by what is called tumour microenvironment (TME) which is a complex and dynamic setting developed by themselves that consist of immune cells, cancer cells and their bordering stroma (**Figure 5**). They communicate with each other by immune mediator such as cytokines and chemokines that normally modulates immune cells towards an immunosuppressive and pro-inflammatory fashion. For example, in the TME the extracellular matrix of cancer cell forms a physical barrier to T cell that do not allow them to migrate, or there are many cytokines related to angiogenesis and epithelial proliferation. The third mechanism related to the capability of **cancer cells to become resistant**. There are different ways they can achieve that, mostly involving genetic alteration. For example, they develop intrinsic resistance to local cell death programs such as apoptosis, upregulate the expression of mitogenic genes or increase mutation of oncogenes such as STAT3 and EGFR.

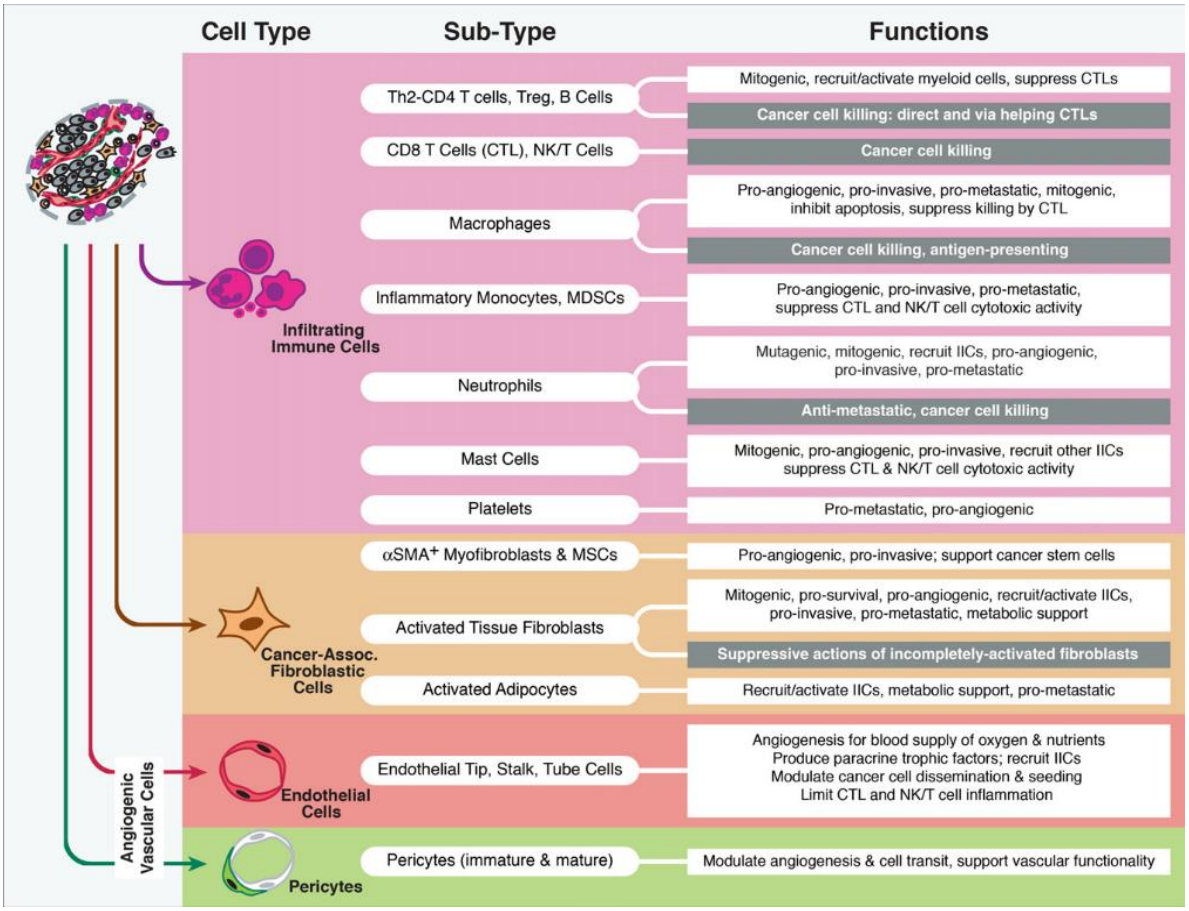

Figure 5. *Different functions of cells subtypes found in TME* (17).

4.2.2. Immunotherapy

The recent breakthroughs in the immunobiology field and the understanding of the cancer-immunity cycle has opened a new field of therapeutic against cancer: immunotherapy.

Immunotherapy (IT) comes as one of the latest and more promising therapies against cancer that might play an important role in polytherapy (18).

The concept of immunotherapy is vast and includes all kind of immune system –other's or our own– used in order to combat cancer. Cancer immunology is challenging because it is difficult to create an immune response to a tissue that is originated in our own body. In order to outpace the mechanisms that tumours develop to evade the immune system, the aim of IT is to "wake up", initiate, enhance, harness or boost the immune system and kill cancer cells but not do much as to damage normal cells and generate an unrestrained autoimmune response (15).

Immunotherapy can be classified into two major groups: *Active immunotherapy* which induces an immune response within the body (*in vivo*) and *passive immunotherapy* in which

the immune response initiated outside the body (*ex-vivo*) and then is transferred into the body.

Active IT is also divided into three types. The first one is *adjuvants,* a non-specific therapy that acts on the innate immune system. It matures DCs so they move to the lymph node and prime T cells. An example of an adjuvant is Bacillus Calmette-Guerin (BCG), a vaccine used to prevent tuberculosis but also effective to treat some non-invasive bladder cancer. The second one is *therapeutic vaccines*, a specific therapy that give an exact target in order to begin the immune response. An example of this is the peptide vaccine that contains an antigen and an adjuvant that when injected in the tumour stimulates DCs. Another example is DC therapy (Provenge – spiuleucel-T) used in some prostate cancer. The idea here is to vaccinate mature DCs so they can move straight to the lymph nodes and do the antigen-presentation (19). The third one is *immune checkpoint inhibitors* one of the most promising therapeutics against cancer. The checkpoint pathways have an important role in down regulating the immune system. During T-cell activation, T-cells begin to express "checkpoints" such as cytotoxic-T-lymphocyte-associated protein 4 (CTLA-4) and programmed-cell-death protein 1 (PD-1) to guard against autoimmunity and to protect tissues from damage by an over-exuberant immune response to an infection. They are both immune-modulating molecules. On the one hand, CTLA-4 regulates the amplitude of the initial immune response, the T cell activation. In order to present the peptide to T-cell, not only DCs needs MHCII – T-cell receptor (TCR) interaction but also the CD80 – CD28 co-stimulation. CTLA-4 counteracts CD28 T-cell activation resulting in a decreased T-cell activation at initial stages of immune response, typically in the lymph nodes. Plus, CTLA-4 enhances the proliferation and activation of regulatory T-cells. On the other hand, PD-1 pathways regulate previously activated T-cells at a later stage of immune response, typically in the peripheral tissues. PD-1 receptor expressed in T-cells have affinity to PD-1 ligands (PD-1L) which are upregulated in all cells when the immune response begins to stop cytotoxic activity of CTL and ensure the immune system does not damage healthy cells. Unfortunately, cancer cells take advantage of this natural inhibitory mechanisms by expressing inhibitory ligands for these checkpoints (PD-L1 for PD-1 and B7 for CTLA-4) and thus, cancer cells become resistant to the immune system. Anti-CTLA-4 and anti-PD1 therapy through monoclonal antibodies have achieved outstanding results but only in some patients. Understanding why there are different responding to these treatments is an object of study of this dissertation and it will be discussed later.

Passive IT which is mainly through T-cell therapy is subdivided into two categories. The first one is *adoptive T-cell therapy* and consist of isolating tumour infiltrated lymphocytes (TILs), expand them *in vitro,* and re-infuse them back to the lymphocyte-depleted patient. However, it has many limitations the main one is that those expanded TILs will be supressed

because of the TME. The second passive IT is *chimeric antigen receptor (CAR) T-cell therapy* which consist in genetically engineered T cells whose receptor has an internal TCR-like part and an external antibody-like part. The main advantage of CAR T-cells is that can overcome immunosuppression because can recognise antigen without using MHC I.

Finally, it is important to underscore that immunotherapy it is more effective and its effects last longer in immunogenic cancers also known as "hot" tumour. Those are characterized on the one hand by generating all the danger signals in the tumoral development site such as pro-inflammatory signals that activate APCs. This is the reason why "hot" tumours have an increased number of activated DCs (able to capture antigens), infiltrated-tumour CTL (able to kill cancer cell) and a high mutational load or neoantigens (peptide mutations in cancer cells that are not affected by central tolerance and thus differ from the human genome increasing the cytotoxic activity of CTL cells and NK). On the other hand, all the immunosuppressive factors are diminished, for example there is an absence of checkpoint molecule expression or absence of inhibitory tumour metabolism. When a tumour is not immunogenic or is "cold", the immune response has not initiated towards it. In those cases, combined therapy is optimal because firstly the "cold" tumour has to turn into a "hot" one and then, the immunotherapy becomes useful because it has targets attack to (18, 19).

The variability of the immune response and the different susceptibility of tumour types makes IT a therapy with limited and irregular efficacy. However, due to its potential, new possibilities to enhance its efficacy – including the gut microbiota – are being studied and they will be discussed later on in this dissertation (7).

4.2.3. The influence of the microbiota in the immune response of the host

One of the most important functions of the microbiota is its role in shaping both innate and adaptive immunity.

As the major crosstalk between the microbiota and the immune response take place in the gut it is important to know its structure. The human gut is a mucosa composed by a single cell layer made up of intestinal epithelial cells (IECs) and intraepithelial lymphocytes. IECs include Paneth cells (secrete antimicrobial products such as lectin REGIIIγ and is important to keep the separation between the microbiota and the epithelial cells) and goblet cells (secrete mucus). Beneath the mucosal layer, there is the lamina propria, a connective tissue layer where it can be found various immune cells such as APCs, innate lymphoid cells, T cells and B cells, some of which are organised in Peyer's patches. The gut-associated lymphoid tissue (GALT) is the largest immune component of all (20, 21).

The microbes can trigger **innate immunity** by two types of signal. The first one is regarding microbial cell components or pathogen-associated molecular patterns (PAMPs) like

lipopolysaccharides (LPS). They are recognised by IECs' and innate cells' pattern recognition receptors (PRR). For instance, a study showed that three different microbiomes from different patient had different immunogenicity of LPS. Depending on its immunogenicity, patients were able to stimulate a toll-like receptor 4 (a kind of PRR) and thereby activate a nuclear factor involved in endotoxin tolerance. The second type of signal to trigger innate immunity is regarding microbes' metabolites. It has been seen that transcriptional programming of innate immune cells is influenced by metabolites. In the case of innate lymphoid cells by tryptophan metabolites and in the case of myeloid cells by short-chain fatty acids (SCFAs). The latter metabolite is involved in many activities in the gut including Treg development and immunoglobulin A (IgA) production by plasma cells (**Figure 6**). IgA are antibodies specialised in the intestinal protection: they block bacterial adherence to epithelial cells, they have effect in bacterial virulence and they can also target specific bacteria specially to those with colitogenic potential, among other activities (9, 20, 21).

In addition to that, the microbiota can also activate **adaptive immunity** (**Figure 6**). PAMPs induce APC maturation like DCs and thus, its activation. Then DCs can travel to mesenteric lymph nodes (mLN), where they activate naïve T cell, and develop therefore an adaptive immune response (Tregs, Th1, Th2, Th17 and even CTL). It will have an enormous importance in the way the adaptive immunity will response. Depending on the subset of activated T cells that will predominate (above all Tregs or Th17), the immune system will be more or less stimulated being crucial to some diseases like cancer. On the one hand, Tregs have a paramount role in gut homeostasis since they induce commensal microbiota tolerance and produce immunosuppressive cytokines such as IL-10, which at the same time TME takes advantage of this in order to scape immunity. On the other hand, Th17 cells have

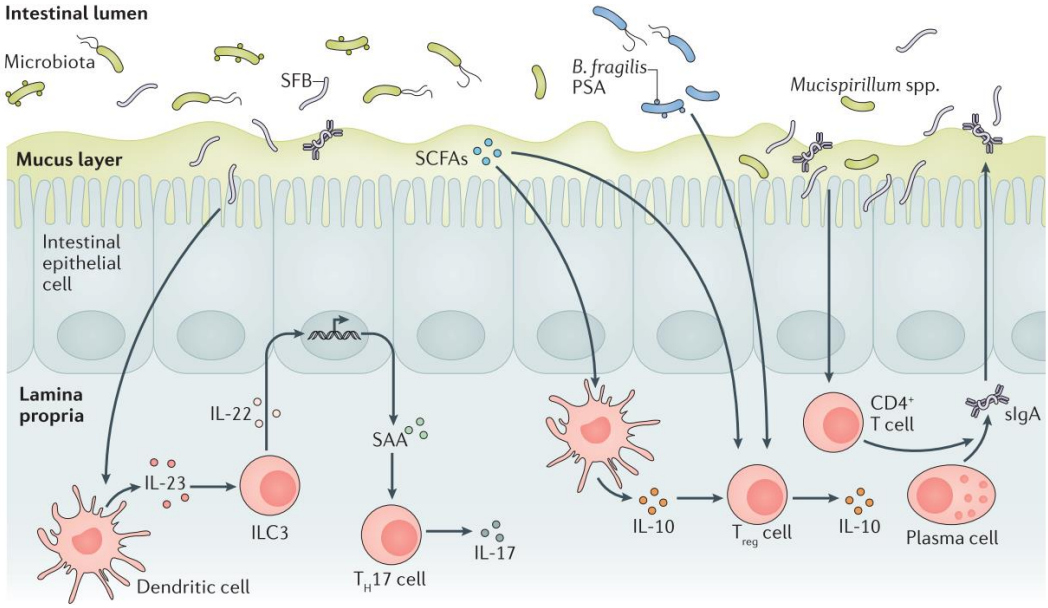

***Figure 6**. The microbiota shapes innate and adaptive immune response (9).*

a crucial role in protecting bacterial and fungal infections. By secreting IL-17 they stimulate IECs to form tight junction and secrete anti-microbial proteins and can recruit neutrophils from blood circulation to induce inflammation (9, 20). Furthermore, there are specific bacterial species that directly develop and differentiate the adaptive immune response. For example, segmented filamentous bacteria (SFB) can induce Th17 cells, whereas *Bacteroides fragilis,* certain strains of *Clostridium spp.* and bacteria secreting SCFA can induce Treg. In addition, SFB and *Mucispirillum spp.* can induce secretion of IgA (**Figure 6**) (9). Moreover, adaptive immunity can be activated by cytokines and interferons, secreted by innate immune effectors in a paracrine or endocrine manner, that give the gut a "immune system tone" to be rapidly present in a pathogen setting, but suppressed in a harmless setting (20).

If the delicate ecosystem of the commensal microbiota in the gut becomes unbalanced it will potentially have severe defects in immunity, with an absence in mucous layer, altered immunoglobulin A (IgA) secretion, reduced size and functionality of Peyer's patches, and draining mesenteric lymph nodes (mLNs) among other things. It has been proved in germ-free mice (20).

Overall, there is compelling evidence that the microbiota helps to shape the immune system.

4.3. Microbiota alterations in cancer

Despite microbiota and cancer relationship is bidirectional and multifactorial, it has been observed that there are **cancer-associated shifts in microbiota** (**Table 1**)**.** For example, head and neck carcinomas have altered composition in oral microbiota. The same situation is repeated in lung carcinomas with bronchial microbiota, in colorectal carcinomas with intestinal microbiota or in cervical carcinomas with vaginal microbiota. These facts can be explained, at least, for three reasons: the first one is that cancer usually progress in an immunosuppressed setting which can deregulate eubiosis. Similarly, the second reason is that cancer also alters the host metabolism which through metabolites can also alter microbiota and thus, the microbiome. The third reason would be that tumour can physically disrupt barriers causing translocation or invasion of certain microbes into tissues that are not supposed to inhabit (8).

Likewise, there are **shifts in the microbiota associated to certain anticancer therapy** such as chemotherapeutic cyclophosphamide or immunotherapeutic monoclonal antibody ipilimumab, which have an impact in treatment result. This association can be explained because these therapies take advantage of the host immune response to fight cancer and the microbiota plays an important role in immune system homeostasis. In the following section it will be discussed in more detail (8).

Table 1. *Epidemiological associations between commensal microorganisms and cancer. ALL, acute lymphoblastic leukemia; CCA, cholangiocarcinoma; HNSCC, head and neck squamous cell carcinoma; HPV, human papillomavirus; rRNA, ribosomal RNA; V, variable region* (8).

Cancer type	Sample size	Analysed specimen	Bacterial identification	Microbial composition alteration	Association	Refs
HNSCC	19 cases, 25 controls	Saliva and tumour samples	16S rRNA V3–V5	*Streptococcus* spp., *Dialister* spp., *Veillonella* spp., *Neisseria* spp., *Aggregatibacter* spp., *Haemophilus* spp. and *Leptotrichia* spp.	Saliva samples had a higher abundance* of *Streptococcus* spp., *Dialister* spp. and *Veillonella* spp. Tumour samples had a lower abundance‡ of *Neisseria* spp., *Aggregatibacter* spp., *Haemophilus* spp. and *Leptotrichia* spp.	12
ALL	51 cases, 51 controls	Faeces	16S rRNA V1–V3	*Anaerostipes* spp., *Coprococcus* spp., *Roseburia* spp. and *Ruminococcus* spp.	Lower abundance‡ of all of these taxa	123
Pancreatic cancer	361 cases, 371 controls	Oral wash	16S rRNA V3–V4	*Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans* and *Leptotrichia* spp.	Higher abundance* of *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*. Lower abundance‡ of *Leptotrichia* spp.	124
CCA	28 *Opisthorchis viverrini*-associated cases, 32 *O. viverrini*-non-associated CCA cases	Human bile duct tissue	16S rRNA V3–V6	*Stenotrophomonas* spp., Bifidobacteriaceae, Enterobacteriaceae and Enterococcaceae associated with *O. viverrini* fluke colonization	Higher abundance* of *Stenotrophomonas* spp. in *O. viverrini*-non-associated CCA	125
HPV-associated cervical cancer	340 cases, 90 controls	Cervical mucus	16S rRNA V4	*Lactobacillus iners* and unclassified *Lactobacillus* spp.	Higher abundance* of *L. iners* and unclassified *Lactobacillus* spp.	13
Breast cancer	25 cases, 23 controls	Nipple aspirated fluid	16S rRNA V4	*Alistipes* spp. and Sphingomonadaceae	Higher abundance* of *Alistipes* spp. Lower abundance‡ of an unclassified genus in the Sphingomonadaceae family	21
Biliary tract cancers	64 biliary cancer cases, 122 liver cancer cases, 224 controls	Serum	Serology: multiplex assay against 15 *Helicobacter pylori* proteins	*H. pylori*	Higher seropositivity for *H. pylori* in patients with cancer	126
Urothelial cancer	8 cases, 6 controls	Urine	16S rRNA	*Streptococcus* spp.	Higher abundance* of *Streptococcus* spp. Higher abundance* of *Pseudomonas* spp. or *Anaerococcus* spp. when *Streptococcus* spp. abundance was low	127
Oral cancer	32 cases, 35 controls	Oral cancer swab compared with mouth swab	16 sRNA V4	*Streptococcus* spp. and *Rothia* spp.	Lower abundance‡ of *Streptococcus* spp. and *Rothia* spp.	128
Lung cancer	8 cases, 8 controls	Sputum and buccal samples	16S rRNA V1–V2	*Granulicatella* spp., *Abiotrophia* spp. and *Streptococcus* spp.	Higher abundance* of *Granulicatella* spp., *Abiotrophia* spp. and *Streptococcus* spp.	129

There are **epidemiologic studies** of the human microbiome and cancer that represent the seed towards the use of the microbiome analysis as a tool to prevent, screen, diagnose or even treat cancer. Even though there are factors that equally trigger carcinogenesis and dysbiosis and that comprehensive studies that causally link microbiota alteration with cancer development need to be done (2, 6).

The influence of microbiota in **breast cancer** is yet to be characterised. However, in a study it was concluded that breast cancer patient had ten times less bacteria (in absolute numbers) in cancer tissue than in adjacent healthy tissue and also concluded that the composition of tumour tissue was high in *Sphingomonas yanoikuyae.* A second study showed that in toll-like receptor 5 (TLR5, a type of PRR) microbial signalling, mice suffered tumour growth whereas in absence of TLR5 signalling they had a reduced tumour progression, suggesting that commensal microbiota could be involved in breast cancer (22). Although little is known about the relationship between microbiota and breast cancer, there is an ongoing trial (NCT02079662) with stage III breast cancer patients initiating

radiotherapy that has as an object of study (as secondary outcomes) the influence of diet in gut and oral microbiome. Another one (NCT03358511), investigates the effects of probiotics in intratumoral CD8+ T cell in patients with stage I–III breast cancer indicating that microbiota might be relevant in breast cancers (20).

Cervical cancer is mainly due to human papilloma virus (HPV) rather than a microbiota dysbiosis. However, dybiosis in vaginal microbiota is associated not only with **vaginal cancer** but also with HPV infection and, thereby, with cervical cancer. Vaginal microbiota is an important barrier in women against pathogens and has a rich *Lactobacillus spp.* composition that are known to have a protective function. In bacterial vaginosis, anaerobic bacteria such as *Gardnerella, Prevotella,* and *Clostridiales* are increased in expense of *Lactobacillus spp* (22).

Primary liver cancer is classified as **hepatocellular carcinoma (HCC)** in 70-90% of the cases. HCC usually occurs after a previous liver damage such as hepatitis infections (by HBV and HCV), alcoholism or obesity, or secondary to liver diseases such as alcoholic liver disease (ALD) or non-alcoholic fatty liver disease (NAFLD). Many studies seem to indicate that the microbiota has a crucial role not only in developing those diseases but also in the progression of HCC. One example is ALD. Chronic consumption of alcohol provokes gram-negative bacteria overgrowth as well as intestinal barrier disruption which lead to the permeability of microbes and its products and toxins to the liver. Consequently, an inflammatory environment is developed and can aggravate liver damage like cirrhosis. Moreover, it has been seen that alcoholic cirrhosis patients have increased gram-negative bacterial translocation in the liver and, in fact, patient that were treated with probiotics showed decreased gut permeability and, thus, decreased incidence of cirrhosis and HCC. Another example of microbiota being involved in hepatic diseases is the case NAFLD. There are two pathways to induce NAFLD. The first one is through obesity, that also increases gut permeability and led gram-negative bacterial products such as LPS to translocate to the liver. As a result, TLR4 and TLR9 signalling pathways can develop NAFLD and also tumour formation. The second one is because of gram-positive bacteria that produce secondary bile acid such as deoxycholic acid (DCA) which is known to produce DNA damage and thus, cancer. In fact, HCC progression in mice could be stopped when treated with vancomycin, a specific gram-positive antibiotic. All in all, in the future HCC prevention and treatment will greatly benefit from microbial modulation therapy since the correlation between detrimental dysbiosis and the disease itself is clear (22).

Microbiota alteration in **colorectal cancer (CRC)** has been widely studied because it was the first cancer to be related to detrimental gut dysbiosis. Perhaps because the gut microbiota shares space with colon and seemed to have more causative impact on CRC than in other extraintestinal cancers. Likewise, differences in microbiota composition in a CRC have been

described in many scenarios: between healthy subjects and CRC patients, between a same patient's tumour tissue and healthy adjacent tissue, and between different stages of one same tumour: from adenoma to adenocarcinoma. The main intestinal changes that many studies agree in CRC patients are increased abundance of *Fusobacteria, Alistipes*, *Porphyromonadaceae, Coriobacteridae, Staphylococcaceae*, *Akkermansia* and *Methanobacteriales* and decreased abundance of *Bifidobacterium, Lactobacillus*, *Ruminococcus*, *Faecalibacterium*, *Roseburia* and *Treponema*. Although it is assumed that microbiota and cancer communication occur in a bidirectional fashion, there are strong preclinical models, at least for CRC, that evidence the causal effects of detrimental dysbiosis in CRC. In a study that compared the gut microbial composition of a colon tumorigenesis model mice (in a pre-neoplastic stage) and a wild type one, born at the same time, it could be seen that the first one showed dysbiosis. It is believed that the mechanisms by which the gut microbiota causes CRC is by inducing host immune response and certainly, there are four ways known to contribute to tumorigenesis. The first one is through proinflammatory cytokine production. The second one, by pattern recognition receptors (PRR) that, when are induced by certain microbial pathogen-associated molecular patterns (PAMPs), they can control epithelial growth and promote eubiosis. The third one is by inflammasomes, multiprotein complexes that, when activated, they secrete inflammatory mediators that similarly to PRR, they avoid tumorigenesis by controlling epithelial growth, promoting eubiosis and in addition, they can supress metastatic growth. The fourth one is by the expression of autophagy genes that promote tumorigenesis and decrease CD8+ T cells. Moreover, it is known that enterotoxigenic *Bacteroides fragilis* (ETBF), *E. coli*, and *Fusobacterium nucleatum* are specially involved in CRC by producing toxins and genotoxins. To finish, using altered microbiota as a non-invasive diagnostic tool seems to be feasible in CRC patient, although there are still many challenges such as reproducibility and generalizability of models across studies that must be solved (2, 13).

4.4. The influence of microbiota in the efficacy and toxicity of certain anti-cancer drugs.

As it was discussed before in this dissertation, there is mounting evidences of the role of the gut microbiota in modulating host immunity and thus having an immunotherapeutic effect against some diseases such as cancer. For instance, the translocation of bacterial products like LPS from the intestinal lumen to the secondary lymphoid organs enhances the efficacy of tumour-specific T cells (10). Therefore, it is reasonable to think that the microbiome could influence efficacy and toxicity to various forms of cancer therapy especially because the microbiota is involved in the biotransformation of some anticancer drugs (**Figure 7**). Results obtained in mice with anti-cancer treatments such as cyclophosphamide, platinum salts or monoclonal antibodies (mAbs), like CTLA-4 or PD-1/PD-L1, showed a reduced therapeutic effect in germ-free mice and broad-spectrum treated mice, suggesting once more the importance of the microbiota in anti-cancer drugs (10). In the following section it will be discussed the fine mechanisms by which the microbiome modulates some anti-neoplastic therapy responses and the different outcomes of each treatment modality.

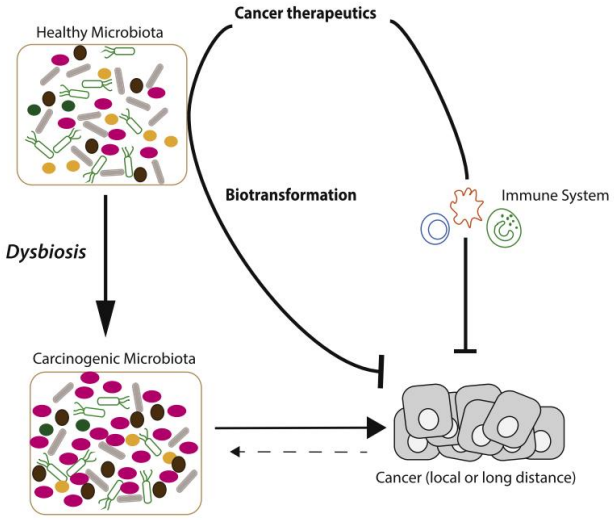

Figure 7. *Healthy microbiota is involved in the biotransformation of anticancer therapeutic drugs which can result to be effective and inhibit cancer (can also act synergically with the immune system) or toxic and change the microbiota towards a procarcinogenic one (13).*

4.4.1. Irinotecan

Irinotecan is an antineoplastic agent quite used in metastatic colorectal cancer. It inhibits the topoisomerase I. In order to understand how the microbiota in the gut modulates its toxicity it is important to first know about its pharmacodynamics. Irinotecan is a prodrug that it is transformed into its active form (SN-38) in the liver and small intestine tissue through carboxylesterase. Afterwards it is processed in the liver by host UDP-glucuronosyltransferases to form inactive SN-38G derivative, which is later excreted via biliary ducts into the GI tract. Because of bacterial β-glucuronidases –present in the gut– SN-38G can turn into its cytotoxic SN-38 form and cause severe diarrhoea intestinal inflammation in many patients that leads to dose reduction or change of treatment.

In order to avoid this major drawback a selective bacterial β-glucuronidase inhibitors must be developed. So far, Kampo medicine and D-saccharic acid 1,4-lactone that slightly inhibit β-glucuronidases have shown to alleviate diarrhoea. Another option would be the usage of antibiotics or probiotics in order to change the proportion of bacterial species expressing β-glucuronidase that have been seen to increase after irinotecan chemotherapy. Concretely, in some members of *Clostridium, Enterobacteriaceae* and *Bacteroidetes* (ie. B. fragilis). In experimental animal, antibiotics effectively treat intestinal inflammation induced by irinotecan therapy, while it modestly decreased irinotecan induced diarrhoea. As the intestinal toxicity of irinotecan in humans is severe and dose limiting, there is much interest in the development of an effective solution to reduce toxicity (7,13).

4.4.2. Cyclophosphamide

Cyclophosphamide (CTX) is a prodrug used against multiple hematologic and solid malignancies that once activated acts as an immunostimulatory and cytotoxic alkylating agent by inducing immunogenic cancer cell death, subverting immunosuppressive T cells and promoting Th1 and Th17 cells. In a study conducted by Viaud *et al.* 2013 (23) it was demonstrated that CTX efficacy depended on commensal bacteria, notably on Gram-positive bacterial species *Lactobacillus johnsonii* and *Enterococcus hirae.* Administration of CTX in mice led to the disruption of small intestinal barrier function, enabling the translocation of these intestinal bacteria into mesenteric lymph nodes and spleen. There, they stimulated the generation of a specific subset of "pathogenic" (p)Th17 cells and memory Th1 immune responses, which are the key of the success of this chemotherapy (**Figure 8**). In order to prove that the gut microbiota was crucial in driving the conversion of naïve CD4 towards Th1 and pTh17 subsets, CTX was administered to germ-free (GF) and specific-pathogen-free (SPF) tumour-bearers' mice. The results showed that in SPF mice the number of lactobacilli and segmented filamentous bacteria (SFB) measured in the small intestine mucosa correlated with the Th1 and Th17 polarization, whereas in GF mice a reduction in pTh17 cells and thereby a resistance to CTX was observed. However, transfer of ex-vivo propagated pTh17 cells (but not Th17 cells) restored the therapeutic effect of CTX in tumour-bearing mice that had been treated with vancomycin, an antibiotic that target Gram-positive bacteria (23). All in all, it can be concluded that CTX anticancer immune response –one of the most important mechanisms of CTX– is due to mainly pTh17 cells that strictly depend on certain species of Gram-positive bacteria found in the gut microbiota.

4.4.3. Immunotherapy: Anti-PD-1/PD-L1 and CTLA4 monoclonal antibodies

Immunotherapy has been one of the most promising and successful achievements in cancer care in the last decade. Especially immune checkpoint blockers (ICB) that have improved the overall survival in many cancer patients. Monoclonal antibodies (mAbs) that block CTLA-

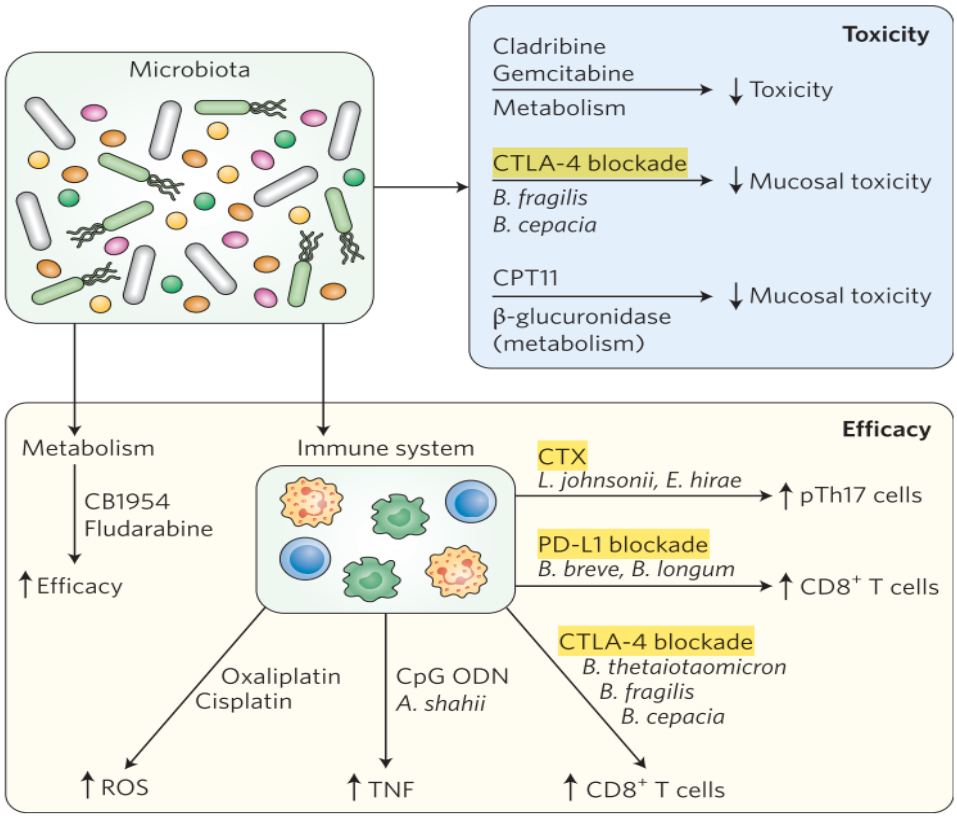

Figure 8. *The influence of microbiota in drug efficacy and toxicity (2).*

4 and PD-1/PD-L1 checkpoints occur to be the ones with most clinical success. Moreover, due to its different mechanism of action, anti-CTLA-4 and anti-PD1/PD-L1 antibodies combination have shown great clinical response in some melanomas in expense of slightly increased toxicity (18). However, their efficacy and toxicity are influenced and regulated by the gut microbiota.

Regarding **anti-CTLA-4 mAbs efficacy** it has been observed that tumour-bearer mice breed in germ-free condition or treated with antibiotics show a decreased therapeutic efficacy: suppressed effector CD4+ T cells and reduced tumour-infiltrating lymphocytes (TIL) and Th1 cells. However, DCs exposed to *Bacteroides fragilis, Bacteroides thetaiotaomicron and Burkholderia cepacia* have been associated with anticancer properties because immunostimulatory effects produced by CTLA-4 blockade require IL-12 in order to induce a Th1 and CTL immune response. Likewise, those exposed DCs induce IL-12 and, thereby, restore CTLA-4 efficacy, which correlates with induced Th1 immune responses in tumour-draining lymph nodes and DC maturation in tumour beds. Moreover, memory Th1 anti-tumoral response is observed against *B. fragilis* and *B. thetaiotaomicron.* Regarding **anti-CTLA-4 mAbs toxicity**, it has been noticed that after its administration, immune related adverse events such as colitis take place and often dictates a treatment suspension. It has been observed that this unfortunate event is more frequent in SPF mice than GF mice,

suggesting that commensal bacteria are involved in anti-CTLA-4 mAbs dysregulation of the equilibrium among IECs, and gut microbiota at the intestinal barrier. Nonetheless, it has been tested that *B. fragilis* and *B. cepacia* –that also restore CTLA-4 mAbs efficacy– not only do not cause colitis but instead provide protection to anti-CTLA-4-induced intestinal lesions by promoting Treg cells in lamina propria. For all those reasons, colonization of the gut by *B. fragilis* and *B. cepacia* could be part of anti-CTLA-4 treatment not only to increase its efficacy but also to reduce its toxicity (**Figure 8**) (24,25).

Regarding **anti-PD-L1 mAbs** there is a study conducted by Sivan *et al.* (2015) (26) that demonstrated that *Bifidobacterium* species promoted antitumor immunity and facilitates anti-PD-L1 efficacy in mice. The study compared subcutaneous melanoma growth in genetically similar mice coming from different mouse facilities, Jackson Laboratory (JAX) and Taconic Farms (TAC) which differ in their commensal microbes. It was seen that tumour-specific T cell responses and intratumoral CD8+ T cell were significantly higher in JAX mice, whereas tumours were growing more aggressively in TAC mice. Later, JAX mice were forced feeding with TAC faecal suspension and vice versa before tumour implantation, in order to test the role of commensal bacteria in regulating antitumor immunity. TAC mice fed with JAX faecal suspension delayed tumour growth and enhanced induction and infiltration of tumour specific CD8+ T cells whilst the reciprocal faecal transfer (from TAC mice into JAX mice) did not show any improvement in neither tumour growth rate nor antitumor T cell responses proving JAX-dominant effects. Comparative analysis showed that 257 taxa were of significantly different relative abundance in JAX mice than in TAC mice, but *Bifidobacterium* species was the only one that demonstrated to be associated with antitumor T cell responses. Finally, it was seen that JAX faecal material alone significantly decreased tumour growth (by amplifying tumour-specific T cell responses and infiltration of antigen-specific T cells into the tumour) as much as systemic PD-L1 mAb. Likewise, PD-L1 therapy alone was significantly more efficacious in JAX mice than in TAC mice. Similar to CTLA-4 microbiota-mediated efficacy, DCs exposed to *Bifidobacterium* result to be associated to anticancer properties because they induce systemic IL-12 which lead to Th1 immune responses in tumour-draining lymph nodes, DC maturation in tumour beds and tumour-specific CD8+ T cell improvement of its effector function (**Figure 8**) (26).

When studies are conducted in patients rather than in mice results tend to differ. It is true that most of them undoubtedly claim the role of the gut microbiota in shaping responses to immunotherapy. Nevertheless, it becomes complicated to determine which bacterial taxa are associated with response or toxicity (24). For example, to test anti-CTLA-4 toxicity, one study concluded that anti-CTLA-4-treated melanoma patients who were colitis-free had an enrichment of *Bacteroidetes* (27), whereas another one concluded that patients with a higher abundance of *Faecalibacterium prausnitzii* and other related *Firmicutes* and low

abundance of *Bacteroidetes* had a higher risk of colitis on anti-CTLA-4 therapy (28). Another example, to study anti-PD-1/PD-L1 efficacy revealed differences between researches. One of them resolved that *Akkermansia* and *Alistipes* species were enriched in responding patients (29), whereas another one concluded that responders to anti-PD-1 mAbs had a higher relative abundance of *Clostridiales*, *Ruminococcaceae* and *Faecalibacterium* and non-responders had a higher relative abundance of *Bacteroidales* (30). Moreover, a third study confirmed that patients who responded to anti-PD-1 therapy had enrichment of *Bifidobacterium longum*, *Collinsella aerofaciens*, and *Enterococcus faecium* (31).

All these variances can be attributed to different techniques used to analyse samples or to different reference databases which advocate standardized approaches for microbiome analysis. However, in the microbiota analysis there are some factors such as diet and lifestyle that have a deeply geographical influence and it is very difficult to manage.

4.5. Therapeutic benefits of bacteria

The microbiome is as important as our own DNA. Constant and dynamic communication between the microbiota and the host occurs through metabolic and immune interactions. Therefore, altered gut microbiota is involved in diverse aspects of disease which offers a wide range of opportunities for intervention. From preventing diseases to developing new therapeutics or optimising health (**Figure 9**) (32).

The microbiota – comparable to hormonal communication – exchanges chemical information with multiple host tissue compartments through metabolic axis and in a multidirectional fashion. Examples of such axis are bile acid production, gut permeability, brain signalling or enteric nervous system modulation. New therapeutic opportunities could arise from targeting some of these metabolic pathways. In fact, pharmacometabolomic is a field that studies pre-interventional metabolic signatures to predict post-interventional outcomes of drug treatment and gives answers to interindividual variations of certain drugs responses that cannot be explained by pharmacogenomics (32).

Microbiota modulation using prebiotics, probiotics or antibiotics have been used in order to prevent diseases or optimise health (dysbiosis is the source or the cause of several diseases such as diabetes, cancer or obesity), although further research have to be done in order to understand the cause-effect relationships of epidemiological associations. Likewise, microbiome modification is a newer and yet-to-be-implemented therapeutic opportunity that revolves around “druggable microbial genome”, a concept where microbial genes involved in host-microbe metabolic and signalling pathways can be specifically targeted in order to control microbial activities to particular host pathways.

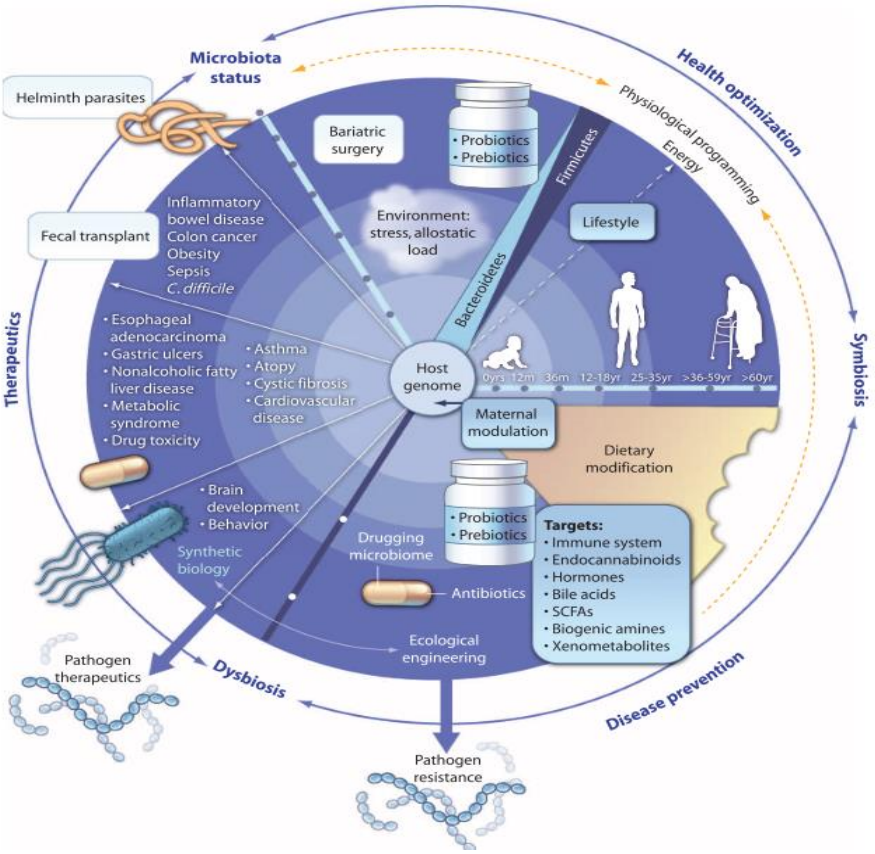

Figure 9. *Therapeutic modulation of the gut microbiota. A general view (32).*

Engineering the microbiota to correct abnormalities in metabolic or signalling pathways involved in disease is another way to improve human health through microbiome modulation (32). Many examples show the major impact of gut microbiome on host metabolism. Polyamines (like vitamin B6) elicits anticancer immune response in chemotherapy settings and reduce toxicity of CTLA-4 mAbs. Short-chain fatty acids provide an energy to colon microbes but are also pharmacologically active, inducing angiogenesis in the gut mucosa or desaminotyrosine (DAT) that protect from influenza virus–mediated lung immunopathology through type I IFN signalling (10).

4.5.1. Oncomicrobiotics and the study of the microbiome

It is undeniable the crosstalk between dysbiosis and oncogenesis, and between the gut microbiota and anticancer therapy both in a bidirectional way. In a cancer setting, due to anticancer therapy or because of oncogenesis, it is common to find the gut in a dysbiotic state. Dysbiosis can be detrimental – when for example, limits therapeutic effects or increases toxicity – or beneficial – when enhance clinical activity. Hence, manipulation of the gut microbiota, as an adjuvant in anticancer therapy to improve therapeutic index, seems to be reasonable (10).

Oncomicrobiotics is a new concept that can be described as a select "cocktail of bacteria and/or bacterial products" aimed to recuperate the natural microbiota or mimic its many beneficial effects that in the case of cancer treatment, it is focused in promoting beneficial immune response. Despite oncomicrobiotics still does not exist, its formulation will supposedly contain clinical products such as probiotics (living microorganisms that provide health to the host), prebiotics (compounds that stimulate growth and activity of bacteria in the gut) and antibiotics. It might be required as an individual or combined therapy. Nonetheless, before choosing the right bacteria or bacterial products to achieve the adjunctive oncomicrobiotics, cancer-associated intestinal dysbiosis must be described (24).

So far, in order to study the gut microbiome and thus all the gut microbiome-related diseases such as cancer, metagenomics – which consists of sampling the genome sequences of a community of organisms inhabiting in a common environment – has been used. This revolutionary method spotlighted the understanding of the relations among the human microbiome, health and diseases. However, metagenomics presented some limitations related to DNA extraction methods and amplification steps and big-data computerized (10). Fortunately, culturomics – the rebirth of culture – was developed in 2008 and meant a breakthrough in microbiology. Culturomics is a high-throughput method that multiplies culture conditions in order to detect higher bacterial diversity and it can cultivate all microbes living in human mucosae. It combines diversified culture media and rapid identification of bacterial colonies using matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) mass spectrometry alone or combined with 16S rRNA sequencing. Compared to metagenomics, which has generated several sequences that have not been assigned to a known microorganism, culturomics allows the culture of many microorganisms including those previously not assigned to a sequence. Moreover, it enables to correlate specific cancer parameters with a concrete microbial species or subspecies rather than with a phyla or genera, that often can include oncogenic and onco-suppressive species and subspecies at the same time. All in all, it is a solid strategy to resolve the gaps of metagenomics. In fact, the number of species isolated from the human gut have been doubled by culturomics. Because of that, nowadays it is possible to develop such a new and ambitious field in therapeutic research such as oncomicrobiotics and in the future it will be possible to discover more diagnosis and therapeutic tools for cancer (33).

4.5.2. Possible strategies to improve gut microbiome in cancer treatment

Cancer is the most challenging disease that humanity faces in this century because in many cases treatment is not efficient and most times early diagnosis is the best 'cure'. Consequently, it is understandable that cancer research not only puts an effort in current anti-cancer therapies but also in different strategies that are indirectly related to cancer cure, some of which involves the gut microbiota modulation (**Figure 10**).

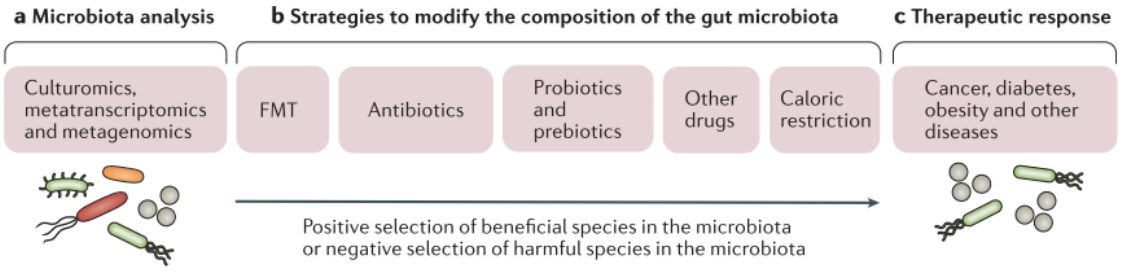

Figure 10. *Interventions on the microbiota in cancer (8).*

Prebiotics are dietary or chemical entities that enhance colonization and expansion of host microorganisms as well as those naturally ingested over life. As they are believed to promote health such as anticancer properties they have been thought as a strategy against cancer. Non-digestible polysaccharides such as dietary fibre or inulin are examples of prebiotics, that can be metabolised by bacteria. These bacterial metabolites such as shot-chain fatty acids (SCFAs) have therapeutic interest rather than prebiotics themselves. Acetate, butyrate and propionate are SCFAs produced in colon by *Clostridium* clusters IV and XIVa. Acetate can be involved in several cancer types' growth. Opposite to acetate, propionate and butyrate have an onco-suppressive effect by inhibiting histone deacetylases and by differentiating and accumulating Treg cells which mediates anti-inflammatory effects and reduce colorectal carcinogenesis risk (8). Prebiotics are linked with **diet** which if changed intensively alter gut microbiota. In a study, where healthy human subjects reduced dietary fibre intake, showed reduce in abundance of *Faecalibacterium prausnitzii* which promotes immunity. Similarly, in another study with inulin supplementation, both *Faecalibacterium* and *Bifidobacterium* species, that enhance host immunity where significantly incremented. Besides, a different study concluded that animal fat free diet decreased detrimental *Bacteroidales.* Finally, antitumor effects of cytotoxic cancer drugs like anthracyclines where seen to be increased when co-administrated with prebiotics in mice. The main advantage of prebiotics and diet as a strategy to improve gut microbiota is that are viable in terms of safety profile and cost (20).

The use of **probiotics** - live microorganisms with health benefits - is a strategy to improve dysbiosis and thus, all its beneficial effects. There are many examples of probiotics that are thought to enhance the immune system and its anticancer activities in very different context (**Figure 11**). However, some of them have limited clinical evidence of effectivity. *Lactobacillus spp.* are known to potentiate the immune system by different mechanisms such as NK cell activation, DCs cell maturation or ferrochrome peptide release. An example of the latter is *L. casei* which induces apoptosis of tumorigenic cells through JNK pathway. Moreover, Prohep, a mixture of *L. rhamnosus* and *E. coli,* showed a potent anti-angiogenic

and anti-inflammatory effect in mice with hepatocellular carcinoma. The anti-inflammatory effect was achieved by shifting gut microbial composition towards certain species that reduced pro-inflammatory Th17 cell while differentiate to Treg cells. *Alistipes shahii* may improve innate immune cell response to immunotherapy. In a study with germ-free mice, those that were inoculated with *A. shahii* showed increased number of infiltrating innate immune cells against colorectal cancer. *Bacteroides fragilis* has a dual role in cancer. The enterotoxigenic strains elicit inflammatory Th17 cells and accelerate carcinogenesis whereas the non-enterotoxins-producing strains have shown great results in an anti-CTLA4 setting; they induce memory T cells and promote matured intra-tumoral dendritic cells (DC). A study demonstrated it with GF mice that received anti-CTLA4 treatment which could not reduce their subcutaneous sarcoma growth. However, the situation could be reverted by inoculating *B. fragilis* and CD4+ T cell previously primed by *B. fragilis*-pulsed DC. Those inoculated mice showed more mature DC inside the tumour than control mice and the reason seems to be the immunogenic polysaccharide A of *B. fragilis'* cell wall. Similarly, *Burkholderia cepacia* also mature intra-tumoral DC *in vitro. B. fragilis* and *B. cepacia* combined have a synergic anticancer effect in CTLA-4 blockade context. *Barnesiella intestinihominis* abundance in colon is increased after cyclophosphamide (CTX) treatment in mice and it is related to more functional CD4+, CD8+ or γδ T cells in spleen and tumour bed. Additionally, combination of CTX with *B. intestinihominis* reduces cancer growth in mice. In CTX context in mice, E*nterococcus hirae* induces Th17 and Th1 CD4+ T cells, stimulates CD8+ T cells and reduce Treg cell because they translocate to lymphoid organs from the small intestine lumen. Colonization of both *B. intestinihominis* and *E. hirae* is inhibited by NOD2-receptor (a type of patter recognition receptor) and thereby their immunostimulatory effects are more pronounced in NOD2-deficient mice. Synergy between *B. intestinihominis* and *E. hirae* is still to be determined. Finally, *Bifidobacterium breve* and *B. longum* stimulated dendritic cell maturation and thus, enhanced tumour specific CTLs priming. Compared to *Bifidobacterium*-free mice, those that contained *B. breve* and *B. longum* had better outcomes to immunotherapy because CTLs function was improved and could easily infiltrated into tumour (8). One of the main advantages of probiotics is that some species show a clear improvement of the immune system to fight against cancer (especially anti-tumour T cell response) and seems more appropriate to boost the gut microbiota than prebiotics. However, more efficacy and safety studies need to be done because on the one hand there are studies that suggest that probiotics with more than one species have larger efficacy than probiotics with a single species. On the other hand, it has been seen that probiotics in some cases can confer tumorigenesis instead of protection against cancer, so it is important to take into account each situation (2, 20). Finally, a future major drawback with probiotics might be that, if they become therapeutically indicated to treat cancer, their current commercial regulation status as

dietary supplement will have to change to drug with all the strict regulation and quality certificates that they require (10).

Since it is known the existence of unfavourable bacteria in the gut, the use of **antibiotics** seems a feasible strategy to shape gut microbiota to improve cancer treatment. However, for a long time, some studies have associated through epidemiological studies that continuous exposure to broad-spectrum antibiotics in life correlates with a higher predisposition to develop cancer (not only colorectal but also lung, prostate and bladder cancer) linking microbiota with carcinogenesis (1, 11). Moreover, the problem with antibiotics is also their lack of specificity which could undermine favourable microbiota (although in the food industry they use bacteriophages as highly selective method to eliminate unwanted bacteria) (10). One example to display antibiotic's tricky efficacy is vancomycin. In an anti-CTLA4 treatment vancomycin is used to eliminate Gram-positive bacteria such as *Clostridiales* in order to Gram-negative *Bacteroidales* to expand and therefore induce Th1 cells immune response which favour CTLA-4 blockade antitumor efficacy. Nevertheless, vancomycin reduces its immunogenic response and thus its anticancerogenic effect in a CTX setting. In conclusion. it could be stated that antibiotics

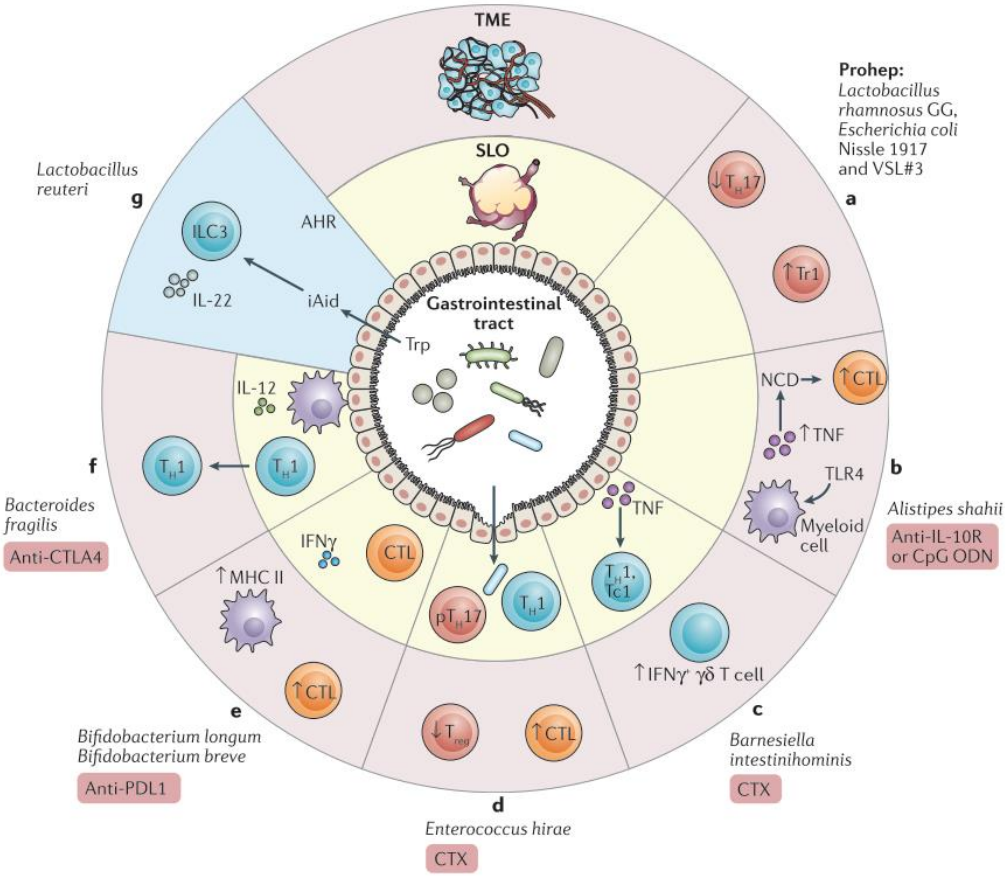

Figure 11. *Potential immune mechanisms that explain the anticancer effects of probiotics (8).*

therapy alone to improve cancer treatment seems risky, however, as adjunctive oncomicrobiotics (combined with pre- and/or probiotics) could be a reasonable strategy to eliminate detrimental bacteria (8).

Faecal microbial transplantation (FMT) consist of exchanging gut microbiota (especially from the lower gastro-intestinal tract) from healthy donors to patient with detrimental dysbiosis. Nowadays, FMT is a strategy used to treat *Clostridium difficile* infection resistant to conventional therapy with a high rate of positive response (80-90%) and a promising one to irritable bowel syndrome. The growing popularity and the earliest advances of this technique (stool from heathy donors now is packed into pills) make FMT appealing to modulate the gut microbiota in order to improve cancer treatment although this idea is still on its beginning. FMT from healthy donors to mice have shown incredible results to many anticancer therapies, however, when FMT from colorectal patients was practiced into germ-free mice dysplasia and polyp formation arisen. FMT needs further research because commensal composition not only modulates the immune response but also plays an important role in chronic diseases such as depression or obesity that could be accidentally transferred. Nonetheless, it is worthwhile to keep trying to implement this strategy because long-term reset of the microbiota can be achieved (2, 8, 10, 20).

Even though these strategies are the most relevant ones, there are others unconventional and yet-to-have scientific evidence strategies that in the future they might be used in cancer treatment. For example, the use of bacterial toxins, pathogen-associated molecular patterns (PAMS), bacterial metabolites (8) or even though the own bacteria as a shuttle delivery method (2).

5. Conclusions

The most relevant ideas of this dissertation can be summarised as follows.

Firstly, the microbiota has an impact in carcinogenesis as well as carcinogenesis can cause or worsen dysbiosis. Some microbes can directly cause cancer as they have intrinsic oncogenic effects or because they alter carcinogenic metabolites. Others induce carcinogenesis indirectly, by destabilising immunosurveillance via immunosuppressive or proinflammatory effects. At the same time, tumours can alter microbiota composition through its tumour microenvironment (TME) which is rich in proinflammatory cytokines and reactive immune cells. Sometimes that leads to cancer-associated shifts of microbiota like in colorectal cancer, hepatocellular carcinoma or breast cancer, which have been epidemiologically associated to some bacterial species.

Secondly, the immune system has a cancer-immunity cycle capable to eliminate cancer cells and keep homeostasis, however, tumours can develop mechanisms to scape this cycle such as loss of antigenicity or immunogenicity. Nonetheless, the microbiota can activate the innate and above all the adaptive immune system, which in turn, depending on the bacterial species can trigger different subset of T cells which will determine the cancer outcome. Improved outcomes (reduction of cancer growth, less progression, etc.) will be related to beneficial dysbiosis, whereas worsened outcomes (tumour growth, increased cytokines, etc.) will be related to detrimental dysbiosis.

Thirdly, the efficacy and toxicity of certain anticancer drugs (chemotherapy, immunotherapy or even radiotherapy) depend on the microbiota and in some cases on particular species. This fact opens a great new opportunity to enhance efficacy or avoid toxicity in cancer patient by previously modulating the microbiota before anticancer treatment.

Fourthly and lastly, understanding the role of the microbiota in the human body in order to modulate it to human health profit is one of the challenges of the 21st century medicine. There are many medicine fields that could greatly benefit from it and oncology is one of them. The mechanisms by which the microbiota causes carcinogenesis need to be known. In this way, it is made sure that current strategies to modify the gut microbiota to prevent or to help curing cancer are appropriate. Likewise, potent techniques like culturomics are required to truly know the significance of the microbiota and all the benefits resulting from it such as the oncomicrobiotics.

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