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Catarina Gonçalves Guerra

Relatórios de Estágio e Monografia intitulada "Microbiome and Cancer" referentes à Unidade Curricular "Estágio", sob a orientação do Professor Doutor João Nuno Moreira, da Dra. Maria Helena Amado e do Doutor António Lucas Nunes apresentados à Faculdade de Farmácia da Universidade de Coimbra, para apreciação na prestação de provas públicas de Mestrado Integrado em Ciências Farmacêuticas.

Setembro de 2019

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Assinatura

Catarina Gonçalves Guerra

(Catarina Gonçalves Guerra)

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Content

Part I

Monography

Microbiome and Cancer

List of Abbreviations

AMPs - Anti-microbial Peptides

APC - Antigen-presenting Cell

CBC - Crypt Base Columnar Cells

CD28 - Cluster of Differentiation 28

CD80 - Cluster of Differentiation 80

CD86 - Cluster of Differentiation 86

CpG-ODN - CpG-oligodeoxynucleotide

CTLA-4 - Cytotoxic T-lymphocyte-associated protein 4

DAMPs - Damage-associated Molecular Patterns

DCs - Dendritic Cells

FMT - Fecal Microbiota Transfer

GALT - Gut Associated Lymphoid Tissue

GI - Gastrointestinal

ICIs - Immune Checkpoint Inhibitors

IECs - Intestinal Epithelial Cells

IgA - Immunoglobulin A

IL-10 - Interleukin-10

IL-17 - Interleukin-17

IL-18 - Interleukin-18

MAMPs - Microbe-associated Molecular Patterns

MDSCs - Myeloid-derived Suppressor Cells

mLNS - Mesenteric Lymph Node

NLRs - NOD-like receptors

PAMPs - Pathogen-associated Molecular Patterns

PD-1 - Programmed Cell Death Protein 1

PD-L1 - Programmed Death-ligand 1

PRRs - Pattern Recognition Receptors

ROS - Reactive Oxygen Species

RTX - Ionizing Radiation Therapy

SCFAs - Short-chain Fatty Acids

T regs - T Regulatory Cells

TBI - Total Body Irradiation

Th1 - T helper 1

Th17 - T helper 17

TIL - Tumor-infiltrating Lymphocytes

TLRs - Toll-like Receptors

TNF - Tumor Necrosis Factor

Abstract

Research regarding human microbiome, especially the gut microbiota, has increased in the last years and several scientists around the world are exploring this topic. The gut microbiota is composed of all the microorganisms that inhabit this part of the human body and has a crucial role in modulating several biological functions as the immunity response, inflammation, hematopoiesis, metabolism, among others. Additionally, it is known that the microbiota can be constituted by different microorganisms taking into consideration the part of the body that inhabits. Moreover, the commensal microorganisms can also vary from person to person considering some characteristics of the host such as the moment of life, the birthing process, the lifestyle (diet as an example), the pathology and treatments, among others.

The gut microbiota has also been associated with several pathologies including inflammatory bowel disease, gastritis, obesity and all types of cancer. In several cases, a certain pathological situation is associated with an alteration of the microbiota's composition, dysbiosis, regarding either the species or the relative frequency of them. Being cancer considered as one of the deadliest diseases worldwide, the exploration and deepening of the influence of microbiota on this disease is crucial and mandatory. Microbiota is known to be involved in cancer initiation, progression and dissemination. Moreover, it can play a crucial role in controlling the adverse effects and improving the efficacy of the therapy.

This monography intends to approach crucial definitions and concepts regarding human microbiome and proposes also to explore its influence in modulating the therapeutics of cancer, including chemotherapy, radiotherapy and immunotherapy. The immune checkpoint inhibitors, as anti-CTLA4 and anti-PDL1 antibodies, a relevant topic at the moment that gave rise to the Nobel Prize in Physiology or Medicine 2018, will also be explored in the present work. Additionally, this monography aims to specify the species involved in each process and their mechanism of action and intends to explore the possibility of targeting the microbiota to improve the antitumoral effect and prevent drug-associated toxicity. Lastly, the present work proposes to describe and explore a recent approach regarding gut microbiota, the fecal microbiota transfer (FMT).

Keywords: microbiome, cancer, immune system, chemotherapy, radiotherapy, immunotherapy, immune checkpoint inhibitors, fecal microbiota transfer.

Resumo

A pesquisa e investigação em torno do microbioma humano, especialmente o microbiota intestinal, tem sofrido um drástico aumento nos últimos anos e diversos cientistas espalhados por todo o mundo exploram este tópico. O microbiota intestinal é composto por todos os microrganismos que habitam esta parte do corpo humano e tem um papel crucial na modulação de diversas funções biológicas como na resposta imunológica, inflamação, hematopoiese, metabolismo, entre outras. O microbiota pode ser constituído por diferentes microrganismos tendo em consideração as diferentes partes do corpo que habitam. Para além disso, os microrganismos comensais podem variar de pessoa para pessoa considerando algumas características do hospedeiro como a idade, o tipo de nascimento, o estilo de vida (por exemplo a dieta), as patologias e respetivos tratamentos, entre outros.

O microbiota intestinal tem sido igualmente associado a diversas patologias incluindo a doença inflamatória intestinal, gastrite, obesidade e todos os tipos de cancro. Em diversos casos, uma certa situação patológica está associada a uma alteração na composição do microbiota, disbiose, quer ao nível da sua composição quer da frequência relativa das espécies. Sendo o cancro considerado uma das doenças mais mortais atualmente, o aprofundamento deste tópico é crucial e obrigatório. Sabe-se que o microbiota está envolvido no início, progressão e disseminação do cancro, mas que, para além disso, desempenha um papel crucial no controlo dos efeitos adversos e no aumento da eficácia da terapêutica.

Esta monografia pretende abordar definições e conceitos cruciais relativos ao microbioma humano e pretende também explorar a sua influência na modulação da terapêutica do cancro, incluindo quimioterapia, radioterapia e imunoterapia. Os inibidores do *checkpoint* imunológico, como os anticorpos anti-CTLA4 e anti-PDL1, um tema relevante do momento que deu origem ao Prémio Nobel da Fisiologia ou Medicina 2018, também vão ser explorados neste trabalho. Além disso, esta monografia visa especificar as espécies envolvidas em cada processo e os seus mecanismos de ação e pretende ainda explorar a possibilidade de ter como alvo o microbiota, para ser possível aumentar o efeito antitumoral e para se poder prevenir a toxicidade de fármacos. Por último, o presente trabalho pretende descrever e explorar uma abordagem recente relativa ao microbiota intestinal, o transplante fecal de microbiota (FMT).

Palavras-chave: microbioma, cancro, sistema imunitário, quimioterapia, radioterapia, imunoterapia, inibidores do *checkpoint* imunológico, transplante fecal de microbiota.

1. Introduction

1.1. The Human microbiome

The research about Human microbiota has massively increased through the last years, and thereby a lot of new information regarding this topic has emerged. The human microbiome is found on the epithelial barrier surfaces of the human's body (mucosa and skin) and it is composed of trillions of several microorganisms including bacteria, archaea, fungi, protozoa and viruses. These organisms inhabit several areas of the human body such as the gastrointestinal (the majority of the microorganism's population), respiratory and genitourinary tract, skin, oral cavity, among others, and each location has a specific population of them, as it is possible to see in figure I. [1–6]

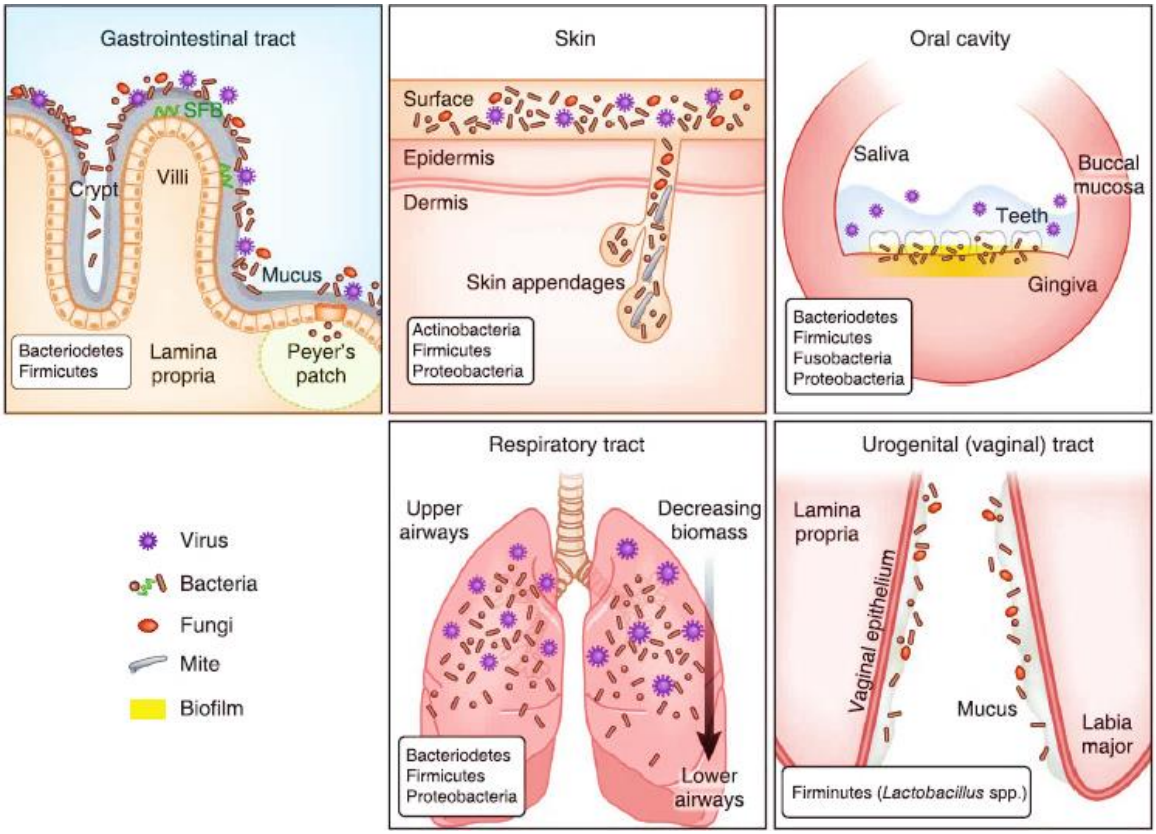

Figure 1- Distribution of microorganisms according to the human's body parts. Adapted from [1].

The human microbiome is separated in four principal phyla: Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria. The first two phyla constitute more than 90% of the gut microbiome's population. The Bacteroidetes are essentially present in the colon and consist of Gram-negative anaerobic bacteria that are able to survive in the presence of oxygen. The Firmicutes phylum are fundamentally present in the gut and vagina and are divided in two main classes: *Bacilli*, composed of obligate or facultative aerobics as *Lactobacillus, Staphylococcus* and *Streptococcus* species, and *Clostridia*, that consists in Gram-positive anaerobic bacteria of which

the *Clostridium* species made part of. Additionally, the phyla Actinobacteria is also composed of anaerobic Gram-positive bacteria and *Bifidobacterium* is one example of that. Lastly, the phylum Proteobacteria is present at the gut of all the vertebrates and it is constituted of Gram-negative aerobic or facultative anaerobic bacteria as *Escherichia, Klebsiella* and *Enterobacter* species. [7,8]

It is important to understand that the species of the human microbiome can vary from person to person since its composition is regulated by several factors as the genetics of the host, birthing process, the lifestyle of the host (for instance nutrition and exercise), prevalence of diseases, treatments and the amount of antibiotics taken. The composition of the microbiome undergoes changes from the beginning of life (from when the organ is developing inside the uterus of the progenitor) till it becomes the mature microbiome of adulthood. Then, the composition of the microbiome tends to remain stable, but it can suffer alterations through food intake, lifestyle modifications, pathologies and respective treatments. [3],[9,10]

The human body plays a symbiotic relationship with microbiome. Symbiosis represents a constant interaction between both parts that allows both of them to live and survive. The symbiotic relationship includes the following biological interactions: mutualism, commensalism, and parasitism. Mutualism consists in an interaction where both species benefit from, while in commensalism only one of them take advantage of the relationship. On the other hand, parasitism is referred to as the interaction where the parasite causes damage to the host. Generally, commensalism is the most common interaction between microbiome-human host, and in the majority of the times, microbiome microorganisms are referred to as commensals. [3], [8], [11,12], [13,14]

The commensals organisms are classified as pathobionts, microorganism that in specific conditions can develop pathological features and became pathological. If somehow the ecology of the gut, for instance, undergoes alterations, bacteria such as *Clostridium difficile* and *Enterococcus* species, can proliferate and acquire the potential to cause disease. This perturbation of the human microbiota, causing an atypical proportion between commensal and pathogenic species, is called dysbiosis, and can modify the interactions between the microbiome and the host, giving rise to several pathological situations. [3], [4], [8], [11-15]

Microbiome plays a crucial role in controlling human's physiology and health and affects metabolism, neurological and cognitive functions, as well as inflammation and immunity of the host. Moreover, dysbiosis is known to be involved in several pathological statuses as obesity, diabetes, diseases of the skin, vaginosis, inflammatory diseases, cancer, among others. Being cancer considered as one of the deadliest diseases worldwide, its relationship with microbiome has been intensely explored. It is important to highlight that microbiome can

control and modify cancer's beginning, development and the body's response to the treatment, regarding efficacy and drug-associated toxicity, subjects that will be approached in the following sections. [2-4], [8], [11-15]

1.2. The Human gut microbiota

As already mentioned, the gastrointestinal tract is the principal location of the microorganisms that constitute the human microbiome and it is composed of around 3×10^{13} bacterial cells. The interactions between the host and the human microbiome influence diverse physiological processes of the host, but the gut microbiota is the principal responsible for that. [3], [16]

The intestinal microbiota interacts with epithelial and stromal cells of the gastrointestinal tract performing an important role in several functions. Gut microbiota takes part in controlling the barrier functions and the immunity response of the local on which it inhabits. Besides, these microorganisms are associated with systemic effects as well, affecting distant organs, although these mechanisms are less well described and understood. Moreover, the microbiota helps to maintain the symbiosis between the microbiota and the host, takes part in controlling and preventing the excessive growth of pathobionts and also avoids the invasion by pathogen microorganisms. On the other hand, gut microbiota is also important in the regulation of the metabolism as a contribution to the metabolism of indigestible dietetic fibre and plays a role in avoiding the development of diseases as obesity. Lastly, it is involved in the production of vitamins. It is important to understand that to preserve the symbiosis between the host and the microbiome and to guarantee the intestinal epithelial homeostasis a correlation between the gut microbiota, the mucosal and the immune system is necessary. If all works correctly, a healthy body can be maintained. [3], [16]

An overview of the principal local and systemic effects of gut microbiota is represented in figure 2. The first box of the image represents the suggested mechanisms through which these effects can be achieved. It is possible to conclude that the microorganisms take part in several physiologic and pathological processes as cancer and other pathologies, on a local and systemic levels, affecting organs that are not directly associated with the gut microbiota. These mechanisms were evaluated considering the microbiota of the gut, but it is expected that the microorganisms of other mucosal barriers will also possess local and systemic effects. [3]

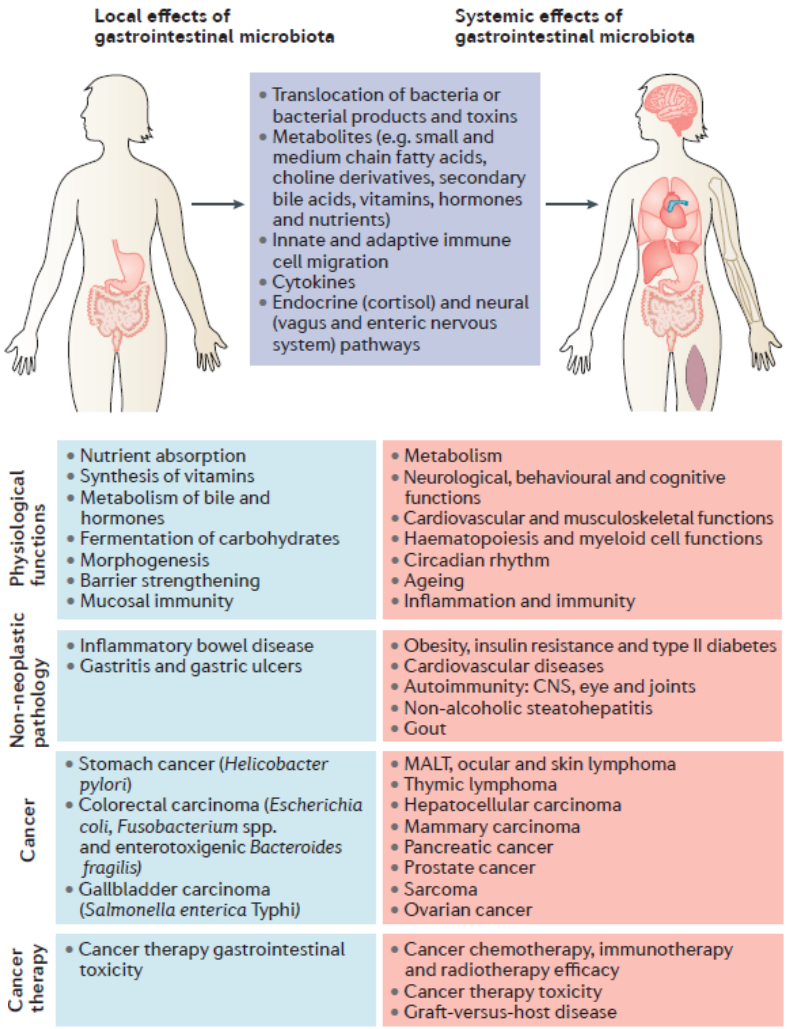

Figure 2 - Gut microbiota's local and systemic effects. Adapted from [3].

1.3. Microbiota and the Immune System

The interaction between gut microbiota and the immune system plays a significant role on both local and systemic levels. Locally, this interaction promotes the tolerance of the microbiota microorganisms and of antigens from diet. Besides, it allows the recognition and destruction of opportunistic bacteria, thus preventing infection. Additionally, microbiota influences the innate and adaptive immunity which contributes to both local and systemic effects. [17]

The gut is constituted of a mucosa barrier and a layer of connective tissue, lamina propria. The mucosa is composed of intestinal epithelial cells (IECs) that include goblet cells, responsible for the secretion of mucus, and Paneth cells, responsible for the secretion of anti-microbial peptides (AMPs). The mucus and the AMPs represent the coating of the epithelium. Besides, the mucosa layer is constituted of intraepithelial lymphocytes as well. The IECs and the lymphocytes are exposed in a single cell layer, allowing the passage and interaction of

microbial metabolic products with the host cells contributing to an easy interaction with the immune system. Lamina propria is composed of the Peyer's patches and immune cells: antigen-presenting cells (APC), innate lymphoid cells, $CD4^+$ and $CD8^+$ T (cytotoxic) lymphocytes and B cells. The Peyer's patches and the referred cells are part of the gut associated lymphoid tissue (GALT) that influences the immune response in both local and systemic levels. Moreover, the gut contains steam cells, as Crypt Base Columnar (CBC) cells, responsible for the differentiation into the desired cell type. [17–19]

Locally, the interaction between microbiota and the immune system is responsible for the tolerance of the microbiota microorganisms, as already mentioned, and for the distinction between the commensals and the pathogenic bacteria. This differentiation is mediated by the Toll-like receptors (TLRs) placed at the membrane of the epithelial cells and innate immunity cells. These receptors are part of the pattern recognition receptors (PRRs). The PRRs are expressed in macrophages, dendritic cells (DCs) and in various nonprofessional immune cells (epithelial, endothelial and fibroblasts) and recognize a structure that is preserved amongst the microorganism species, the pathogen-associated molecular patterns (PAMPs), or the microbe-associated molecular patterns (MAMPs), leading to the detection of organism's presence. The PRRs also recognize the damage-associated molecular patterns (DAMPs), which are endogenous components released from injured cells. There are four categories of PRRs: TLRs, C-type lectin receptors – transmembrane proteins, Retinoic acid-inducible gene (RIG)-I-like receptors and NOD-like receptors (NLRs) – cytoplasmic proteins. [16,17], [20]

The gut local immunity passes by the recognition of the PAMPs, as lipopolysaccharide and flagellin, by the TLRs present at the epithelial barrier and at the innate immunity cells as monocytes, macrophages and DCs, leading to their activation. These cells are responsible for the release of inflammatory mediators and to promote the adaptive immunity response, described below. The activity of TLRs in the first weeks of life is reduced in order to allow the development of a stable gut bacterial community. As time passes by, their activity increases, contributing to the maintenance of the intestinal homeostasis. [16,17], [20]

After the recognition of the microorganisms by the TLRs of DCs and subsequent activation and maturation of DCs, the gut microbiota can control the adaptive immune response and can induce the migration and differentiation of populations of T cells: T helper 1 (Th1), T helper 2, T helper 17 (Th17) and T regulatory cells (T regs). The recognition of the microorganisms can be made by the passage of the dendrites through the mucosal barrier (direct way) or after the phagocytosis and transport by the M cells (indirect way). These cells are specialized IECs that do the transportation of the intestinal antigen and deliver it into the GALT. After that, DCs travel to the mesenteric lymph node (mLNs) and induce adaptive

immunity by promoting the transformation of T cells into T $CD4^+$ cells, specially T regs and T helper 17. Besides, DCs can also induce the formation of CD8+ T cells as well. After that, DCs can enter to the bloodstream, or they can go back to the gut mucosa, but normally when they are activated by the gut microbiota, do not enter the circulation or distant lymphatic nodules. [16,17], [21]

T cells need several stimuli to be activated and differentiated. Firstly, it is necessary a binding between the T cell receptor and the antigen associated with the major histocompatibility complex of the APC, as DCs. It is also required a costimulatory signal, the binding of the cluster of differentiation 80 (CD80) and cluster of differentiation 86 (CD86) ligands present in the APC with cluster of differentiation 28 (CD28), a receptor of the T cells. After that, the T cells are activated and their differentiation is mediated by the synthesis of interleukins, and other chemicals mediators, by macrophages, DCs, and some of them by non-recognized cellular sources. For example, the synthesis of interleukin-12 by macrophages and DCs and of interferon-gamma by the natural kill cells promote the differentiation of the activated T cells into Th1. [16,17], [21]

The T cells modulate the gut homeostasis, especially T regs, that contribute to the mucosal microbial tolerance due to the synthesis of interleukin-10 (IL-10), an immunosuppressive cytokine (anti-inflammatory activity). On the other hand, Th17 are responsible for the production of interleukin-17 (IL-17) that stimulates the synthesis of AMPs (part of the innate immunity response – responsible for the dead of several microorganisms) by the Paneth cells and responsible for recruiting neutrophils from the systemic circulation. The Th17 cells origin the pro-inflammatory activity, the opposite effect of the T regs cells. The activated T cells can promote the activation of the B cells. Activated B and T cells can leave the mLNs of the gut and circulate through the bloodstream to enable immune responses at distant locations. *Bacteroides fragilis*, an important gut commensal, is responsible for promoting the decrease of the production of IL-17 and increase the IL-10 synthesis. These bacteria have a mucosa protective function against inflammatory reactions initiated by bacterial antigens. [17], [20]

On the other hand, bacteria can produce metabolites that control the gut homeostasis. The short-chain fatty acids (SCFAs) can promote the synthesis of Immunoglobulin A (IgA) by B cells into the lumen of the intestine. Additionally, several different mucosal antigens and several cytokines as transforming growth factor beta, interleukin-4, IL-10, interleukin-5, and interleukin-6 can stimulate this immunoglobulin synthesis. IgA, essentially secretory IgA, is one of the most important local innate immunity barriers, and it is the first line mechanism against pathogens and toxins. The gut microbiota induces the production of small amounts of this

immunoglobulin. IgA has several functions highlighting the blockage of the bacteria's adhesion to the epithelial cells and its role in controlling the virulence of the bacteria. This molecule's regulation is important to maintain the intestinal homeostasis. [17], [20]

At the same time, it is important to refer that some other bacteria or specific microorganisms possess the capability of passing straightly into the bloodstream causing systemic immune alterations. The enunciated interactions are represented in figure 3. [17], [20]

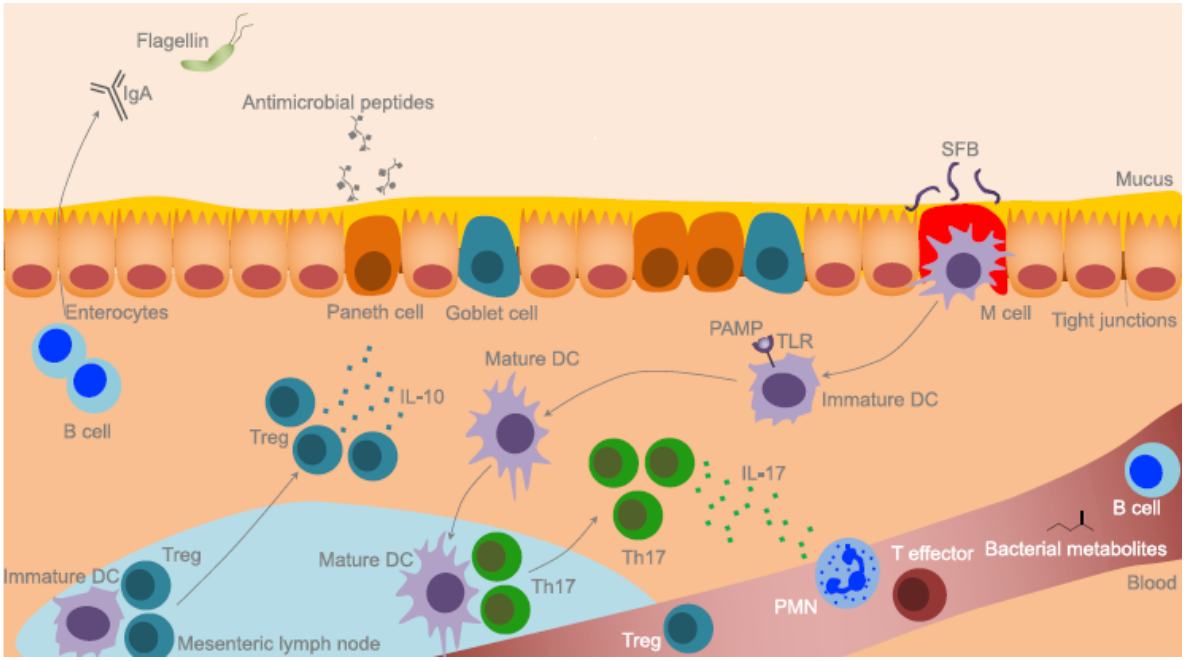

Figure 3 - Microbiota and Immune System interactions. Adapted from [17].

Dysbiosis can promote a decrease in the immunity response and subsequently origin alterations at the gut barrier. These effects allow the passage of the gut bacteria into the mLNs and consequently into the bloodstream. Additionally, several pro-inflammatory cytokines can be produced promoting a Th17 cells activation giving rise to an influx of neutrophils and an inflammatory response both on local and systemic levels. [17], [20]

2. Microbiome's influence on Cancer initiation, progression and therapy

As already mentioned, microbiome has the ability to control and modify several physiologic and pathological processes of the host, such as its inflammation process and immunity response. Dysbiosis can promote pathological processes, leading to the alteration of the interaction between the commensal microorganisms and the host cells, which results in alterations at the barriers and development of inflammatory diseases. These alterations can

possibly modulate the beginning and evolution of cancer and host's response to the therapy. [3]

Several studies were performed with mice incapable of synthesizing or responding to interleukin-18 (IL-18), a mediator responsible for intestinal mucosal protection. The absence of IL-18 resulted in a dysbiosis process that increased the susceptibility of developing colitis or cancer of colon. Besides that, the gastrointestinal (GI) tract microbiota can also influence cancer development at distant organs, as proved in several animal studies. For example, *Helicobacter hepaticus* can lead to a colon's infection, that not only increases the chance of acquiring cancer of colon, but also, mammary and prostate cancers. On the other hand, the existence of *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans,* at the oral mucosa, has been related to the development of pancreatic cancer while the phylum *Fusobacterium*, part of the human gut and oropharynges microbiota, has been revealed beneficial, decreasing the chance of the development of this pathology. [3], [22,23]

Apart from the fact that microbiome is related to the initiation and progression of cancer, it is suggested that it has a contribution to the therapeutic part as well, since its targeting can reduce the drug-associated toxicity. Moreover, microbiome may be able to reduce the resistance of the organism to the therapy as well, in order to promote a better response to the treatment, improving the drug's therapeutic effect. These topics will be discussed in the following sections. [3], [22,23]

2.1. Chemotherapy

The GI microbiota plays an important role in controlling the drug pharmacokinetics since it regulates the absorption and bioavailability of several drug products and it contributes to the metabolism of drugs. On the other hand, it is known that the xenobiotics themselves (chemical substances not produced by the body) can modulate the microbiota's composition and gene expression, contributing to the regulation of the drug metabolism as well. [3], [24]

There are several classes of drugs used in chemotherapy based on their mechanisms of action: alkylating agents, heavy metals, antimetabolites, antineoplastic antibiotics, spindle poisons (mitotic inhibitors) and topoisomerase inhibitors. The majority of them act by disrupting DNA integrity and the cancer cells division, but they can also affect the mitochondria, among other parts of the cell. [3]

The alkylating agents promote the addition of an alkyl group to the guanine base of the DNA, avoiding the connection of the two strands of the molecule, not allowing the formation of the double helix. This affects the DNA molecule, preventing cell division and promoting its

subsequent death. There are five categories of this agents: nitrogen mustards (cyclophosphamide as an example), nitrosoureas, alkyl sulfonates, triazines and ethylenimines. [25–31]

The heavy metals class of drugs encompass the platinum agents as cisplatin, carboplatin and oxaliplatin, and their interaction with the DNA molecule promote the crosslink between the strands, avoiding the DNA synthesis. On the other hand, antimetabolites are structural analogs of critical molecules that promote the growth of the cell. They are constituted of substances like vitamins, nucleosides, amino acids and compete with the endogenous substance intervening in the DNA synthesis. This class of drug products has several categories such as folic acid antagonists (methotrexate as an example), adenosine deaminase inhibitor and purine and pyrimidine antimetabolites (as gemcitabine). [25–31]

Antineoplastic antibiotics, another group of anticancer drugs, have a similar mechanism of action as quinolones, but the principal difference is that the former act in cancer cells while the latter in bacterial cells. Its mechanism of action passes by its insertion into the DNA strands or by promoting the production of the radical superoxide leading to the rupture of the DNA molecule, avoiding cell's division. An example of this class of drugs is doxorubicin. [25–31]

On the other hand, the spindle poisons (mitotic inhibitors), like paclitaxel, bind to tubulin (a protein that makes part of microtubule cytoskeleton) and promote the activation of the spindle assembly checkpoint leading to a long arrest in the mitosis phase promoting the cell's dead. Lastly, the topoisomerase inhibitors, like irinotecan, inhibit the topoisomerase I enzyme. This enzyme is responsible for reducing the DNA twists and supercoiling and it is essential in transcription, replication and repair of the molecule. The topoisomerase inhibitors end up causing the cell's death. [25–31]

Several drugs have shown to be affected by the GI tract microbiota and among the chemotherapy drugs, a couple of them are clearly described, but several are still being explored. In a study that evaluate the interaction between bacteria and thirty chemotherapy drugs, the efficacy of ten was inhibited by certain bacteria, while the same microorganisms increased the efficacy of six of them. It is known that chemotherapy brings a lot of toxicity effects since it is not specific, acting in both cancer and normal cells. Due to that fact, it is important to explore ways of reducing these effects or improving the pharmacological outcome. The microbiota has been studied to evaluate the potential in achieving the previously described goals. This area is constantly growing, and the following sections will approach the gut microbiota influence in several chemotherapy drugs. [3]

2.1.1. Irinotecan

Irinotecan, a topoisomerase inhibitor used for colorectal and lung cancer, is metabolized by liver and small intestine carboxylesterases (I and II) and gives rise to SN-38, the active metabolite. Additionally, it suffers detoxification by UDP-glucuronosyltransferases, at the liver, before being excreted into the gut, giving rise to SN-8-G, the inactive form. [3], [32]

Microbiota can influence irinotecan's activity regarding metabolism and enzyme degradation. Bacteria β-glucuronidases, responsible for the breakdown and metabolization of several macromolecules, placed at the gut, can promote the conversion of SN-8-G in SN-38 (the active form). This leads to intestinal toxicity and consequently, diarrhea. On the other hand, studies that involved rats with tumors treated with irinotecan, showed that this drug itself can alter the microbiota composition. Irinotecan is responsible for increasing the number of β-glucuronidases bacteria such as *Escherichia coli*, *Staphylococcus* spp. and *Clostridium* spp. (*Clostridium* cluster XI for example) and decreasing the *Lactobacillus* spp., responsible for the inhibition of β-glucuronidases and *Bifidobacterium* spp. that have protective properties to the mucosal barrier. These alterations of the microbiota can be the origin of the diarrhea and the inflammatory process. Due to these facts, it is important to conclude that microbiota has influence in this drug transformation, but irinotecan itself can alter the microbiota composition, enhancing the toxicity effects. [3], [24], [32]

By controlling the microbiota, it is possible to regulate the response to the therapy. Studies executed in animals showed that antibiotics responsible for decreasing the β-glucuronidase bacteria, can reduce the intestinal inflammatory process, decreasing the toxicity effects of this drug. Moreover, clinical trials testing probiotics (living microorganisms – food supplements) revealed a decrease in the incidence of diarrhea as well. The probiotics used were composed of *Bifidobacterium* spp., *Lactobacillus* spp. and *Streptococcus thermopilus*. These bacteria are known for their decreased activity of gut β-D-glucuronidases, thereby could be used for the diarrhea's prevention in patients administered with irinotecan. [3], [32,33]

Since the toxicity of irinotecan is severe and can be involved in the determination of the dose to be administered, the development of inhibitors of the β-glucuronidases bacteria is being explored to attempt a resolution for this problem. [3], [32]

2.1.2. Gemcitabine

Gemcitabine (2′,2′-difluorodeoxycytidine) is an antimetabolite, nucleoside analogue, indicated for the treatment of several types of cancer, but it is essentially used in pancreatic

cancer. Gemcitabine is a prodrug that is metabolized to its active metabolite, the triphosphate form of gemcitabine. [34]

The effect of several bacteria in this drug was evaluated. The *Escherichia coli* influence in the activity of this drug was studied *in vitro* and *in vivo*, in mice. The evaluated bacteria revealed a possible inhibition of the effect of gemcitabine. A more recent study evaluated the possibility of intratumor bacteria in promoting tumor resistance to this drug. Through colon cancer models, it was verified that bacteria, essentially *Gammaproteobacteria* and *Mycoplasma*, can promote the metabolization of gemcitabine into its inactive form 2′,2′-difluorodeoxyuridine, leading to the therapy resistance. These bacteria are able to produce a long isoform of the bacterial enzyme cytidine deaminase that is the preferential mechanism responsible for that metabolism. On the other hand, the effect of antibiotics in reverting this resistance was also evaluated. Mouse colon cancer models with resistance to gemcitabine provoked by *Gammaproteobacteria*, where treated with ciprofloxacin. Through this test was possible to verify that the resistance was reverted with the use of the antibiotic. [24], [34]

Moreover, gemcitabine is essentially used for the treatment of pancreatic ductal adenocarcinoma. Samples of the tumor removed from patients with ductal adenocarcinoma, allowed to conclude that *Gammaproteobacteria* is present at the tumor environment, possibly compromising the therapy. All the *in vitro* and *in vivo* results revealed a complex interaction between the microbiota and gemcitabine both at the efficacy of the drug and at the resistance mechanism. All these findings will possibly contribute to a better outcome of this therapy, although there is a lot more to explore to perhaps improve the therapy response of gemcitabine. [34]

2.1.3. Doxorubicin

Doxorubicin is an antineoplastic drug, part of the antineoplastic antibiotics group, used to treat several types of carcinomas, sarcomas, and it is one of the most prescribed drugs in this area. This drug has multiple mechanisms of action, but its use is limited thanks to the toxicity in healthy tissues. Doxorubicin can induce some adverse effects as diarrhea and vomiting, although its principal side effect involves the cardiac tissue. Moreover, doxorubicin is known to induce alterations at the barrier and consequently promote the translocation of bacteria, especially Gram-positive microorganisms (*Lactobacillus johnsonii*, *L. murinus* and *Enterococcus hirae*). [3], [24], [35]

Some of the adverse effects, as cardiomyopathy and intestinal mucositis, are related to alterations of the oral and intestinal microbiota and it is suggested that a modification in the

microbiota's ratio influences the toxicity. The receptors involved in the toxicity mechanism are not described yet. However, a receptor is known for being involved in a positive effect. The bacterial muramyl dipeptide, a peptidoglycan common to all bacteria, can stimulate de NLR2 receptor (present in Paneth cells and CBC cells), promoting a protective effect and avoiding the damage caused by doxorubicin at the gut mucosa. [3], [35]

Under other conditions, recent studies approaching *Raoultella planticola,* a normal microorganism of the gut, developed with model species *Caenorhabditis elegans*, revealed that these bacteria are strong inactivators of doxorubicin. *R. planticola* bacteria metabolize doxorubicin, through deglycosylation, into the metabolites 7-deoxydoxorubicinol and 7-deoxydoxorubicinolone. The models evaluated demonstrated that *R. planticola* is involved in a decrease of the toxicity caused by doxorubicin. The same group analyzed other bacteria and the results showed that *Klebsiella pneumoniae* and *Escherichia coli* can also degrade doxorubicin. [35]

In conclusion, there are not enough studies that allow the understanding of what really happens when doxorubicin is administered, but some results prove that a crosslink between doxorubicin and microbiota exists, what awakes the interest around this topic. It is important to continue the investigation regarding these subjects in the future to possibly find a way of improving the efficacy of the therapy and to reduce the toxicity effects. [3], [24], [35]

2.1.4. Platinum-based antineoplastic drugs – Oxaliplatin and Cisplatin

The platinum agents as cisplatin, carboplatin and oxaliplatin are antineoplastic drugs that form adducts with DNA leading to the rupture of the molecule. These drugs can cause several adverse effects, but essentially a severe GI tract toxicity, promoting alterations of the barrier's functions. Due to this fact, microorganisms can pass into the mLNs and access the bloodstream, promoting the development of septicemia and inflammation. [3], [36]

It is known that reactive oxygen species (ROS) are responsible for the cytotoxic effects and the DNA damage of this type of drugs. It was expected that the ROS were produced by the tumor cells. However, the reduction of GI tract microbiota in several animal studies revealed a decrease in the synthesis of these chemical species by the tumor-infiltrating myeloid cells, revealing a possible relationship between both. Studies developed with Lewis lung cancer mouse model treated with a platinum agent, cisplatin, and antibiotics (vancomycin, ampicillin and neomycin), responsible for the destruction of the gut microbiota, revealed a superior tumor size than mice administered only with cisplatin. Additionally, the survival rate of the former was inferior than the latter. On the other hand, mice administered with cisplatin and

probiotics containing *Lactobacillus acidophilus* demonstrated a reduced tumor size and an enhanced survival rate. These data suggest that the administration of probiotics containing *Lactobacillus acidophilus,* natural bacteria of the gut, among others, can restore the drug effects by promoting the ROS synthesis by the myeloid cells. The ROS synthesis is made through activation of the myeloid differentiation primary response 88, an adaptor of the PRRs, by the *Lactobacillus acidophilus* MAMPs. Other studies approaching oral administration of probiotics containing *L. acidophilus* and *Bifidobacterium bifidum* to patients receiving cisplatin and radiotherapy showed a decrease in the toxicity effect. [3], [36]

In conclusion, these studies demonstrate that gut microbiota species as *L. acidophilus* can promote the antineoplastic activity of this group of drugs and also decrease the incidence of toxicity effects by regulating the ROS production both in healthy tissues and tumor cells. [3]

2.1.5. Methotrexate

Methotrexate is part of the antimetabolites group of antineoplastic drugs, more specifically the folic acid antagonists, and it is known for causing mucositis by provoking intestinal toxicity. The intestinal toxicity is the major factor impacting the selection of the dose to the treatment. However, the toxicity mechanism is poorly understood. Recent studies revealed that the receptors involved in this toxicity effect are the TLR4, activated by DAMPs or microbial metabolites. [3], [24]

The alteration of the diversity of gut microbiota caused by this drug is related to the intestinal side effects. Recent *in vitro* and *in vivo* studies revealed that, after the administration of methotrexate, the microbiota population suffered a strong alteration. *Bacteroides fragilis* were the bacteria that showed the most drastic reduction. To evaluate the influence of these bacteria on the methotrexate treatment, a probiotic containing *Bacteroides fragilis* was administered to mice. It was possible to verify a reduction of the inflammatory process caused by methotrexate, decreasing the incidence of the intestinal side effects. [24], [37]

On the other hand, different studies revealed the impact of the receptor TLR2 in the methotrexate therapy. TLR2, when activated by the *Lactobacillus reuteri* MAMPs, is responsible for protecting the intestinal mucosa against the adverse effects of this drug. The protection effect occurs due to the increase of the efflux of drugs from gut cells by the stimulation of the ABC transporter multidrug resistance protein 1 - P-glycoprotein 1 transporter. [3], [24], [37]

Overall, it is possible to observe that the gut microbiota has a strong influence in the intestinal mucositis caused by methotrexate and some bacteria can promote a protective effect. It is important to explore these subjects in a deeper way to possibly reduce the adverse

effects of the treatment, allowing the administration of a higher dose and an overall improvement of the therapy. [37]

2.1.6. Cyclophosphamide

Cyclophosphamide is an alkylating agent, more specifically a nitrogen mustard, and it is known to provide immunogenic tumor cell death. The immunogenic tumor cell death starts with an autophagy process that exposes signals that can stimulate the immune system at the surface of the cell, promoting an antitumor immune response by the stimulation of APC, triggering the formation of Th17, Th1 and CD8+ T cells. Moreover, this drug has shown its capability to modulate the composition of the microbiota when administered in mice with tumors. Cyclophosphamide decreases the number of several bacteria but increases the Gram-positive ones, as *Enterococcus hirae,* and it is responsible for disrupting the barrier integrity, promoting the migration of the bacteria into the mLNs. [3], [24]

Several studies approaching animals with tumors demonstrated that the bacteria migration, as *Lactobacillus* spp., caused by cyclophosphamide, promotes the activation and maturation of DCs. Consequently, an increase of the CD8 + T cells, Th17 cells and the memory Th1 cells, and a decrease of the T regs cells occurs which leads to an antitumor adaptive immune response. Mice with absence of Gram-positive bacteria or administered with antibiotics showed a decrease in the pathogenic Th17 cells and a reduction of the efficacy of cyclophosphamide. This situation showed to be reverted in mice without microbiota by the transference of the Th17 cells. These studies reveal that the efficacy of the cyclophosphamide therapy is dependent on the gut microbiota. Moreover, studies involving patients with lung or ovarian cancer receiving chemotherapy demonstrated that the existence of specific Th1 cells of *E. hirae* and *Barnesiella intestinihominis* is related to a more promising prognosis of the pathology, revealing a close relationship between gut microbiota and cancer. [3], [24], [38]

All these studies allow to conclude that the gut microbiota influences the immunogenic cell death mechanism and control the antitumor effect of cyclophosphamide, demonstrating a connection between the microorganisms and the efficacy of the drug. [3], [24], [38]

2.2. Radiotherapy

Radiation is a physical agent used to kill the tumor cells. The therapy is called the ionizing radiation therapy (RTX) and kills cancer cells by placing high-energy radiation in these cells. There are two types of radiation incidence based on the incident radiation: the external beam radiation in which the radiation is emitted by a machine external to the body and the

internal radiation therapy (brachytherapy), where the radioactive material is deposited in the body close to the area that is supposed to be treated. The radiotherapy is genotoxic and acts by causing direct damage to the cells, interfering with the DNA molecule, or by an indirect path, through the production of free radicals, like ROS, after the ionization or excitation of the cell's water. These interactions end up causing the dead of the cell. [39]

The radiation can affect both cancer and normal cells, but in normal cells the repair mechanisms occur in a faster way, and the majority of the cells can return to its normal functions. In contrast, in cancer cells, the repair mechanisms are not that efficient, and the cells end up dying. On the other hand, this therapy can also cause damage to non-irradiated cells, as the bystander effect on neighboring cells and the inflammatory and immunity responses. The principal mechanisms responsible for the damage to the non-irradiated cells are the release of extracellular mediators as ROS, oxide nitric, cytokines and exosomes and of DAMPs. Moreover, the non-irradiated cells can also suffer with the instability of the genome. [3], [39]

The interaction between RTX and microbiota in controlling the efficacy of the drug is not well understood. However, it is known that radiotherapy is extremely complex and has both immunostimulant and immunosuppressive effects and it is expected that the gut microbiota has influence in the immunostimulatory part of this therapy (since it plays this role associated with some chemotherapy drugs – cyclophosphamide as an example). [3]

The microbiota role regarding the toxicity effects of radiotherapy is more explored. This therapy leads to an alteration in the composition of the microbiota which contributes to several adverse effects as mucositis, diarrhea, colitis, among others. A study developed in mouse radiation proctitis model revealed that the gut microbiota composition in mice after irradiation is different. It was noticed an increase of P*roteobacteria* spp. These bacteria synthesize several inflammatory cytokines as interleukin-1b and tumor necrosis factor (TNF), thereby enhancing the susceptibility of the gut mucosal inflammation in these animals. Besides, a modification of the intestinal barrier is also observed. All these alterations can affect the innate and adaptive immunity response. [3], [40]

Additionally, several studies in mice approaching bacteria that activate the TLR2, as *Lactobacillus rhamnosus*, a normal inhabitant of the gut microbiota, revealed that this type of bacteria have a protective effect on the intestinal toxicity caused by chemotherapy or radiotherapy. Moreover, probiotics containing *L. acidophilus, B. bifidum* and *Lactobacillus casei* demonstrated to reduce diarrhea induced by the therapy. Lastly, the effect of probiotics as *Lactobacillus brevis* was also evaluated in a clinical study that concerned patients with neck and

head cancers treated with radiotherapy and chemotherapy. Results revealed that the probiotic reduces the mucositis occurrence. [3], [40]

On the other hand, it was observed that the toxicity of the incident radiation was influenced by the time of the day in which the therapy was performed. It is known that the circadian rhythm influences the apoptosis induced by the radiation. In addition, the circadian rhythm can modulate alterations of the gut microbiota, the synthesis of SCFAs and the immunity response. Thereby, it is expected that some of the variations on the radiotherapy sensitivity are promoted by the alterations of gut microbiota caused by the circadian rhythm. [3]

In conclusion, it is necessary a better understanding and more information regarding the microbiota effect in radiotherapy efficacy and toxicity. Besides, it is important to evaluate more deeply the influence of this therapy in normal cells that suffer with the incidence radiation and in non-irradiated cells. The acquisition of more data and further knowledge in this area will allow to increase the efficacy of the therapeutic and to reduce the adverse effects of the therapy. [3], [39,40]

2.3. Immunotherapy

The research regarding immunotherapy and cancer has increased and evolved in the last years. It is known that the inflammatory cells play a complex role in cancer, and an abnormal innate and adaptive immune response is observed in this pathology. The immune system is responsible for enhancing the carcinogenesis by inducing immunosuppression, since inhibitory cells as the T regs cells and the myeloid-derived suppressor cells (MDSCs) are in a superior number in a tumor environment. Consequently, this aberrant response promotes the proliferation of the carcinogenic cells and enhances the formation of metastasis. [41,42]

It is known that the conventional cancer therapies are not efficient in all the cases since the tumor develops treatment resistance and there is a strong probability of recurrence. Due to these facts, the interest in immunotherapy has enhanced. Recent studies showed that this therapy has revealed potential to treat solid and hematopoietic tumors and to induce positive responses in patients who did not react to the conventional therapies. [3]

The core of immunotherapy regarding cancer are the T cells. These cells play a crucial role in antitumor immunity response what defines the goal of immunotherapy, that is to induce an antitumor effect mediated by the activated T cells. There are several types of immunotherapy including the adoptive T cell transference, CpG-oligodeoxynucleotide (CpG-ODN) intratumor therapy and immune checkpoint inhibitors (ICIs). The efficacy of

immunotherapy is limited in the way that it depends on the immune response of each patient and on the sensibility of each type of tumor. [3], [41]

The influence of gut microbiota in immunotherapy has been explored in the last few years and several new information has emerged. Due to this fact, has been possible to improve the outcome of the treatment by targeting the commensal microorganisms. However, there is a lot more to explore regarding these topics. [3]

2.3.1. Adoptive T cell transfer

The adoptive T cell transfer therapy is based on the isolation and reinfusion of T cells into the patients. This subject has been explored during the last years and several strategies were evaluated. The first approach consisted in the promotion of the *ex-vivo* growth, expansion and reinfusion of autologous lymphocytes with antitumor characteristics. This T cells can be isolated from tumors, the tumor-infiltrating lymphocytes (TIL). On the other hand, other T cells, obtained from the peripheral blood, can be activated to obtain the desired antitumor effects. These cells can be genetically modified to origin the T-cell receptors T cells or chimeric antigen receptors T cells to improve the specificity of the T cells into the tumor antigens. It is important to highlight that the host can be prepared to receive the cells. It is possible to remove immune suppressor cells as T regs and MDSCs, improving the efficacy of the transferred T cells. The last developed approach involved the *in vivo* stimulation of T cells with antibodies, which includes the checkpoint inhibitors, discussed in the following sections. [43,44]

There are a couple of reports approaching the crosslink between microbiota and adoptive T cell transfer. The first one involved adoptive T cells transfer, total body irradiation (TBI), the gut microbiota and antibiotics. This study revealed that the effectiveness of the transferred adoptive T cells after a TBI, in mice, was compromised by the administration of antibiotics. The TBI is used as a preparation for the adoptive T cell transfer and it is responsible for the migration of the gut commensals into the mLNs. The migrated microbiota promotes the proliferation of the transferred T cells and improves their antitumoral activity. These facts demonstrate why the efficacy of adoptive T cell transfer is reduced in the absence of microbiota, when destructed by the antibiotics. Moreover, it is known that the receptor responsible for the gut microbiota activity in this therapy is the TLR4. Mice with a deficiency in the TLR4 did not respond to the therapy while mice with normal TLR4 and administered with an agonist of this receptor, the lipopolysaccharide, demonstrated a better response to the therapy. [3], [45]

Additionally, a clinical study evaluated the adoptive T cells transfer response in patients with metastatic melanoma and the influence of a conditioning regimen, as the myeloablative radiotherapy, in the efficacy of the therapy. The patients revealed an improved response when the previously described regimen was applied. The mechanism described in the previous paragraph can explain the results obtained in this clinical evaluation. [3], [45,46]

In conclusion, it is important to explore in a deeper way the influence of the microbiota in this therapy and the effect of previous radiation as well. The microbiota manipulation can serve to improve the efficacy of the adoptive T cell transfer therapy, as already described, and the exposure to a previous radiation seems to have several positive effects. These evidences are important for the future since immunotherapy is in constant growing. [3], [45,46]

2.3.2. CpG-oligodeoxynucleotide intratumor therapy

The CpG-ODN is an antitumoral drug and functions as an agonist of the TLR9. The CpG-ODNs induce the synthesis of pro-inflammatory cytokines, as IL-12 and TNF, by the myeloid cells. These cytokines are responsible for the transformation of an anti-inflammatory state of the infiltrating tumor macrophages and DCs to a pro-inflammatory state. The pro-inflammatory state is responsible for an adaptive immunity response of antigen specific antitumor T cells that is able to destroy the tumor cells, as described in studies carried out with mice. Besides, the pro-inflammatory cytokines promote a hemorrhagic necrosis. The effect of this drug can be enhanced by avoiding the immunosuppressive and anti-inflammatory activity of IL-10, secreted by immunosuppressive cells as T regs and MDSCs, by concomitant administration of IL-10 receptor antibodies. [3], [47]

The relationship between the CpG-ODNs and the gut microbiota has been evaluated. Studies approaching germ-free mice or mice administered with a mixture of different antibiotics and treated with CpG-ODNs and anti-IL-10R, revealed that this therapy was not efficient in these circumstances and the tumor ended up growing. The absence of microbiota compromised the synthesis of the pro-inflammatory cytokines, by the myeloid cells, in response to the therapy. Moreover, the hemorrhagic necrosis did not occur nor the adaptive specific response, leading to the failure of the treatment. [3], [47]

In addition, it is possible to verify that the response of the myeloid cells to the CpG-ODNs also depends on the TLR4, as in adoptive T cell transfer. Mice without TLR4 reveal a decrease in the responsiveness of the myeloid cells to the therapy. Moreover, germ-free mice with normal levels of TLR4 and administered with lipopolysaccharide, a ligand of the TLR4, and CpG-ODNs, revealed that is possible to revert the response of the myeloid cells to the

treatment. It is possible to conclude that the GI tract microbiota, through activation of the TLR4, stimulates the myeloid cells, as TIL, to be responsive to TLR9, receptor activated by CpG-ODNs, which represents one of the principal connections between gut microbiota and CpG-ODNs therapy. [3], [47]

On the other hand, the production of TNF, by the myeloid cells, can be regulated by the presence of certain microbiota bacteria. In mice, the Gram-negative bacteria, *Alistipes* genus, present normally in the human body, and the Gram-positive, *Ruminococcus*, normal inhabitant of the human gut microbiota, are related to an improvement in the TNF synthesis. Mice were firstly administered with antibiotics and then recolonized with *Alistipes shahii.* The capability of the myeloid cells to synthesize the TNF was reestablished after the introduction of the bacteria. Otherwise, the administration of *Lactobacillus fermentum,* part of the *Lactobacillus* genus, in mice, revealed a decreased production of TNF. The *Lactobacillus* genus which includes species as *Lactobacillus murinum*, *Lactobacillus intestinalis* and *L. fermentum*, part of the constitution of the human gut, influence in a negative way the production of TNF. [3], [24], [47]

Overall, the inexistence of the gut microbiota results in the absence of the stimulation of the myeloid cells for responding to the CpG-ODNs. However, different bacteria can origin different effects on the efficacy of the therapy, as previously described. In conclusion, the response of the host can be regulated by the manipulation of the gut microbiota. It is possible to modify the composition of the microbiota or change the ratios of species by administration of prebiotics (food not digestible that endorse the growth of helpful gut microorganisms), probiotics and antibiotics, for example, and control the therapeutic efficacy. [3], [24], [47]

2.3.3. Immune checkpoint inhibitors

It is known that the immune system is able to recognize the cancer cells, but their destruction is compromised, several times, due to the escape of the cancer cells from the immune system. Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), expressed by T cells, is a CD28 homologous that links to CD80 and CD86 receptors with a much stronger affinity than CD28. The CTL-4, when linked to its receptor, is responsible for preventing the T cell activation (T regs and T effector cells), working as a negative immune regulation, at an initial stage, inside the lymph nodes. A class of immune checkpoint inhibitors are composed of CTLA-4 inhibitors, that by decreasing the inhibitory signs, promote the recognition and destruction of the cancer cells by the immune system. [21], [48]

On the other hand, cancer cells are responsible for the overproduction of proteins as the Programmed death-ligand 1 (PD-L1). The PD-L1 proteins bind to the Programmed cell death protein 1 (PD-1), leading to the inactivation and blockage of proliferation of the immune system cells, as T cells, putting them to *sleep*. The PD-1 regulates T-cell activation at a later stage, blocking the effector phase. Another group of ICIs are the PD-1 and PD-L1 inhibitors that are also important to promote the dead of the tumor cells. The figure 4 demonstrates the phenomena described earlier. [48]

The ICIs have been evaluated in several studies approaching animals and also in clinical trials. They revealed a strong anticarcinogenic activity in patients with several types of cancer, but in some cases, the response was not clear which makes the variability of the response one of the biggest concerns of this therapy. Moreover, the immune checkpoint inhibitors are responsible for several adverse effects as colitis and inflammation of the hypophysis, specially by the CTL-4 antibodies, and pneumonitis and thyroid impairment, particularly by the ones that interfere with the PD-L1 and PD-1 connection. [3]

The effect of the gut microbiota in modulating the response and efficacy of the immune checkpoint inhibitors in cancer therapy has been recently reported and will be described in the following sections.

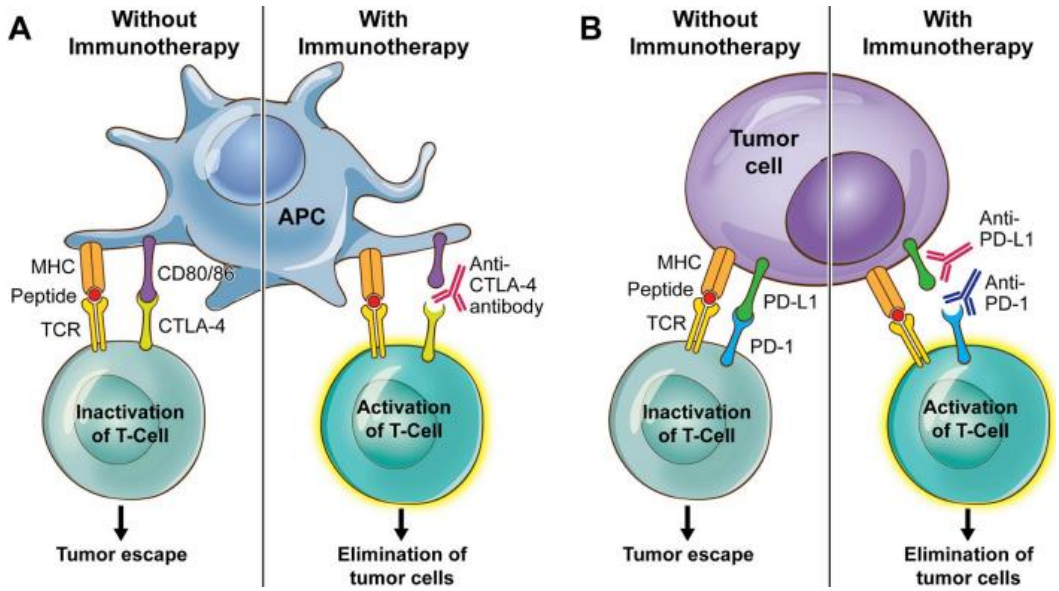

Figure 4 - Role of CTLA-4 (A) and PD-1(B) in the immunity response and regulation by the immune checkpoint inhibitors. – Adapted from [21].

2.3.3.1. Anti-CTLA4 monoclonal antibodies

The influence of gut microbiota in the anti-CTLA4 therapy has been explored in recent years and a couple of reports emerged. Several studies demonstrated that the presence of microbiota is required to the antitumoral effect of anti-CTLA4. Studies developed with germ-

free mice or mice administered with antibiotics revealed that the anti-CTLA4 therapy had a poor effect on subcutaneous tumors of these animals, which is possibly associated with the lack of microbiota. Moreover, one of the adverse effects of this group of drugs, colitis, is a consequence of the damage caused at the ileum and colon and it is related to an alteration of the gut microbiota composition. [3], [16], [49]

Mice with tumors treated with anti-CTLA4 showed a decrease in the bacteria of orders Bacteroidales (including *Bacteroides thetaiotaomicron* and *Bacteroides uniformis*) and Burkholderiales (as *Burkholderia cepacian*). However, the bacteria of ordo Clostridiales increased in these cases. The *Bacteroides fragilis*, bacteria responsible for regulating the immune system response, revealed regular and stable values in the evaluated mice. When the administration of *B. thetaiotaomicron* or *B. fragilis* was tested in mice without microbiota, a recovering of the therapeutic effect of the anti-CTLA4 was observed. This occurred due to the maturation of DCs and the trigger of a CD4+ T cells response, specially the Th1 cells, induced by the bacteria. On the other hand, the oral administration of a combined therapy of *B. fragilis* and *B. cepacia*, not only restored the therapeutic effect of anti-CTLA4 but also decreased the colitis incidence. [3], [16], [24]

Moreover, several studies with mice evaluated the effect of antibiotics in the therapeutic effect of anti-CTLA4. The antibiotics modify the commensal's composition causing an impact on the effect of the drug. For example, mice administrated with vancomycin, responsible for the reduction of the Gram-positive bacteria and conservation of the Gram-negative ones, as Bacteroidales and Burkholderiales, revealed a better response to the anti-CTLA4 therapy. [3], [16], [24]

The gut microbiota can also be related to the toxicity effects of ICIs, as already mentioned. Clinical trials with patients administered with anti-CTLA4 revealed that the higher abundance of bacteria of the order Bacteroidales is related to a reduction of the colitis incidence, similar to what was already described with mice analysis. On the other hand, the lack of bacteria involved in polyamine transport and vitamin B biosynthesis was related with a much higher risk of the occurrence of the adverse event. Other recent studies revealed that patients with higher abundance of *Faecalibacterium prausnitzii* and low quantity of Bacteroidales showed a higher incidence of colitis. [3], [16], [24]

Additionally, a recent report described the effect of fecal microbiota transfer (FMT), a topic that is going to be approached in the following sections, to germ-free mice. FMT of patients with melanoma treated with anti-CTLA4 was made into germ-free mice. The first conclusion of this study was that the microbiota composition of the patients changed with the administration of anti-CTLA4. Each patient had a different gut microbiota, and only some of

them allowed the colonization by *B. thetaiotaomicron* or *B. fragilis* on mice. These patients demonstrated to have a microbiota composition more favorable for the treatment. Consequently, as already described in mice tests, the colonization by these bacteria enhanced the efficacy of this class of drugs. [3], [16]

Overall, the therapy itself can change the composition of the gut microbiota, but in some circumstances this modification can enhance the therapeutic effect of anti-CTLA4. Otherwise, in situations where the treatment with anti-CTLA4 alters the composition of the gut microbiota in a negative way, its manipulation can change the course of the therapy. [3], [16]

2.3.3.2. Anti-PDL1 monoclonal antibodies

The relationship between anti-PDL1 drugs and gut microbiota has been evaluated in the last years and several conclusions can be withdrawn from a couple of reports. In contrast to anti-CTLA4, the anti-PDL1 therapy does not promote intestinal toxicity nor seems to have an extreme dependence of the existence of gut microbiota, however this last statement is doubtful since the publication of recent reports. [3]

A study developed with two groups of mice showed that these two groups, that came from different facilities, composed of different commensal groups, exhibited different rates of tumor growth. The anti-PDL1 drug revealed an antitumoral effect in both groups, but one of them demonstrated faster tumor growth. This was correlated with lower tumor infiltration of CD8+ T cells, inducing a weaker immunity response, not totally avoiding the tumor progression. Then, several analyses were developed to understand if this effect was related to the microbiota differences. The two groups of mice were cohoused before the implantation of the tumor. This process eliminated the differences in the tumor progression suggesting that an environmental factor was responsible for the differences. Mice with the unfavorable microbiota were possibly colonized by the microorganisms responsible for the improved antitumoral response. Other studies were performed and all the data suggested that the gut microbiota was able to modulate the antitumoral immunity and the response to the therapy. [16], [24], [50]

Additionally, the crosslink between the improved effect of anti-PDL1 and specific gut microbiota bacteria was evaluated. The responsiveness to the therapy in mice transplanted with subcutaneous melanoma seemed to be related to the abundance in the GI tract microbiota of *Bifidobacterium* bacteria, as *Bifidobacterium breve, Bifidobacterium longum* and *Bifidobacterium adolescentis.* The administration of a probiotic containing a mixture of *Bifidobacterium* spp. bacteria, by itself or combined with anti-PDL1, revealed an improvement

of the antitumor immune response caused by an increase of the CD8+ T cells. However, the effect of the administration of *Bifidobacterium* spp. was revoked in mice without CD8+ T cells. Mice without CD8+ T cells showed that the effect of the *Bifidobacterium* spp. in these cases was not observed, revealing that the bacteria effect is dependent on the CD8+ T cells. These facts revealed that the PDL-1 therapy improves the antitumor immune response and the mechanism responsible for that does not involve a specific microbiota response but instead an antitumoral T cell response. [3], [24], [50]

In conclusion, these studies do not allow the complete understanding of the relationship between the gut microbiota and the anti-PDL1 treatment. The mechanism that contributes to an improvement in the antitumor immune response regarding anti-PDL1 treatment seems to not be specifically associated with gut microbiota. Nevertheless, the response to the anti-PDL1 therapy can be improved in animals which had a previously antitumoral immune response, especially when *Bifidobacterium* spp. is abundant in the intestinal microbiota. [3], [50]

Several uncertainties still emerge when this subject is approached, therefore it continues to be explored. The administration of antibiotics was evaluated in mice with melanoma and fibrosarcoma treated with PD-1 monoclonal antibodies or with a combination of the former and CTLA-4 monoclonal antibodies. The antibiotics demonstrated to decrease the clinical efficacy of ICIs therapy and the survival of mice with advanced cancer. The effect of the antibiotic's administration was also evaluated in patients with non-small cell lung cancer and renal cell carcinoma treated with PD-1/PD-L1 monoclonal antibodies. The results revealed that the progression-free survival and overall survival were inferior in the group of patients receiving the antibiotics. In this context, since antibiotics can alter the gut microbiota composition, dysbiosis can possibly influence the efficacy of ICIs. Additionally, in situations where the patients do not respond to ICIs therapy, one of the reasons for that to happen may be related to dysbiosis. [16], [51]

Additionally, the FMT was also explored. FMT from patients responsive to the therapeutic into germ-free mice or mice administered with antibiotics improved the efficacy of PD-1 antibodies. Conversely, the FMT from patients who did not respond to the therapy into mice revealed the opposite effect. [51]

Lastly, the analysis of the patient's stool samples demonstrated a connection between the efficacy of ICIs and high amounts of *A. muciniphila*, one of the most abundant bacteria on the ileum, in the gut microbiota. A clinical trial confirmed that these bacteria were increased in patients with the best clinical response. At the diagnosis this number was already high and ended up being beneficial to the treatment. Moreover, oral administration of probiotics

containing *A. muciniphila* and *Enterococcus hirae* to mice who received the FMT from non-responsive patents, reverted the efficacy of PD-1 antibodies by increasing the secretion of IL-12 by DCs and promoting the recruitment of CD4+ T cells (T cells expressing small intestine-associated chemokine receptor CCR9 and/or the T helper 1-associated chemokine receptor CXCR3 - *A. muciniphila*) into the tumor environment. Besides, the administration of *A. muciniphila* also affects the T regs cells promoting a decrease of this population. [16], [51]

Other recent study investigated the stool samples from metastatic melanoma patients. Through analysis of the data was possible to verify a significant association between the gut microbiota composition and the response to the therapy (anti-PD1). The most common bacteria in the responsive patients were *Bifidobacterium longum, Collinsella aerofaciens*, and *Enterococcus faecium*. Moreover, an FMT was also made from responsive patients to germ-free mice. The FMT allowed an improvement of DCs activation and an increment of the T cell (Th1) response, enhancing the therapeutic effect of anti-PDL1 in mice. Overall, the results suggest that the gut microbiota can possibly influence the antitumor immune response. [16], [52]

In conclusion, all the data showed that the composition of the gut microbiota influences the efficacy of PD-1 monoclonal antibodies and the cancer immunity response. However, several uncertainties are still present and unresolved as the mechanism by which the *A. muciniphila* modulates the immune system, that remains unclear. Nevertheless, the manipulation of the gut commensal microorganisms to avoid the resistance to ICIs becomes more possible over the years and will be useful to solve difficulties associated with the heterogeneity of the response, sometimes associated with alterations of the microbiota's composition. [16], [51]

3. Fecal Microbiota Transfer (FMT)

The majority of reports regarding microbiota describe results obtained from the analysis of the stool samples from patients, before and after the therapy, including the PD-1 immune therapy. The analysis of bacteria requires several complex tools, and the process by itself is considered as a hard one. There are several groups evaluating the stools and trying to figure out the gut bacteria identities. However, the bacteria from responsive patients vary from study to study. This occurs probably due to differences in the analysis process, including the manner of how the samples are collected and examined. Additionally, this may be related to the difficult in understanding whether the responsible for the result is a single species or a group of bacteria. [48]

There are several ways of controlling and targeting the gut microbiota including diet, probiotics, prebiotics, antibiotics, and fecal microbiota transfer. FMT is used to re-establish the intestinal microbiota diversity and it is considered the most innovative and extreme method due to its high capability of interfering with the gut composition. The fecal transplantation is known for being effective in the treatment of several pathological situations as fatal infections by *Clostridium difficile* and has been recently explored as an adjuvant in cancer treatments. Several recent preclinical studies explored the FMT from responsive patients to mice with tumors and the results suggested that a certain component of the stools is contributing to a better response to the cancer therapy, possibly the gut microbiota. It is suggested that the equilibrium of the gut microbiota may be related to a reduction of the tumors. A more recent approach, a clinical trial, involves the FMT from responsive patients into the non-responsive ones, to evaluate if the effect is similar to what occurs in mice. [48], [53]

In recent years, several clinical trials are evaluating the effect of FMT in the treatment of several diseases. However, the clinical trials regarding FMT and cancer are in a reduced number. The first clinical trial approaching FMT and cancer is being performed, currently in the recruiting phase, and involve the transfer of *bacterial cocktails* derived from the stools of responsive melanoma patients to anti-PD1 therapy, into non-responsive ones. FMT process will start with the collection of fecal matter or stool from a donor. Then, the material will be combined with a saline or other solution and strained. Formerly, the selected patients whose tumors did not respond to anti-PD1 immunotherapy will suffer a biopsy of the tumor. Lastly, the administration of the bacteria will be made by two different approaches: via colonoscopy and by oral administration of capsules containing the bacteria. These capsules can be administered as the standard drug-containing capsules by the standard method. The stool capsules are a well-known method of FMT that has been explored and used for several years. Afterwards, several treatments with an anti-PD1 drug, pembrolizumab, will be carried out. The response of the patients will be constantly evaluated. The responsive patients will continue the treatment of the anti-PD1 drug for two years. [22], [48], [54]

Another clinical trial is being performed regarding these topics, also currently in the recruiting phase, and will evaluate the FMT effect in treating the adverse effects, diarrhea and colitis, caused by ICIs treatment in patients with genitourinary cancer. [48], [55]

All these trials are not completed yet, but at least, will be useful to understand more of the science behind the microbiota and immunotherapy and will advance new correlations between cancer, gut microbiota and response to immunotherapy. [48], [55]

4. Conclusion and Future perspectives

The microbiome plays a complex role in several physiological functions, as inflammation and immunity and some of the mechanisms by which it affects the host have been described. However, there is a lot more to know and discover regarding these topics, since several mechanisms are not described yet. Nevertheless, microbiota, especially gut microbiota, has a strong influence on several diseases, being cancer one of them. Gut microbiota is able to control the cancer initiation, progression and dissemination and regulates the response to the therapy, chemotherapy, radiotherapy and immunotherapy, and toxicity effects as well.

The majority of the studies that allowed the identification of bacteria in each process involve animals, essentially mice and rats, but the translation of the new discoveries and conclusions to the human organism remains a challenge. There are several tumor animal models but the majority of them fail to truly reproduce what really occurs in a human carcinogenic process. [3] Moreover, the composition of the animal's microbiota varies, and animals obtained from different facilities reveal significant differences leading to a huge influence in physiology and pathological processes. Due to these facts, the correlation with the human organism becomes a hard process. Fortunately, in recent years, the clinical trials approaching these topics have increased, promoting a strong scientific advance.

There are several clinical trials taking place at the time that will be useful to understand in a deeper way the science behind the microbiota and pathologies. As soon as the ideal composition of microbiota for each treatment has been unraveled, the next stage is to understand how the human microbiome can be altered. [3] A recent approach for the modification of the composition in cancer patients involves FMT and a couple of clinical trials are evaluating its feasibility and its effects on the therapy and adverse events. Additionally, the administration of prebiotics, probiotics and antibiotics can also regulate the microbiota's composition. For example, administration of *Bifidobacterium* spp. and *Lactobacillus* spp. is related, in several cases, with a decrease in the adverse effects of different cancer therapies. An overview of some of the effects of microbiome in several cancer therapies, approached in the present work, is represented in the appendix A.

In the future, the goal is to use the microbiome as a diagnostic and treatment tool. [3] However, the most important aim is to determine bacterial species that can control the therapy in a way that allows the reduction of the adverse effects and that improves the efficacy of the therapy. Microbiome is considered a promising strategy in cancer treatment and the key goal is to improve the life quality of the patients, always considered the main goal of the therapy.

Bibliography

1. BELKAID, Y.; NAIK, S. - **Compartmentalized and systemic control of tissue immunity by commensals.** Nature Immunology. ISSN 15292908. 14:7 (2013) p. 646–653.

2. NOECKER, C. *et al.* - **High-resolution characterization of the human microbiome.** Translational Research. ISSN 18781810. 179:7 (2016) p. 7–23.

3. ROY, S.; TRINCHIERI, G. - **Microbiota: A key orchestrator of cancer therapy.** Nature Reviews Cancer. ISSN 14741768. 17:5 (2017) p. 271–285.

4. BELKAID, Y.; HARRISON, O. J. - **Homeostatic Immunity and the Microbiota.** Immunity. ISSN 10974180. 46:4 (2017) p. 562–576.

5. HUPP, R. J.; FERNEINI, E. M. - **Head, Neck and Orofacial Infections: Bacteriology of the Head and Neck Regions.** 1ª Edição. Elsevier Science, 2016. ISBN 9780323289450.

6. CHO, I.; BLASER M. J. - **The Human Microbiome: at the interface of health and disease.** Nat Rev Genet. 13:4 (2012) p. 2-28.

7. BELIZÁRIO, J. E.; NAPOLITANO, M. - **Human microbiomes and their roles in dysbiosis, common diseases, and novel therapeutic approaches.** Frontiers in Microbiology. ISSN 1664302X. 6:10 (2015) p. 1–16.

8. SILVESTRE, R.; TORRADO, E. - **Metabolic Interaction in Infection: Microbiome and Gut Dysbiosis.** 1ª edição. Springer, 2018. ISBN 9783319749327.

9. COYTE, K. Z.; SCHLUTER, J.; FOSTER, K. R. - **The ecology of the microbiome: Networks, competition, and stability.** Science. ISSN 10959203. 350:6261 (2015) p. 663–666.

10. MOYA, A.; FERRER, M. - **Functional Redundancy-Induced Stability of Gut Microbiota Subjected to Disturbance.** Trends in Microbiology. ISSN 18784380. 24:5 (2016) p. 402–413.

11. ELOE-FADROSH, E. A.; RASKO, D. A. - **The Human Microbiome: From Symbiosis to Pathogenesis.** Annual Review of Medicine. ISSN 0066-4219. 64:1 (2013) p. 145–163.

12. FLOCH, H. M.; RINGEL, Y.; WALKER, W. A. - **The Microbiota in Gastrointestinal Pathophysiology: The Upper Gastrointestinal Tract-Esophagus and Stomach**. 1ª edição. Academic Press, 2017. ISBN 9780128040249.

13. PARFREY, L. W.; KNIGHT, R. - **Spatial and temporal variability of the human microbiota.** Clinical Microbiology and Infection. ISSN 14690691. 18:4 (2012) p. 5–7.

14. CANI, P. D. - **Human gut microbiome: Hopes, threats and promises.** Gut BMJ.

ISSN 14683288. 67:9 (2018) p. 1716–1725.

15. RAFFEL, T.R.; MARTIN, L. B.; ROHR, J. R. - **Parasites as predators: unifying natural enemy ecology.** Trends in Ecology and Evolution. ISSN 01695347. 23:11 (2008) p. 610–618.

16. LI, W. *et al.* - **Gut microbiome and cancer immunotherapy.** Cancer Letters. ISSN 18727980. 447: 12 (2019) p. 41–47.

17. GOPALAKRISHNAN, V. *et al.* - **The Influence of the Gut Microbiome on Cancer, Immunity, and Cancer Immunotherapy.** Cancer Cell. ISSN 18783686. 33:4 (2018) p. 570–580.

18. WU, D. *et al.* - **How do cancer cells grow and spread?** Pharmaceuticals. ISSN 14248247. 3:1 (2014) p. 1–4.

19. ROOKS, M. G.; GARRETT, W. S. - **Gut microbiota, metabolites and host immunity.** Nature Reviews Immunology. ISSN 14741741. 16:6 (2016) p. 341–352.

20. TAKEUCHI, O.; AKIRA, S. - **Pattern Recognition Receptors and Inflammation.** Cell. ISSN 00928674. 140:6 (2010) p. 805–820.

21. SOULARUE, E. *et al.* - **Enterocolitis due to immune checkpoint inhibitors: A systematic review.** Gut BMJ. ISSN 14683288. 67:11 (2018) p. 2056–2067.

22. POUTAHIDIS, T. *et al.* - **Pathogenic Intestinal Bacteria Enhance Prostate Cancer Development via Systemic Activation of Immune Cells in Mice.** PLoS ONE. ISSN 19326203. 8:8 (2013) p. 1–9.

23. FAN, X. *et al.* - **Human oral microbiome and prospective risk for pancreatic cancer: A population-based nested case-control study.** Gut BMJ. ISSN 14683288. 67:1 (2018) p. 120–127.

24. ALEXANDER, J. L. *et al.* - **Gut microbiota modulation of chemotherapy efficacy and toxicity.** Nature Reviews Gastroenterology and Hepatology. ISSN 17595053. 14:6 (2017) p. 356–365.

25. SHALOAM, D.; TCHOUNWOU, P. B. - **Cisplatin in cancer therapy: Molecular mechanisms of action.** European Journal of Pharmacology. 740 (2014) p. 364–378.

26. FRERES, P.; JERUSALEM, G.; MOONEN, M. - **Anticancer Treatments and Cardiotoxocity: Categories of Anticancer Treatments. 1ª edição.** Academic Press, 2016. ISBN 9780128025093.

27. MCQUEEN, A. C. *et al.* - **Biomedical Sciences: Antimetabolites.** 1ª edição. Elsevier Science, 2019. ISBN 978-0-12-801238-3.

28. BERTINO, J. - **Encyclopedia of Cancer: Alkylating Agents.** 2ª edição. Elsevier Science, 2002. ISBN 9780080547886.

29. HENA, S.; ZNAD, H. - **Fundamentals of Quorum Sensing, Analytical Methods and Applications in Membrane Bioreactors: Membrane Bioreactor for Pharmaceuticals and Personal Care Products Removal From Wastewater.** 1ª edição. Elvesier Science, 2019. ISBN 9780444640642.

30. MATSON, D. R.; STUKENBERG, P. T. - **Spindle poisons and cell fate: A tale of two pathways.** Molecular Interventions. ISSN 15340384. 11:2 (2011) p. 141–150.

31. YONG, W. P.; SOO, R.; INNOCENTI, F. - **Cancer Drug Design and Discovery: Pharmacogenomics and Personalized Medicines in Cancer Treatment.** 2ª edição. Academic Press, 2014. ISBN 9780123965219.

32. STRINGER, A. M. *et al.* - **Faecal microflora and β-glucuronidase expression are altered in an irinotecan-induced diarrhoea model in rats.** Cancer Biology and Therapy. ISSN 15558576. 7:12 (2008) p. 1919–1925.

33. MEGO, M. *et al.* - **Prevention of irinotecan induced diarrhea by probiotics: A randomized double blind, placebo controlled pilot study.** Complementary Therapies in Medicine. ISSN 18736963. 23:3 (2015) p. 356–362.

34. GELLER, L. T. *et al.* - **Potential role of intratumor bacteria in mediating tumor resistance to the chemotherapeutic drug gemcitabine.** RESEARCH Cancer. 357:1160 (2017) p. 1156–1160.

35. YAN, A. *et al.* - **Transformation of the Anticancer Drug Doxorubicin in the Human Gut Microbiome.** ACS Infectious Diseases. ISSN 23738227. 4:1 (2018) p. 68–76.

36. GUI, Q. F. *et al.* - **Well-balanced commensal microbiota contributes to anti-cancer response in a lung cancer mouse model.** Genetics and Molecular Research. ISSN 16765680. 14:2 (2015) p. 5642–5651.

37. ZHOU, B. *et al.* - **Induction and Amelioration of Methotrexate-Induced Gastrointestinal Toxicity are Related to Immune Response and Gut Microbiota.** EBioMedicine. ISSN 23523964. 33 (2018) p. 122–133.

38. DAILLÈRE, R. *et al.* - **Enterococcus hirae and Barnesiella intestinihominis Facilitate Cyclophosphamide-Induced Therapeutic Immunomodulatory Effects.** Immunity. ISSN 10974180. 45:4 (2016) p. 931–943.

39. BASKAR, R. *et al.* - **Biological response of cancer cells to radiation treatment.** Frontiers in Molecular Biosciences. ISSN 2296889X. 1:11 (2014) p. 1–9.

40. ALEXANDER, J. L.; KOHOUTOVA, D.; POWELL, N. - **Science in Focus: The Microbiome and Cancer Therapy.** Clinical Oncology. ISSN 14332981. 31:1 (2019) p. 1–4.

41. VAN DEN BULK, J.; VERDEGAAL, E. M. E.; MIRANDA, N. F. - **Cancer**

immunotherapy: broadening the scope of targetable tumours. Open Biology. ISSN 20462441. 8:6 (2018) p. 1–10.

42. GONZALEZ, H.; HAGERLING, C.; WERB, Z. - **Roles of the immune system in cancer: From tumor initiation to metastatic progression.** Genes and Development. ISSN 15495477. 32:19–20 (2018) p. 1267–1284.

43. KALOS, M.; JUNE, C. H. - **Adoptive T Cell Transfer for Cancer Immunotherapy in the Era of Synthetic Biology.** Immunity. ISSN 10747613. 39:1 (2013) p. 49–60.

44. HOUOT, R. *et al.* - **T-cell-based immunotherapy: Adoptive cell transfer and checkpoint inhibition.** Cancer Immunology Research. ISSN 23266074. 3:10 (2015) p. 1115–1122.

45. PAULOS, C. M. *et al.* - Microbial translocation augments the function of adoptively transferred self / tumor -specific CD8+ T cells via TLR4 signaling. The Journal of Clinical Investigation. 117:8 (2007) p. 2197–2204.

46. DUDLEY, M. E. *et al.* - **Adoptive cell therapy for patients with metastatic melanoma: Evaluation of intensive myeloablative chemoradiation preparative regimens.** Journal of Clinical Oncology. ISSN 0732183X. 26:32 (2008) p. 5233–5239.

47. IIDA, N. *et al.* - **Commensal bacteria control cancer response to therapy by modulating the tumor microenvironment.** Science. ISSN 10959203. 342:6161 (2013) p. 967–970.

48. THE CONVERSATION - **Could your gut microbes hinder your cancer treatment ? A new first-in-human trial investigates**. [Consultado em 7 de Agosto de 2019]. Disponível na internet: https://theconversation.com/could-your-gut-microbes-hinder-yourcancertreatment-a-new-first-in-human-trial-investigates-99728

49. VÉTIZOU, M. *et al.* - **Anticancer immunotherapy by CTLA-4 blockade relies on the gut microbiota.** Science. ISSN 10959203. 350:6264 (2015) p. 1079–1084.

50. SIVAN, A. *et al.* - **Commensal Bifidobacterium promotes antitumor immunity and facilitates anti-PD-L1 efficacy.** Science. ISSN 10959203. 350:6264 (2015) p. 1084–1089.

51. ROUTY, B. *et al.* - **Gut microbiome influences efficacy of PD-1-based immunotherapy against epithelial tumors.** Science. ISSN 1095-9203. 359:6371 (2018) p. 91–97.

52. ZHA, Y. *et al.* - **The commensal microbiome is associated with anti–PD-1 efficacy in metastatic melanoma patients.** Science. ISSN 0036-8075. 359:6371 (2018) p. 104–108.

53. CHEN, D. *et al.* - **Fecal microbiota transplantation in cancer management: Current status and perspectives.** International Journal of Cancer. ISSN 10970215. 2031 (2019) p. 2021–2031.

54. ClinicalTrials.gov - **Fecal Microbiota Transplantation (FMT) in Metastic Melanoma Patients Who Failed Immunotherapy**. [Consultado em 8 de Agosto de 2019]. Disponível na internet: https://clinicaltrials.gov/ct2/show/NCT03353402?term=sheba+medical+center&cond=Cancer&draw=4&rank=201

55. ClinicalTrials.gov – **Fecal Microbiota Transplantation in Treating Immune-Checkpoint Inhibitor Induced-Diarrhea or Colitis in Genitourinary Cancer Patients**. [Consultado em 8 de Agosto de 2019]. Disponível na internet: https://clinicaltrials.gov/ct2/show/NCT04038619?term=fecal&cond=Cancer&draw=2&rank=33

Appendices

A – Effects of the gut microbiota in several cancer treatments

Table 1- Effects of the gut microbiota in several cancer treatments. Adapted from [24].

Mechanism	Chemotherapy	Bacteria	Effect
Translocation	Cyclophosphamide, doxorubicin	Gram-positive microorganisms (*Lactobacillus johnsonii*, *L. murinus* and *Enterococcus hirae*)	Commensal bacteria cross the intestinal barrier to enter secondary lymphoid organs[26,27]
Immunomodulation	Cyclophosphamide	*Lactobacillus*, segmented filamentous bacteria	Gram-positive commensals mediate accumulation of T_H17 and T_H1-cell response[26]
	CpG oligodeoxynucleotides	*Ruminococcus*, *Alistipes*	Priming of tumour-associated myeloid inflammatory responses[28]
	Oxaliplatin	Not known	Mediated by TLR4 and reactive oxygen species production by myeloid cells[28]
	Anti-PD-L1	*Bifidobacterium*	Tumour-specific T-cell induction and increased T cells in tumour microenvironment[31]
	Methotrexate	Not known	Gut microbes regulate against chemotherapy-induced small bowel injury via TLR2 signalling and drug transporter p-gp[36]
Metabolism			
Enzymatic degradation			
	CPT-11 (Irinotecan)	β-glucuronidase-expressing gut bacteria	Bacterial β-glucuronidase cleaves glucuronide from inactive metabolite, releasing active metabolite (SN-38) in the gut[45]
Reduced diversity and ecological network function			
	Methotrexate	Anaerobes, streptococci, *Bacteroides*	Reduced diversity and shifts in relative abundance associated with chemotherapy-induced diarrhoea[55]

Part II

Report of the Internship in Community Pharmacy

Luciano & Matos Pharmacy

List of Abbreviations

INN - International Nonproprietary Name

MICF - Master's degree in Pharmaceutical Sciences

OOS - Out-of-stock

OTC - Over-the-counter

SWOT - Strengths, Weaknesses, Opportunities and Threats

1. Introduction

The Master's degree in Pharmaceutical Sciences (MICF), by the Faculty of Pharmacy from the University of Coimbra, requires the execution of a Curricular Internship in a Community Pharmacy. This last stage before the conclusion of the Pharmaceutical degree allows a first contact between the students and the real work world and it is an important experience to acquire and improve several technical and soft skills.

The Community Pharmacy is an extremely important sector of the Pharmaceutical area. The Community Pharmacy is considered as the principal professional opportunity for a Pharmacist since it is the area that includes the majority of the professionals. Moreover, a Community Pharmacist plays a crucial role in society. The Pharmacist, in the majority of times, represents the first contact between the patients and health institutions, since it is recognized as a reliable professional able to clarify uncertainties and solve difficulties regarding the health subjects. Furthermore, the Pharmacist can play a role in the latter stage of the health cycle, promoting the correct and rational use of the drug products before the initiation of the therapeutic plan. Therefore, the Pharmacist owns a crucial and responsible position in society regarding the health subjects and it is considered as a health public agent, ensuring the health and well-being of the patients.

In conclusion, an internship approaching this sector is considered of extreme importance. It allows the consolidation of the knowledge obtained through the Pharmaceutical science degree and, on the other hand, allows the following of the quotidian of a Community Pharmacist. The present report intends to describe the work developed during this internship performed at the Luciano & Matos Pharmacy under the guidance of the Pharmacist Maria Helena Amado.

2. Luciano & Matos Pharmacy

The Luciano & Matos Pharmacy is placed at Praça 8 de Maio, in Rua da Sofia, in Coimbra. Its activity started in 1929 and in 1995 it was acquired by the current owner, Technical Director and Pharmacist Maria Helena Amado. In 2009, the Pharmacy joined the Holon Group, a group of independent and autonomous Pharmacies that share the same values, brand and image. The next figure represents the Luciano & Matos Pharmacy facilities.

Figure 1 - Facilities of the Luciano & Matos Pharmacy. Adapted from https://core.ac.uk/download/pdf/151539292.pdf.

The Luciano & Matos Pharmacy is recognized as one of the most rigorous Pharmacies of Coimbra and surroundings and its prestige is noticed through all the country. This Pharmacy has developed an excellence work through all the years and its activity is always based on the maximum quality services focusing on the well-being of the patient. The Luciano & Matos Pharmacy is known for its excellence and differentiated counselling to the patients, for the high-quality production of compounded drugs, among others. In conclusion, Luciano & Matos Pharmacy is recognized as one of the best Pharmacies in the area so an internship in this place was considered a great opportunity.

3. SWOT analysis

The evaluation of this internship was performed with an analysis of the Strengths, Weaknesses, Opportunities and Threats (SWOT) of the internship. This tool was created in the nineteen-sixties and it is essentially associated with the business world as a comparative method between one company and its competition. Moreover, this analysis can be used for individual purposes to evaluate a specific person's situation. [1]

SWOT tool consists in a subjective analysis that takes into consideration the individual's opinion and has both internal and external reflections. Strengths and Weaknesses are part of the internal analysis, while Opportunities and Threats belong to the external one. The following SWOT analysis represents a critical point of view of the internship developed at Luciano & Matos Pharmacy. [1] The overview of this SWOT analysis is represented in the appendix A.

3.1. Strengths

3.1.1. Pharmacy's location and diversity of the patients

The Luciano & Matos Pharmacy is located in the heart of Coimbra's downtown. This area is surrounded by a noticeable heterogeneity of the population. Firstly, it is a very touristic zone, frequented by people from all around the world. Additionally, being this Pharmacy one of the oldest and most prestigious, patients from the periphery of the city use public transports, common in the area, to dislocate themselves to the Pharmacy. Furthermore, the inhabitants of the urban zone also frequent this pharmacy, including the ones with more economic difficulties, such as drug addicts, beggars, among others. Moreover, the Pharmacy is located near to health institutions, covering an even wider range of patients.

The heterogeneity of the population allowed the development of several technical and soft skills. The majority of the Pharmacy's patients are elderly people, and most of them are taking prescription drugs for administration of chronic medication. This allowed an improvement of the knowledge regarding these drug products, including names, brands, specifications and precautions associated with each product.

On the other hand, the contact with tourists promoted an improvement of the English skills and allowed the contact with a couple of new products, especially over-the-counter drugs (OTCs), as cosmetics, food supplements and herbal medicine products. This provided an enhancement in the counselling of these drugs, promoting the gain of experience regarding these classes of products.

Lastly, the contact with socially disadvantage groups of the population allowed the development of other specific interpersonal relationships. The Luciano & Matos Pharmacy participates in the "Kit Troca Seringas" program, that consists in the exchange of used syringes for new ones in order to avoid the spread of infections caused by HIV and Hepatitis viruses. This campaign attracts the disadvantage groups of the population into the Pharmacy. In these cases, it was necessary to adapt the communication and counselling, to the literacy of the patients and to their economic situations, always taking into consideration the equality in the access of the drug products.

In conclusion, the heterogeneity of the population contributed to the contact with several different situations and circumstances and required specific approaches for each condition, allowing the development of several technical and soft skills and, overall, promoting a better preparation for the world of work.

3.1.2. Preparation, guidance and diversity regarding the executed tasks

As soon as the internship at Luciano & Matos Pharmacy started, it was possible to realize that everything regarding this experience had been previously thought and that the plan for the internship was clear since the beginning. Every task was preceded by a brief explanation of what needed to be done and the initial times were always carried out with help and guidance of the Pharmacy's team.

The first stage of this internship consisted in the organization and storage of the health products, as well as the order's intake. This area of the Community Pharmacy is extremely important, since it has a huge control in the Pharmacy economics and can affect its profit and finances. The next stage of the internship constituted the first contact with the patient and consisted of the determination of biochemical parameters as glycemia, blood pressure, triglycerides and cholesterol. The time spent with the patient allowed to understand its clinical history, therapeutic plan, the treatment compliance and possible adverse reactions. This information complemented with the tests results allowed a more careful and accurate counselling. The subsequent executed task involved the counselling of patients while dispensing prescription and OTC drugs. The last stage of the internship was the production of compounded medicines. Moreover, a couple of pharmacy marketing services were taken into consideration as well. All these tasks were interconnected.

In conclusion, it was possible to execute a huge variety of tasks, constantly guided by the Pharmacy's team. Every time that a difficulty appeared, its clarification was promptly made by the collaborators. Through this internship, it was possible to contact with all the different functions of a Community Pharmacist, never promoting the sensation of a monotonous job.

3.1.3. Pharmacy Dispensing Robot

The Pharmacy Dispensing Robot (automatic system of storage) is a recent innovation regarding Community Pharmacy. This machine allows an efficient storage of the drug products taking into consideration the size and shape of the packaging. The robot also controls the stock numbers and the expiry dates following the first in-first out principle - the products with the shorter expiry date are the first to be sold.

The Pharmacy Dispensing Robot brought several advantages to the Community Pharmacy world. This machine promoted a close contact with the products at the beginning of the internship, allowing a faster familiarization with the packaging, brands and names. Although, as time passed by, the storage started to become an "automatic task", and the idea

of how a product is, in terms of packaging, becomes lost. This ends up causing difficulties to the counter service in the manner that, some patients required "the rose box of metformin" for example, and it was hard to discover which brand was. On the other hand, this machine has considerably decreased the number of mistakes and exchanges regarding the drug products. Through the utilization of the robot, the dispensing of the wrong medicine by confusion with another product by its similar shape, color, name, or dose does not occur so frequently. Moreover, the time that the product takes to arrive to the counter service promotes the communication between the Pharmacist and the patient.

Overall, although having the disadvantage described, the utilization of a Pharmacy Dispensing Robot was considered a strength of this internship. This machine brought several advantages over the course of the internship, promoting more confidence and safety while dispensing the drug product, and allowing the focus of the intern's attention into other important subjects of the dispensing drug product service.

3.2. Weaknesses

3.2.1. Production of compounded medicines

Luciano & Matos Pharmacy is recognized for its production of high-quality compounded medicines. A compounded medicine is considered as "the process of combining, mixing, or altering ingredients to create a medication tailored to the needs of an individual patient". [2] Moreover, in Portugal, a compounded medicine is considered as "any masterful formulation or officinal preparation prepared and dispensed under the responsibility of a pharmacist", being a "masterful formulation" a medicine prepared considering a medical prescription and an "officinal preparation" a medicine prepared using Pharmacopoeia as basis. [3]

Luciano & Matos Pharmacy is certificated by the ISO 9001:2015 and Good Pharmacy Practices and has appropriate laboratory facilities with the necessary equipment to produce these drug products. Initially, a brief explanation about the laboratory rules and the compounded medicines in general was given and a couple of auxiliary material was dispensed. It was possible to realize the close relationship between the Pharmacy and the "Laboratório de Estudos Farmacêuticos", a laboratory that belongs to "Associação Nacional das Farmácias Portuguesas" that is able to clarify any uncertainty regarding any compounded drug. Later on, it was possible to produce two different compounded products: a Castellani solution, prescribed for fungal infections, and a Sulfur ointment, used for scab treatment. Associated with the preparation of the products was the filling of the "Compounded medicine preparation sheet" including the calculation of the price to be applied, the retailing selling price (PVP in

Portugal), calculated considering the decree nº769/2004, and the filling of the label (appendices B and C). Besides, it was also possible to produce extemporaneous solutions, mainly antibiotics, for pediatric use.

The preparation of the compounded medicines allowed the consolidation of knowledge acquired in several curricular units and the development of new skills. However, being Luciano & Matos Pharmacy considered as one of the most important and recognized Pharmacies in this area with a preparation of several compounded medicines, at least one product per day, the desire to explore this component and being able to produce more of these drug products was always present.

3.2.2. Relationship between the MICF gaps and the world of work

As expected, no university degree allows the total preparation of the student to the world of work. Thus, the principal goals of the Curricular internship in Community Pharmacy are to improve and develop several technical and soft skills, able to fulfill some of the degree's gaps. However, there are a couple of subjects that could be improved in this Pharmaceutical Degree.

One of the most substantial difficulties during this internship was the struggle with the association of the trade name with the active pharmaceutical ingredient name or the international nonproprietary name (INN). During the Pharmaceutical Degree, curricular unities as Pharmacology, that approach several drug products, use most of the times the INN designation. This difficulty was felt with more intensity at the beginning of the internship since several patients refer to the drug products as the trade name and sometimes with a wrong pronunciation, which complicates the job in finding the correct medicine. This topic could be more explored during the curricular plan, introducing these names in a slow manner, promoting a more comfortable feeling during the initial times of the internship. As time passed by, and with the help of the mentors and of IT tools (as Sifarma 2000® and internet in general), this difficulty was slowly bypassed.

At the initial times, the principal worry during the dispensing service was the IT tool, Sifarma 2000®, associated with all the required procedures (manual recipes, the co-funding organisms, among others) instead of the counselling by itself. The software Sifarma 2000® is already explored in the MICF content, but in a very superficial way. This topic could be more exhaustively approached. On the other hand, all the co-funding organisms should be considered during the degree since the lack of experience made these processes more extended than usual.

As soon as these difficulties were solved and some experience was gained, the counselling started to occupy a superior amount of time and attention during the pharmaceutical service. However, there were several areas where this counselling was not that solid, as Dermocosmetics or Veterinary products. It is important to highlight that the experience and the contact with the reality and different situations are the main contributors to a comfortable counselling, what can only be achieved by experience. Nevertheless, several subjects could be further explored to promote a more solid base.

Lastly, the interpersonal relationships were a difficulty as well. As already mentioned, this Pharmacy is frequented by a wide range of patients, from doctors to socially disadvantaged groups and the approach in each service needs to be adapted to the situation, always focusing on the ethics and deontology. The MICF content could explore in a deeper way the different communications approaches, the management of conflicts, among other subjects that could promote a better performance during the internship. Although, this is something that comes with the experience as well, and the internship promoted its development and improvement, as time passed by.

Overall, the theoretical content of the Pharmaceutical Degree has some gaps, as expected, that could be improved and fulfilled. However, one of the goals of the internship is to exactly fulfill these gaps, and the experience is the main contributor for that.

3.3. Opportunities

3.3.1. Working with a versatile team

The Luciano & Matos Pharmacy belongs to the Holon Group of Pharmacies. The aim of this group is to optimize the way that pharmacies carry out their day-to-day activities, providing a high-quality service focused on the patients. Moreover, the Holon Group provides a personalized pharmaceutical care always focused on services of excellence. This group offers several services as the Holon Nutrition Service, Holon Diabetic Foot Service, Holon Dermopharmacy Service, among others, provided by different health professionals such as Nurses, Pharmacists and Nutritionists. All these services promoted the opportunity of contacting with different rigorous health professionals, allowing the integration of knowledge and experiences. Additionally, some of these professionals performed training sessions for the interns, allowing the improvement of knowledge in these areas.

On the other hand, the Luciano & Matos Pharmacy team is composed of several different professionals but all of them share the same values. The team has Pharmacists and Pharmacy Technicians and it is important to highlight their professionalism and dynamics. The

team provided an extremely thoughtful integration process and their availability to clarify uncertainties that occurred during the internship was always present. Additionally, this team is very rigorous and expect the best of their interns, which promoted a constant interest in doing the best possible job and being in constant improving. Moreover, this team allowed the improvement of the communication in a professional environment.

In conclusion, the contact with several rigorous health professionals constituted a great opportunity for the intern to expand and improve its knowledge in several areas and to push itself to always provide the best services for the patient.

3.3.2. Contacting with a Community Pharmacy environment

As already mentioned, the Community Pharmacy is an extremely important sector of the Pharmaceutical area. Working in the Luciano & Matos Pharmacy allowed the contact with a different pharmaceutical reality and with different tools used in this environment.

This internship provided the contact with the IT tool, Sifarma 2000®, which is the principal software used by the Pharmacies of Portugal. This tool helps in the management processes, as the stock and orders organization, and promotes an easier dispensing drug product service, with the shortcuts and scientific information, among others. All of this assures that the focus of the Pharmacist is essentially the counselling. The opportunity to contact with this software constituted an advantage for the intern, allowing the development of experience in its operation, that could be important for the future work.

The internship at Luciano & Matos Pharmacy also promoted the contact with the continuous improvement, more specifically, the Kaizen methodology. Kaizen means "good change" and consists of involving the workers in activities with the goal of having ideas to improve and solve problems. [4] From time to time a meeting of fifteen minutes was organized to discuss the sales and how far the monthly goals were of being achieved, communications that needed to be made to the team, mistakes that were done and problems that needed to be solved. This tool ends up promoting the communication between the members of the team, the motivation and optimization of time and processes. As an intern, the opportunity to follow these meetings allowed the understanding by a close eye of the functionality of a Pharmacy and the importance of the continuous improvement to the Pharmacy's organization, providing the development of important basis for the future.

Overall, this internship provided a more deepen understanding regarding the Pharmacy's dynamics and quotidian and an improvement of the knowledge concerning the principal tools used in this environment.

3.4. Threats

3.4.1. Lack of medicines in the Pharmacy

During the internship, several drug products were out-of-stock (OOS). There are several reasons for that to happen, including a failure in the production, distribution, or raw material, parallel exportation, among others. It is not possible to predict the time that the product will stay OOS, which concerns even more the patient.

This problem can be solved, sometimes, by a couple of different forms. If the drug product does not require a prescription, it can easily be substituted by other product that will produce the same effect. If it is a prescription drug and generics are available, it is possible to suggest one of the generics, although several patients still have uncertainties regarding this topic. If there is a different dose of the same active pharmaceutical ingredient, capable of being split, after a discussion with the physician it is a legitimate alternative. The real problem begins when there is no other therapeutic alternative for that pathological situation. In these cases, only the physician can prescribe something that can be adapted for the situation, or, in a couple of situations, that alternative may not exist.

The lack of medicines in the Pharmacy was considered a threat in the manner that it did not allow the full performance of the Pharmacist function, that in certain cases prejudiced the patient, not entirely satisfying its needs. Besides, this problem was not well understood by the patients, and a couple of times the guilt was assigned to the Pharmacy and the team, even though the effort to try and change that opinion had been done.

3.4.2. Sale of prescription drugs without a prescription

The request of prescription medicines without the prescription was one of the principal threats regarding this internship. This was a scenario witnessed almost every day at the Pharmacy's environment. The principal reasons declared by the patients for these requests were: the low price of the drug product (lower than the price to be paid for a medical consultation), the time that the patient will lose that corresponds to the loss of an entire day of work and lastly, the lack of the state contribution regarding these medicines.

During the internship, the risks of this action were highlighted several times. The majority of the patients had not been to a doctor's appointment in a long time, thereby, uncertainties related to if the drug product was still appropriate, or if it was the right one at the moment had emerged. An adjustment in the therapeutic plan is often required, to be the correct one for the clinical situation of the moment. Changes in the dose of the medicine or

in the dosage or even a substitution of a drug product are recurrent scenarios in the clinical practice, and these patients could possibly need of one of these adjustments.

This topic was considered a threat in the manner that, although several warnings, the reaction of the population was not always the best and associated with the lack of trust in the interns counseling, patients may sometimes call into question the functions and knowledge of the interns. Besides, the refuse to sell some of the solicited drug products was not well-received by a couple of patients. In conclusion, it is crucial to highlight the importance of the presentation of the prescription, especially for the preservation of the well-being of the patient.

4. Conclusion

The internship at Luciano & Matos Pharmacy was an extremely important experience. Apart from the consolidation of knowledge acquired during the academic path, this internship allowed the development of new technical and scientific knowledge and the growth of several skills, as the communication with the patients and with other health professionals and the active listening. All of this contributed to the preparation to the world of work, promoting the development of essential characteristics to a professional career within pharmaceutical related jobs.

The team of the Luciano & Matos Pharmacy was helpful since day one. The collaborators are versatile and rigorous professionals and every task was preceded by a brief explanation. Moreover, the ability to clarify uncertainties during the experience was always present. On the other hand, the location of the Pharmacy and the heterogeneity of the patients were an added value to this internship. There were only a few negative points regarding this experience: the low preparation of compounded drugs and the gaps existent in the MICF that can affect in a negative way the performance during the internship. Although the negative part, this internship was considered more than a positive experience.

The internship allowed to confirm the idea of a Pharmaceutic as a diversified professional, capable of providing differentiated services and able to interact in several different areas as the order's intake, the determination of the biochemical parameters, the production of compounded drugs and the counselling of the patient by promoting the therapeutic compliance, the non-pharmacological approaches or the precautions to take for instance. Despite the activity executed by the Pharmacist, its focus is always on the patient and the goal is to promote the rational use of the medication. All of this proves the important and crucial role of the Pharmacist in society.

Bibliography

1. TEOLI, D.; AN, J. - **SWOT Analysis**. Treasure Island (FL): StatPearls Publishing [Consultado em 9 de Agosto de 2019]. Disponível na internet: https://www.ncbi.nlm.nih.gov /books /NBK537302

2. FDA - **Compounding and the FDA: Questions and Answers**. [Consultado em 10 de Agosto de 2019]. Disponível na internet: https://www.fda.gov/drugs/humandrugcompounding/compounding-and-fda-questions-andanswers

3. **Decreto-Lei nº 95/2004**, de 22 de Abril. Diário da República: Série I-A, nº 95. [Consultado em 9 de Agosto de 2019]. Disponível na internet: https://dre.tretas.org/dre/171095/ decretolei-95-2004-de-22-de-abril

4. MAZZOCATO, P. *et al.* - **Kaizen practice in healthcare: a qualitative analysis of hospital employees' suggestions for improvement.** BMJ Open. ISSN: 2044-6055. 6:7 (2016) p. 1–2.

Appendices

A – SWOT analysis

	Positive	Negative
Internal	**Strengths:** Pharmacy's location and diversity of the patients; Preparation, guidance and diversity regarding the executed tasks; Pharmacy Dispensing Robot.	**Weaknesses:** Production of compounded medicines; Relationship between the MICF gaps and the world of work.
External	**Opportunities:** Working with a versatile team; Contacting with a Community Pharmacy environment.	**Threats:** Lack of medicines in the Pharmacies; Sale of prescription drugs without a prescription.

B – Prescription for the Sulfur ointment treatment

Receita Médica Nº

SNS

Utente: IS

MM

Telefone: R.C.:

Entidade:

Nº. de Beneficiário:

LPEUPS CENTRO

Especialidade: DERMATO-VENEREOLOGIA

Telefone:

"U989892"

Identificação Ótica

R DCI / Nome, dosagem, forma farmacêutica, embalagem, posologia Nº Extenso

1 Manipulado:Enxofre-24 Gr. Vaselina-Q.B.P. 300 Gr.F.S.A. e Mde Em Boião 2 Duas

Posologia aplica ao deitar -10 noites e banho de manhã

2

3

4

por computador - iReceita-EHR, v1.2 - CimpleCare, Lda

-idade: 30 dias

-03-19

(Assinatura do Médico prescritor)

Guia de tratamento para o utente

Receita Médica Nº

Local de Prescriç

Médico:

Utente: IS

Código Acesso:

Código Direito opção:

(Informação a utilizar para a dispensa de medicamentos na farmácia)

DCI / Nome, dosagem, forma farmacêutica, embalagem, posologia Nº

1 Manipulado:Enxofre-24 Gr. Vaselina-Q.B.P. 300 Gr.F.S.A. e Mde Em Boião 2

Posologia aplica ao deitar -10 noites e banho de manhã

2

3

4

Encargo para o utente de acordo com os medicamentos comercializados que cumprem a prescrição médica

1

2

3

4

Para obter mais informações sobre o preço dos medicamentos:

- Consulte «Pesquisa Medicamento» no sítio do INFARMED (www.infarmed.pt);
- Contacte a Linha do Medicamento 800 222 444 (Dias úteis: 09.00-13.00 e 14.00-17.00)
- Fale com o seu médico ou farmacêutico.

Data: 2019-03-19

Processado por computador - iReceita-EHR, v1.2 - CimpleCare, Lda

C – Compounded medicine preparation sheet for the Sulfur ointment and respective label

farmácia Luciano & Matos
FARMÁCIAS HOLON

FICHA DE PREPARAÇÃO DE MEDICAMENTOS MANIPULADOS

Medicamento: Pomada de Enxofre a 8%, em vaselina

Teor em substância(s) activa(s); 100g (ml ou unidades) contêm 8 g (ml) de enxofre

Forma farmacêutica: pomada **Data de preparação:** 20/03/2019

Número de lote: 4419 **Quantidade a preparar:** 300 g

Matérias-primas	Nº de lote	Origem	Farmaco-peia	Quantidade para 100g	Quantidade calculada	Quantidade pesada	Rubrica operador	Rubrica supervisor
Enxofre precipitado	161539-P-1	Acofarma	Ph. Eur. 8.8	8g	24g	24,002g	Catarina	
Vaselina líquida	01-091418	LabChem	Ph. Eur. 9	10g	30g	29,958g	catarina	
Vaselina sólida	R1099M8	JMGS, Lda	Ph. Eur.	q.b.p. 100g	q.b.p. 300g	246,004g	Catarina	

Preparação:

	Rubrica do operador
1. Verificar o estado de limpeza do material.	Catarina
2. Pesar a vaselina líquida diretamente para o recipiente unguator.	Catarina
3. Pesar o enxofre e colocar diretamente no recipiente unguator, e misturar com vareta.	Catarina
4. Adicionar vaselina sólida ao recipiente Unguator.	Catarina
5. Executar a mistura no Unguator.	Catarina
6. Fechar o recipiente e rotular.	Catarina
7. Lavar e secar o material utilizado.	Catarina
8.	
9.	
10.	
11.	

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farmácia Luciano & Matos
FARMÁCIAS HOLON

FICHA DE PREPARAÇÃO DE MEDICAMENTOS MANIPULADOS

Aparelhagem usada: Balança BL.01
Unguator

gako unguator
1 300S
The Original - Made in Germany

Embalagem:

Tipo de embalagem: recipiente unguator

Capacidade do recipiente: 300/390 ml

Material de embalagem	Nº de lote	Origem
Recipiente unguator	11602920	Gako

Operador: Catarina

Prazo de utilização e Condições de conservação:

Condições de conservação: Conservar em local seco e fresco, no recipiente bem fechado e ao abrigo da luz.

Operador: Catarina

Prazo de utilização: 30 dias

Operador: Catarina

Rotulagem:

1. Proceder à elaboração do rótulo de acordo com o modelo descrito em seguida.
2. Anexar a esta ficha de preparação uma cópia, rubricada e datada, do rótulo da embalagem dispensada.

Modelo de rótulo

Identificação da Farmácia
Identificação do Director Técnico
Endereço e telefone da Farmácia

Identificação do Médico prescritor
Identificação do doente

DENOMINAÇÃO DO MEDICAMENTO

Teor em substância(s) activa(s)
Quantidade dispensada
Referência a matérias-primas cujo conhecimento seja eventualmente necessário para a utilização conveniente do medicamento
Posologia
Via de administração

Data de preparação
Prazo de utilização
Condições de conservação
Nº de lote
Manter fora do alcance das crianças
Advertências (precauções de manuseamento, etc.)
Uso externo (caso se aplique) (em fundo vermelho)

Operador: Catarina

farmácia Luciano & Matos
FARMÁCIAS HOLON

FICHA DE PREPARAÇÃO DE MEDICAMENTOS MANIPULADOS

Verificação:

ENSAIO	ESPECIFICAÇÃO	RESULTADO	Rubrica do operador
Cor	Amarelada	*CONFORME*	
Odor	Característico do enxofre	*CONFORME*	
Aspecto	Homogéneo	*CONFORME*	
Quantidade	300g + 5%	*CONFORME*	

Aprovado ☑ Rejeitado ☐

Supervisor: _____ 20/03/2019

Nome e morada do doente:

[redacted]

Nome do prescritor:

[redacted]

Anotações:

farmácia Luciano & Matos
FARMÁCIAS HOLON

FICHA DE PREPARAÇÃO DE MEDICAMENTOS MANIPULADOS

Cálculo do preço de venda

MATÉRIAS-PRIMAS:

Matérias-primas	Embalagem existente em armazém		Preço de aquisição de uma dada quantidade unitária (sem IVA)		Quantidade a usar	Factor multiplicativo	Preço da matéria-prima utilizada na preparação
	Quantidade adquirida	Preço de aquisição (s/ IVA)	Quantidade unitária	preço			
Enxofre precipit.	1000g	8,57 €	1g	0,00857 €	x 24g	x 1,9	= 0,39 €
Vaselina branca	900g	3,76 €	1g	0,003369 €	x 246g	x 1,6	= 1,64 €
Vaselina líquida	840g	2,83 €	1g	0,00337 €	x 30g	x 1,9	= 0,19 €
		€		€	x	x	= €
		€		€	x	x	= €
						Total Matéria-Prima (A)	= 2,22 €

HONORÁRIOS DE MANIPULAÇÃO:

	Forma Farmacêutica	Quantidade	F (€)	Factor multiplicativo	Valor
Valor referente à quantidade base	pomada	100 g	5,03 €	x 3	= 15,09 €
Valor adicional		200 g	x 5,03 €	x 0,01	= 10,06 €
				Total da Manipulação (B)	= 25,15 €

MATERIAL DE EMBALAGEM:

Materiais de embalagem	Preço de aquisição	Quantidade	Factor multiplicativo	Valor
Unguator 300/390 ml	2,80 €	x 1	x 1,2	= 3,36 €
	€	x	x	= €
			Total de Material de Embalagem (C)	= 3,36 €

P. V. P. DO MEDICAMENTO MANIPULADO:

Soma de (A) + (B) + (C)	Factor multiplicativo	Valor
30,73 €	x 1,3	= 39,95 €
	I. V. A.	+ 2,40 €
	(D)	= 42,35 €

DISPOSITIVOS AUXILIARES DE ADMINISTRAÇÃO:

Dispositivo	Preço unitário	Quantidade	Valor
		(E)	€

PREÇO FINAL: (D) + (E) **42,35 €**

Operador: Catarina, **Supervisor:** _____

farmácia Luciano & Matos
FARMÁCIAS HOLON

Direção Técnica de
Maria Helena Costa Neves Correia Amado
Praça 8 de Maio, 40 – 42 • 3000-300 Coimbra
Telef. 239 822147/8 - Fax 239 824112

Lote nº 4419 **Data**: 20/03/2019 **Preço**: 42,35€

Utente: [redacted]

Médico: [redacted]

Enxofre – 24 g
Vaselina q.b.p. 300 g

Contém vaselina branca e vaselina líquida.

Medicamento para aplicação cutânea.

Posologia: **Aplicar ao deitar nas áreas atingidas e tomar banho de manhã, 10 noites seguidas.**

[redacted] Manter fora do alcance das crianças.

Conservar à temperatura ambiente, no frasco bem fechado e ao abrigo da luz.

Prazo de utilização: 30 dias

20/3/19
Catarina

Part III

Report of the Internship in Pharmaceutical Industry

Bluepharma Industria Farmacêutica SA

List of Abbreviations

EMA - European Medicines Agency

FDA - U.S Food and Drug Administration

LAIs - Long-Acting Injectables

SWOT - Strengths, Weaknesses, Opportunities and Threats

1. Introduction

The last stage before the conclusion of the Master's degree in Pharmaceutical Sciences is the Curricular Internship. This internship allows a first contact between most of the students and the real work world, constituting a kickoff experience before the initiation of their own careers. Moreover, curricular internships are essential to consolidate the knowledge obtained through the entire academic path and to acquire new technical and soft skills indispensable to ingress and progress in a professional career within pharmaceutical related jobs.

During the academic career, the desire to contact with Pharmaceutical Industry, in particular with Research and Innovation technology fields, was always present. During the Pharmaceutical science degree the contact with curricular units as Pharmaceutical Technology (I, II and III) and Pharmaceutical Nanotechnology that demonstrated the constant innovation and advances in science, arisen the interest in these challenging and interesting areas. Since Bluepharma Pharmaceutical Industry SA is recognized as one of the most innovative pharmaceutical companies in Portugal, an opportunity of doing an internship in this company was undeniable.

The current report intends to describe the work developed during the internship of three months performed at the Innovative Research Department of Bluepharma, through the analysis of the Strengths, Weaknesses, Opportunities and Threats (SWOT) of the internship.

2. Bluepharma

Bluepharma Indústria Farmacêutica SA is a Portuguese Pharmaceutical Industry located in Coimbra, Portugal. Bluepharma was founded in February of 2001, after the acquisition of the industrial facilities of the Germany's multinational company Bayer, placed in São Martinho do Bispo. Nowadays, it is one of the biggest and well succeeded Pharmaceutical Industries in Portugal and its prestige is recognized in the most competitive markets, such as Europe and USA. [1-3]

Bluepharma has several years of experience in manufacturing drug products and its services are always based in maximum quality and excellence. The principal mission of the company is to offer a better quality of life to the population, through providing high quality medicines and competitive prices, and promoting the rational use of drug products. All of these qualities can be achieved thanks to the foundations of the company: the focus on training and promoting the growing of the technical staff and the enthusiasm and vision of the management group. [1-3]

Figure 1 - Bluepharma Pharmaceutical Industry. Adapted from https://www.noticiasdecoimbra.pt/wp-content/uploads/2017/09/bluepharma.jpg.

The core business of Bluepharma is mainly drug products production. Its activity is based on development and/or manufacturing of pharmaceutical products for itself and/or for external clients (positioning the company as a CMO - Contract Manufacturing Organization and/or CDMO - Contract Development and Manufacturing Organization) and production and sale of generic products. This industry has a strong internationalization component since the majority of the production is exported, around 87%. Moreover, Bluepharma develops new and innovative dosage forms, allowing topics as research and innovation to be in constant growing. [2,3]

This internship took place at the Research and Innovation Technology sector of the Research and Innovation Department of Bluepharma, under the supervision of the head of the sector, António Nunes, PharmD, PhD and the Director of the Department, Cláudia Silva, PharmD, PhD. The main aim of this Department is essentially the development of new formulations and delivery systems. [1,2], [4]

3. Work plan

The work developed during this internship was focus on the development of innovative dosage forms, such as the Long-Acting Injectables (LAIs), complex pharmaceutical formulations. The tasks carried out during the 3 months internship consisted in bibliographic research and data organization regarding characterization methods, formulation and manufacturing processes of these type of drug products.

4. SWOT analysis

4.1. Strengths

4.1.1. Integration and welcoming process

Bluepharma has an integration process that is extremely welcoming and thoughtful to make sure the new employees do not feel excluded. The principal goal is to guarantee that the new staff feels comfortable in the Industry's ambiance since day one. It is important to enhance that this process is similar between new interns and employees.

The integration begun with the presentation of the new employees to the rest of the collaborators (not all, but at least the ones the student will work within the internship period). After that, the process passed by the attribution of a tutor. This person was responsible for the presentation to the team and to introduce the places that would be useful in the future. Lastly, the tutor gave a brief explanation regarding the company's organization. All of these stages assured that the new intern did not feel lost. Besides, the new intern was also able to feel as part of the team, contributing to a good work environment since the beginning.

The second part of the integration process consisted in the participation in several lectures that introduced the company, its several departments, its functions and the mode of working in a more detailed way. The presentations approached the following topics: Integrated Management System; Research, Development and Innovation; Bluepharma's Evolution; Pharmacovigilance; Regulatory Affairs; Continuous Improvement; Good Manufacturing Practices and Information Systems. These lectures are usually part of the fourth week of the new employee in the company and approached base concepts about the company. Those concepts, if transmitted in the first weeks, would have been more useful, because would have avoided several doubts that occurred at the initial times. Despite all that, this allowed the acquisition of a more clear vision about the company, contributing to the integration process as well.

Overall, the integration process was great, carefully thought, and is great to recognize that Bluepharma and all of its collaborators care about this topic, contributing to the feeling of being welcomed in the company. It is important to enhance the crucial role of the Research and Innovation Department, the mainly responsible for the integration, not excluding the importance of the Human Resources Department through all this process.

4.1.2. Regular meetings

During all the internship, numerous meetings were carried out to approach different issues and topics. In the first week, a meeting with the mentor was organized and a brief explanation about the company was given. Besides that, this meeting was useful to discuss the work plan to be developed during the internship. This plan ended up suffering alterations related to Industry's priorities. Then, in almost every week a meeting was planned to evaluate the work done through the week and to approach the work plan to the next week. This constant guidance was important so that the work could improve and go in line with what was expected. The only negative point regarding this topic was that, in weeks where the job in the industry was more intense, the weekly meeting did not occur, leading to a more difficult and less organized week.

Besides the weekly meetings, this department was part of other several meetings: the monthly sector meeting, the quarterly meeting of the Department and the laboratories meeting. The opportunity of being part of a couple of them was given. The quarterly meeting of the Department involved all the elements of the Innovative Research Department and approached the goals for the current year, the projects, and how far were they of being achieved. The Laboratories meeting, the last one that was possible to attend to, was elaborated with the presence of all the elements of the laboratories. The goal was to discuss recurrent problems of these working areas.

All of these meetings allowed the acquisition of more information about the projects developed at the time and a better understanding regarding the dynamics and ambitions of the company and its flaws and challenges.

4.1.3. Evolution through the internship

This internship allowed the development of several technical and soft skills. The majority of the work performed during the internship involved bibliographic research on the topic of new and innovative dosage forms. Overall, promoted the gaining of general knowledge on how to search for relevant information, in particular form US and European authorities. Moreover, allowed the development of the autonomy and the improvement of interpersonal relationships skills.

As already mentioned, this internship had a very strong bibliographic research component. At initial times, knowing where to look for information or how to achieve the material needed was difficult. While time passed by and with the help of the mentors, this obstacle was overcome. At the end of the internship, the research skills suffered a considerable

improvement. On the other hand, this topic led to the development of the autonomy skills. Every time that a doubt appeared or a challenge emerged, the first goal was to solve the problem alone. The motivation was always present but the soft skill was developed during the internship. Moreover, due to the intensity of the work developed at Bluepharma and the priorities of the company, the opportunity to clarify some doubts that occurred was not always present, promoting the development of the autonomy as well. However, it is important to highlight the encouragement given to interrupt and discuss any subject since the beginning.

Additionally, the team of the Innovative Research Department contained several collaborators and a different environment, promoting the development of diverse soft skills, as the interpersonal ones. The communication in a professional environment was improved. Moreover, the team spirit, teamwork and mutual aid between the Research and Innovation Department collaborators were exceptional and contagious, promoting the upgrading of these characteristics as well.

4.2. Weaknesses

4.2.1. Low laboratorial execution

The selection of the trainees to perform the internships at Bluepharma is made through interviews. During the interview, the interest in Innovative Research laboratories had always been demonstrated, and that ended up being the department selected for the internship. With that, an expectation of working in a laboratorial environment was created. However, all the work developed in this ambit was theoretical, without the laboratorial component.

Through the internship, this question was approached several times, and the main reason for that to not happen was the timing. The internship took place at some point where the priorities of the industry were the execution of other type of works that were not the laboratorial ones. It is important to understand that the priorities of a Pharmaceutical industry are in constant changing and several challenges are associated with that. On the other hand, the confidentiality of the projects also had impact in the choice of the work to be developed.

The internship ended up not allowing the improvement the skills of laboratorial execution but was useful in the enhancement of other skills, as already mentioned. Moreover, the work developed during the internship was never undervalued and this project was important to understand what the initial part of a research projects is and being a part of that was important for the future.

4.2.2. Low variety of the executed tasks

This internship had the duration of only three months. During the first week, the principal task was to read internal documents and Standard Operating Procedures of the company, to understand its organization, functions, and safety rules. Besides that, some technical information regarding apparatus and techniques was read as well. The next mission performed was the bibliographic research to support the initial development of innovative dosage forms, such as complex pharmaceutical formulations, the LAIs. This task included the structuration of the information using Excel tools and preparation and delivery of a PowerPoint presentation.

The developed work was essentially focused on research and compilation of information not allowing the execution of differentiated tasks. However, this was related to the project that was defined for this internship that did not require the execution of several different tasks.

In conclusion, even though the low variety of executed tasks, it was possible to experience a huge component of the research and innovation areas, the search of information. This component is important in the manner that allows the compilation of information useful to reduce the error of the next stages of the product's development and useful to know what was already made and what needs to be explored in the next phases of the work.

4.3. Opportunities

4.3.1. Improvement of the English skills

During the internship the contact with the English language was constant. As already mentioned, this work had a very solid research component and all of the available information was written in this language.

All the documents read, including official documents of European Medicines Agency (EMA) and U.S Food and Drug Administration (FDA), articles, Summary of Products Characteristics and Pharmacopoeia material were written in English, improving the reading and understanding components. On the other hand, the research regarding techniques and apparatus endorsed the development of some technical language as well. Moreover, the compilation of the information and the PowerPoint presentation were written in English, also promoting an improvement of this component.

Overall, this internship allowed the development and improvement of this skill, both in reading, understanding and writing, and promoted the enlargement of the vocabulary as

well, motivating the writing of the "Documento Único" in this language. Since English is the universal language, being comfortable with it is important for any future situation. Additionally, all of the Pharmaceutical careers have contact with this language, so having the opportunity of improving this skill was important for the work that will be developed in the future.

4.3.2. Working in an industrial environment

As already mentioned, the wish to explore the Pharmaceutical Industry's environment had always been present. Besides that, the academic units do not approach a clear vision of the complexity and dynamics of a Pharmaceutical Industry. Due to these facts, as soon as the opportunity of doing an internship at Bluepharma appeared, the chance of filling this gap and deepen the knowledge in this area emerged.

At first, this internship promoted a more clear vision of the crosslink and collaborations between different areas of the industry. This could only be achieved thanks to the direct contact with the company. The Research and Innovation Department is very versatile and is divided in two groups: the galenic and the analytical. Through this experience was possible to understand how the development of a drug product is made in terms of the crosslink between the two groups. The Kaizen (topic discussed in the next section) was important in the manner that helped to understand the roles of each group in different stages of the process.

In addition, since an internship in research in the University's environment had already been made, a comparative analysis between the two experiences could be made. Due to this fact, working in a Pharmaceutical Industry environment showed to be more demanding, requiring the performance under several rules and numerous protocols and, in general, demonstrated to have more regulations. Besides that, the internship developed at Bluepharma promoted a more open mind and a different approach of what can be done in the pharmaceutical research area.

In conclusion, having the opportunity of working in this environment allowed the understanding of several remaining doubts and a more clear vision of how it works and what can be done in a Pharmaceutical industry, including the possible professional careers of a Pharmacist.

4.3.3. Contacting with Continuous Improvement

The Continuous Improvement is a crucial area in Bluepharma Industria Farmacêutica SA. Bluepharma has collaborators responsible for developing this area and for applying the

Lean and Kaizen methodologies. Lean's methodology goal is to achieve the Continuous Improvement through the reduction of processes variations and of activities that do not show added value. [5]

One example of a Kaizen practice implemented at Bluepharma is the Kaizen board – daily Kaizen. This board is a visual tool that helps teams to organize the ideas of improvement and to see the status of ongoing tasks. This is used to expose several categories that are evaluated from time to time. Some of the categories approached in the Innovative Research Lab board - daily Kaizen were: work plan (work that is expected to be done through the week to each worker), learned lessons, equipment management and improvement actions – Plan, Do, Check, Act (PDCA), among others. Every day, a kaizen meeting was organized to discuss some of the board categories. In these meetings was evaluated the compliance of the work plan. Besides that, these meetings were used to approach communications that needed to be made to the team and to discuss constraint and challenges that occurred. This tool ends up promoting the communication between the members of the team. The Kaizen meetings were executed every day by every Bluepharma team.

This internship allowed the perception of the importance that is given to Continuous Improvement in Pharmaceutical Industries. Besides that, since a contact with Kaizen was already obtained at the Community Pharmacy, a comparison between the two experiences was possible. Bluepharma's methodology is applied in a bigger scale, with more details. Moreover, the meetings were different in the two cases since they needed to be adapted to each environment. The Pharmacy's Kaizen meetings were not made every day and were used to discuss the sales and how far the monthly goals were of being achieved. Both meetings approached communications that needed to be made to the team, mistakes that were committed and problems that needed to be solved. Although the differences between the two realities, the aims turned out to be the same in terms of trying to improve, learn, encourage the communication and avoid mistakes.

4.4. Threats

4.4.1. Duration of the internship

The duration of the internship (three months) is imposed by the Faculty of Pharmacy of the University of Coimbra. Its short duration has several implications on the level of detail that is given to the work. Besides that, may not allow the full understanding of the company. Due to these facts, the duration of the internship can be considered a threat.

Internships of three months did not allow a deepening of the theme and of the executed tasks. The level of detail of the developed work is relatively low. Moreover, the short duration of the internship was not enough to fully understand the dynamics and main roles of all the departments and sectors that co-exist in the pharmaceutical industry.

Overall, although the short duration, the plan established at the beginning was fulfilled. This demonstrates that an internship in the research and innovation area of a pharmaceutical industry with this available time is possible. Moreover, its duration did not prevent the acquisition of important qualities and knowledge imperative to ingress the world of work. Due to that fact, the internship was considered pertinent and crucial.

4.4.2. Lack of guidelines/available information approaching extended release formulations

The work developed during this internship was based on the LAIs. These are injectable formulations that promote a prolonged or sustained drug release over a period of time (from days to months). [6]

The work plan to this internship was the research about characterization methods, bio relevant *in vitro* release tests, formulations and manufacturing processes of LAIs. The major difficult associated with these tasks was founding available information. The majority of the documents that had data with interest for this case were covered due to confidentiality. On the other hand, the regulatory authorities EMA and FDA have several guidelines, especially FDA which has specific guidelines for pharmaceutical industries approaching several pharmaceutical dosage forms. However, guidelines approaching LAIs or extended release formulations were not available at the time.

As time passed by, and with the help of the mentors, this difficult was bypassed due to different approaches that were made. In conclusion, this obstacle made the work complex but challenging, promoting the critical and constant thinking, and did not prevent its conclusion since the final goals were achieved.

5. Conclusion

The Pharmacist has several roles through all the life cycle of a drug product. As a versatile professional, has numerous opportunities in the Pharmaceutical Industries world. Due to that fact, an internship in this area would be extremely useful. Bluepharma was chosen for that experience thanks to the location and prestige in Research and Innovation areas.

Since day one, this company welcomed the interns and the integration process was carefully thought enabling a fast integration. Working in an Industry's environment allowed the perception of how it works, its functions, departments, its dynamics and challenges. Besides, allowed a better perception of the Pharmacist potential careers in Industries. There were only a few negative points regarding this internship, as the low laboratorial execution, that did not allow the improvement of the laboratorial skills, as desirable, and the low variety of the executed tasks. However, the developed project was relevant and challenging and associated with qualified professionals allowed the development and consolidation of other several skills.

The internship at Bluepharma was a truly significant experience that promoted a personal and a professional growth allowing the preparation to the world of work. Moreover, research and innovation areas are more and more important every day in the Pharmaceutical area, thus having the opportunity of making part of this and following its quotidian was a unique experience.

Bibliography

1. BLUEPHARMA - **About us.** [Consultado em 10 de Julho de 2019]. Disponível na internet: https://www.bluepharma.pt/about-us.php

2. BLUEPHARMA - **Bluepharma Group**. [Consultado em 10 de Julho de 2019]. Disponível na internet: https://www.bluepharma.pt/about-bluepharmagroup.php

3. BLUEPHARMA - **History**. [Consultado em 10 de Julho de 2019]. Disponível na internet: https://www.bluepharma.pt/about-history.php#0

4. BLUEPHARMA - **Innovative Research.** [Consultado em 10 de Julho de 2019]. Disponível na internet: https://www.bluepharma.pt/innovation/research.php

5. MAZZOCATO, P. *et al.* - **Kaizen practice in healthcare: a qualitative analysis of hospital employees' suggestions for improvement.** BMJ Open. ISSN: 2044-6055. 6:7 (2016) p. 1–2.

6. CHAUDHARY, K.; PATEL, M. M.; PRITI, M. J. - **Critical Reviews™ in Therapeutic Drug Carrier Systems. Long-Acting Injectables: Current Perspectives and Future Promise**. ISSN: 0743-4863. 36:2 (2019) p. 137.

Appendices

A – SWOT analysis

	Positive	Negative
Internal	**Strengths:** Integration and welcoming process; Regular meetings; Evolution through the internship.	**Weaknesses:** Low laboratorial execution; Low variety of the executed tasks.
External	**Opportunities:** Improvement of the English skills; Working in an industrial environment; Contacting with Continuous Improvement.	**Threats:** Duration of the internship; Lack of guidelines/available information approaching extended release formulations.