**Bruna Filipa Campos Correia**

Licenciada em Biologia – Biologia Molecular e Genética

# Unveiling the clinical relevance of low density neutrophils in Breast Cancer patients

Dissertação para obtenção do Grau de Mestre em
Genética Molecular e Biomedicina

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**Vogal:** Professora Doutora Maria de Guadalupe Gonçalves Cabral, NOVA Medical School, Faculdade de Ciências Médicas da Universidade Nova de Lisboa

**Novembro, 2020**

UNIVERSIDADE NOVA DE LISBOA

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## Abstract

Neutrophils are prominent immune components of solid tumors, which can protect against the onset of cancer (N1) or have protumor activity (N2). Circulating neutrophils, divided into high density neutrophils (HDN) and low density neutrophils (LDN), functionally mirror those N1 and N2 cells, respectively. LDN, practically absent in non-pathological conditions, have been extensively studied in cancer, due to their increased frequency in this disease and their protumor phenotype. However, this has been mainly demonstrated in animal models and proper validation in humans is an urgent need. In this thesis, we enlightened the clinical impact of LDN in breast cancer (BC) patients. We observed that LDN were practically absent in healthy donors' blood, while significantly increased in the blood of BC patients, particularly with metastatic disease. Within the population of non-metastatic patients, LDN were more prevalent in patients with poor response to neoadjuvant chemotherapy than in patients with good response. The association of a higher incidence of circulating LDN and the worse prognosis of BC patients could be explained by the protumor features exhibited by these cells. Namely, there are more LDN expressing the immunosuppressive markers PD-L1 and CCR4, than HDN. Additionally, LDN also showed increased expression of activation markers; robust formation of neutrophil extracellular traps; augmented phagocytic activity and higher capacity to release reactive oxygen species, which altogether contribute for tumor development and metastization. Moreover, the percentage of LDN in BC patients' blood was positively correlated with the immunosuppressive CCR4+ regulatory T cells and negatively correlated with activated cytotoxic T lymphocytes, corroborating the impairment on the antitumor immune responses by LDN, which was further demonstrated *ex vivo*. Hence, this thesis reveals the potential of LDN as a clinical meaningful biomarker of BC response to treatment and opens new avenues for developing targeted immunotherapies.



**Keywords:** Low density neutrophils, Breast cancer, Biomarker, Neoadjuvant chemotherapy

## Resumo

Os neutrófilos são componentes imunes relevantes nos tumores sólidos, podendo proteger contra a progressão do cancro (N1) ou ter atividade pro-tumoral (N2). Na circulação, os neutrófilos podem dividir-se em neutrófilos com alta densidade (NAD) ou baixa densidade (NBD), refletindo as funções de N1 e N2, respetivamente. Os NBD, praticante ausentes em contextos não-patológicos, têm sido extensivamente estudados em cancro devido ao seu fenótipo pro-tumoral e à alta representatividade em doentes oncológicos. Contudo, a maioria dos estudos existentes são em modelos animais e, como tal, é ainda necessária a validação com amostras humanas. Nesta tese, demonstrámos o impacto clínico dos NBD em doentes com cancro da mama (CM). Observámos que esta população estava praticamente ausente em indivíduos saudáveis e que, em contrapartida, se encontrava significativamente enriquecida no sangue das doentes, especialmente nas metastáticas. Verificámos que, na população das doentes não-metastáticas, os NBD eram mais prevalentes no sangue de doentes sem resposta à quimioterapia neoadjuvante, comparativamente às doentes que responderam. A relação entre a elevada incidência de NBD e um pior prognóstico pode explicar-se pelas características imunossupressoras destas células. Nomeadamente, existem mais NBD a expressar os marcadores imunossupressores PD-L1 e CCR4, comparativamente com os NAD. Adicionalmente, os NBD apresentaram nível elevado de expressão de marcadores de ativação; capacidade fagocítica aumentada; elevada produção de espécies reativas de oxigénio e aptidão para formar armadilhas extracelulares dos neutrófilos, que no geral contribuem para o desenvolvimento tumoral e metastização. Observámos também que a percentagem de NBD no sangue de doentes correlacionava-se positivamente com as células T reguladoras CCR4+ e negativamente com os linfócitos T citotóxicos ativados, corroborando a capacidade dos NBD de inibirem respostas imunes anti-tumorais, o que demonstrámos também *ex vivo*. Assim, nesta tese, evidenciámos o potencial dos NBD como biomarcador de resposta à quimioterapia neoadjuvante em CM e abrimos novos caminhos para o desenvolvimento de imunoterapias direcionadas.

**Palavras-Chave:** Neutrófilos de baixa densidade, Cancro da mama, Biomarcador, Quimioterapia neoadjuvante

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## List of Abbreviations

| | |
|---|---|
| ARG1 | Arginase 1 |
| BC | Breast cancer |
| BMI | Body mass index |
| BSA | Bovine serum albumin |
| CCL- | C-C chemokine ligand |
| CCR4 | C-C chemokine receptor 4 |
| CD- | Cluster domain |
| cm | Centimeter(s) |
| CTLA-4 | Cytotoxic T lymphocyte-associated antigen 4 |
| CTLs | Cytotoxic T lymphocytes |
| DAPI | 4′,6-diamidino-2-phenylindole |
| DCFH2-DA | 2′,7′-Dichlorofluorescin diacetate |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| EDTA | Ethylenediamine tetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| ER | Estrogen receptor |
| FACS | Fluorescence activated cell sorting |
| FBS | Fetal bovine serum |
| FITC | Fluorescein isothiocyanate |
| g | Gramme(s) |
| G-CSF | Granulocyte colony-stimulating factor |
| GM-CSF | Granulocyte macrophage colony-stimulating factor |
| h | Hour(s) |
| HBSS | Hank's balanced salt solution |
| HDN | High density neutrophils |
| HER2 | Human epidermal growth factor receptor 2 |
| HOCl | Hypochlorous acid |
| HRP | Horseradish peroxidase |
| IFN-$\gamma$ | Interferon gamma |
| IL- | Interleukin |
| LB | Lysogeny broth |
| LDN | Low density neutrophils |
| M | Molar concentration |
| mAbs | Monoclonal antibodies |
| MDSCs | Myeloid-derived suppressor cells |
| MFI | Median fluorescence intensity |
| mg | Milligrams(s) |

| | |
|---|---|
| min | Minute(s) |
| mL | Milliliter(S) |
| mm | Millimeter(s) |
| MMP- | Metalloproteinase |
| MPO | Myeloperoxidase |
| NACT | Neoadjuvant chemotherapy |
| NE | Neutrophil elastase |
| NETs | Neutrophil extracellular traps |
| ng | Nanogram(s) |
| NLR | Neutrophil-to-lymphocyte ratio |
| NO | Nitric oxide |
| ºC | Degrees Celsius |
| PBMCs | Peripheral blood mononuclear cells |
| PBS | Phosphate buffered saline |
| pCR | Pathological complete response |
| PD-1 | Programmed death protein 1 |
| PD-L1 | Programmed death ligand 1 |
| PFA | Paraformaldehyde |
| PMA | Phorbol 12-myristate 13-acetate |
| PMN | Polymorphonuclear |
| PR | Progesterone receptor |
| ROS | Reactive oxygen species |
| rpm | Rotations per minute |
| TANs | Tumor-associated neutrophils |
| TBM | 3,3',5,5'-tetramethylbenzidine |
| TGF-β | Transforming growth factor beta |
| Th | Helper T cells |
| TILs | Tumor infiltrating lymphocytes |
| TIME | Tumor immune microenvironment |
| TNBC | Triple negative breast cancer |
| TNF-α | Tumor necrosis factor alpha |
| TRAIL | Tumor necrosis factor-related apoptosis-inducing ligand |
| Tregs | Regulatory T cells |
| VEGF | Vascular endothelial growth factor |
| µg | Microgram(s) |
| µL | Microliter(s) |

# Chapter I – Introduction

## 1. Cancer

Cancer develops when a cluster of cells breaks out of the mechanisms that control the cell cycle. These abnormal cells gain the ability to proliferate in an uncontrolled manner and to escape apoptosis, becoming immortal. Such features are a result of the accumulation of mutations, most of them on oncogenes or tumor-suppressing genes, as the ones involved in cell cycle regulation or DNA repair mechanisms (1).

Back in 2000, Hanahan *et al* suggested a compilation of six essential alterations manifested by malignant cells, referred to as the hallmarks of cancer, to facilitate the understanding of cancer biology (2). The six proposed hallmarks were: self-sufficiency in growth signals, insensitivity to antigrowth signals, evasion of programmed cell death, limitless replicative potential, self-sustained angiogenesis and capacity for tissue invasion and metastasis (2). Each of these hallmarks corresponds to a novel biological feature acquired during tumor development, being all crucial to the success of the process.

Years later, the authors revised the original hallmarks, as they were not sufficient to explain the mechanisms of tumor behaviour. In this revision, the authors proposed two enabling characteristics along with two new emerging hallmarks (3). The acquisition of the essential functional features that allow cancer cells to thrive is only possible due to the mentioned enabling characteristics, which are the genome instability and consequent mutability typical of malignant cells and the tumor-promoting consequences driven from the inflammatory response associated with malignant lesions (3). Moreover, the two proposed emerging hallmarks are key attributes involved in the pathogenesis of cancer: the reprogramming of the cellular energy metabolism to support continuous cell proliferation and the development of active mechanisms of evasion from immune destruction (3) (Figure I.1).

Thereby, over the past few years, it has become clear that tumor biology is much more complex and dynamic than initially thought. The importance of the immune system in carcinogenesis was brought to light and cancer can no longer be considered only by the malignant cells *per se*. Instead, it is necessary to acknowledge the interactions between cancer cells and the other cell populations present in the tumor microenvironment, namely the immune cell populations, as well as the role of effector molecules released by the cells present. Indeed, cancer progression is dependent not only on the features of tumor cells but also on the interplay occurring in the tumor microenvironment. In this thesis, a focus on the immune compartment of the tumor microenvironment was made and, as such, the tumor immune microenvironment (TIME) will be detailed below.

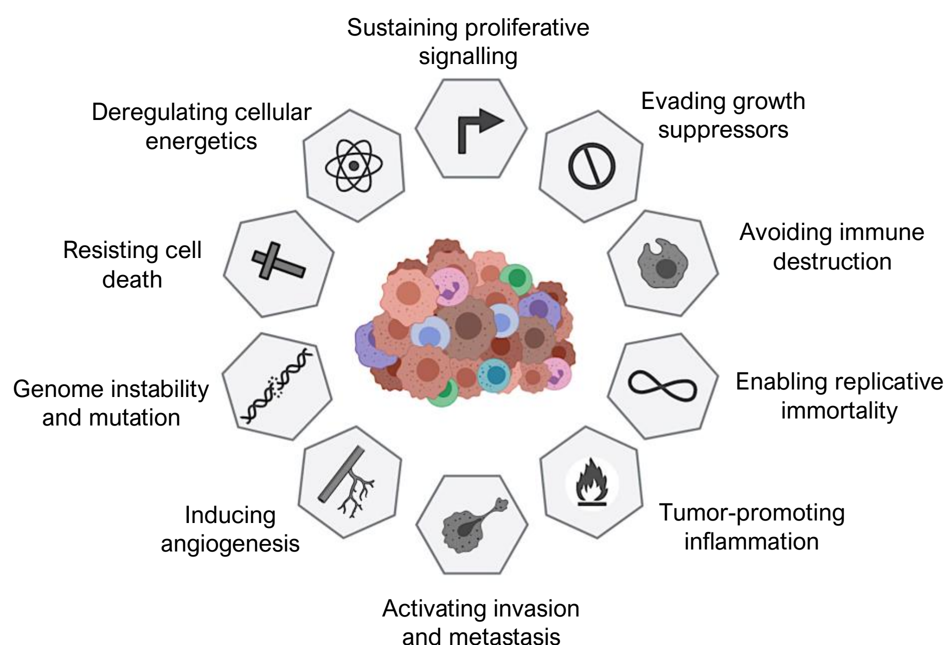



**Figure I. 1 – Hallmarks of cancer.** Adapted from Hanahan *et all*, Cell 2011 (3).

## 2. Tumor Immune Microenvironment (TIME)

The TIME is fundamental in the determination of tumor behaviour and success, affecting also therapeutic efficacy (4).

The immune infiltrate includes lymphocytes, such as cytotoxic T lymphocytes (CTLs) and T regulatory cells (Tregs); and myeloid cells, namely tumor-associated neutrophils (TANs) and myeloid-derived suppressor cells (MDSCs) (5). All of these populations portray key roles in carcinogenesis, being able to either promote or inhibit tumor growth and progression (5).

CTLs are a subset of T cells that express the CD8 molecule, which have the ability to recognize antigens when presented by MHC class I molecules (6). Within the TIME, CTLs are known as frontline agents against tumor cells, having high cytotoxic capacity (6). CTLs can kill tumor cells through different mechanisms, being one of them the granule exocytosis pathway, where perforin and granzyme B are released (7). Perforin induces the formation of pores in the membrane of cancer cells, allowing the traffic of granzymes, which will then act as a protease that will cleave target substrates in the cytoplasm, ultimately leading to apoptosis (7). CTLs can also eliminate tumor cells through the Fas/FasL system, responsible for inducing tumor cell apoptosis (7). Furthermore, CTLs can also secrete tumor necrosis factor (TNF-) $\alpha$ and interferon (IFN-) $\gamma$ which will induce cytotoxicity against tumor cells (8). The elimination of tumor cells by CTLs is the first step of the immunoediting process (9,10). After the elimination phase, tumor cells and the immune populations achieve an equilibrium, where elimination of certain tumor variants still occur, while new variants with resistance to immune surveillance arise. This stage of tumor dormancy can go on for years in

which the tumors remain undetected. The third and last stage is the escape, where less immunogenic tumor variants that developed immunosuppressive mechanisms to impair the antitumor immune responses, are able to proliferate and lead to progressively growing tumors (9,10).

Tregs are a subset of CD4+ T cells with immunosuppressive features (6). Tregs have the ability to suppress immune responses, including CTLs' function, supporting tumor development (11). The suppression mediated by Tregs may be achieved through the cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) (12) or through the release of immunosuppressive cytokines, as transforming growth factor (TGF-) β and interleukin (IL-) 10 (13,14).

MDSCs have a suppressive role, by the release of reactive oxygen species, nitric oxide and arginase, which will inhibit T cell responses (15). These cells can also stimulate angiogenesis and tumor cell extravasation, through the release of vascular endothelial growth factor (VEGF) and metalloproteinases, respectively (15).

Neutrophils can also be present in the TIME and since these cells are the main focus of this thesis, their role will be further explored in the upcoming section.

Aside from the cell populations, the cytokines released in the TIME are also key participants in carcinogenesis. These molecules are secreted by immune cells as well as by tumor cells. Cytokines are essential to provide the necessary conditions for tumor progression or elimination, to regulate signalling pathways and for cell recruitment. Some of the main cytokines in the TIME that will be further explored in this thesis are IFN-γ, TGF-β, IL-10, IL-17 and C-C chemokine ligand (CCL-) 17 (16,17).

IFN-γ is a pro-inflammatory cytokine produced by tumor suppressor cells, including CTLs and acts mainly as an anti-tumor factor, due to its immunostimulatory effect (6).

TGF-β can have ambiguous roles in the immune system; however, in cancer, it is mostly considered an immunosuppressive player (18). In fact, TGF-β produced by cancer cells can impair the action of different immune cells (19), such as CTLs, or even induce polarization towards immunosuppressive phenotypes in macrophages and neutrophils (19,20), overall leading to dampened antitumor immune responses.

IL-10 is an immune-modulatory cytokine with anti-inflammatory activity. IL-10 is produced not only by the majority of immune cells, including T cells, macrophages and granulocytes (16,21), but also by tumor cells (22). IL-10 has been described to promote tumor growth, resistance to apoptosis and angiogenesis in different cancer types, such as melanoma, lung and breast cancer (23–25).

IL-17 is a pro-inflammatory cytokine mainly produced by a subtype of CD4+ T cells – the Th17 (26). The pro-tumorigenic roles of IL-17 have been detected in multiple cancer types, such as breast, prostate, colon and gallbladder cancer, and include the promotion of tumor proliferation, angiogenesis, chemotherapy resistance and metastasis (27–30).

CCL17, produced by neutrophils and macrophages, acts as a chemoattractant primarily involved in the recruitment of CCR4+ T cells (31). The accumulation of Tregs in tumors in a CCL17-dependent manner have been described in lung and gastric cancer (17,31,32).

## 3. Neutrophils

### *3.1 Neutrophils in the immune system*

Neutrophils are part of the polymorphonuclear (PMN) leucocytes and are the most abundant type of white blood cells, corresponding to 50-70% of the circulating leucocytes (33). In homeostasis, neutrophils enter the circulation and migrate to tissues, where they constantly patrol for signs of pathogens (34). These leucocytes comprise the first line of defence of the innate immune system and are quickly recruited to the sites of infection or inflammation, where they have a primary role of resistance against pathogens and in acute inflammation (35).

There are three main recognized means by which neutrophils can exert their antimicrobial activity (Figure I.2). At the infection sites, when neutrophils recognize invading microorganisms, they can phagocytose them. Once the pathogens are encapsulated in phagosomes, the cells activate the release of toxic substances, such as proteolytic enzymes, bactericidal proteins and reactive oxygen species (ROS), into the phagosome, which altogether will lead to the death and destruction of the pathogen (33,36). ROS are produced by neutrophils in an oxidative burst, characterized by a rapid increase in oxygen uptake, increase of glucose consumption and abrupt ROS production and release (36,37). Besides being released from the neutrophil granules into the phagosome, the antimicrobial proteins and ROS can also be released into the extracellular medium, in a process referred to as degranulation, and act against pathogens extracellularly (33,38). In addition to these two ways of action, activated neutrophils may also undergo the process of NETosis, in which they can eliminate extracellular microorganisms by releasing neutrophil extracellular traps (NETs). NETs are composed of decondensed chromatin material and also histones, cytoplasmatic proteins and granular enzymes, such as myeloperoxidase (MPO) and neutrophil elastase (NE), that are attached to the DNA (33,39). The NETs are capable of binding to pathogens, preventing their spread and facilitating their phagocytosis, as well as being able to directly kill them (33,40).

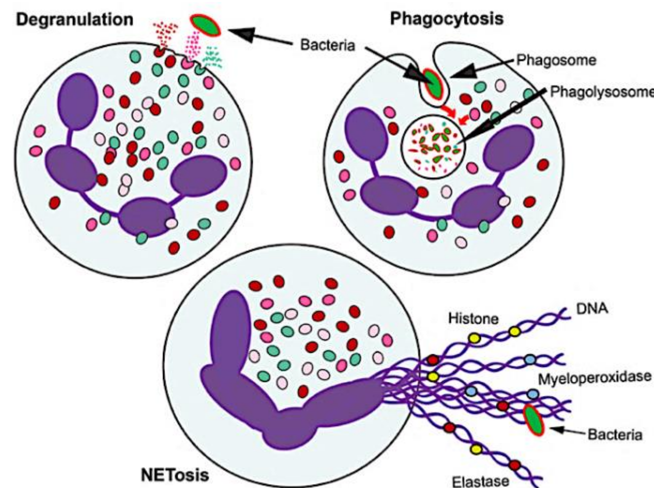



**Figure I. 2 – Antimicrobial mechanisms of neutrophils – Phagocytosis, Degranulation and NETosis.** Adapted from Rosales, Frontiers in Physiology 2018 (34).

According to the classical view, neutrophils represent a homogenous population with the restricted functions mentioned above. However, recent research has been demonstrating that neutrophils have, in fact, phenotypic heterogeneity and a more diverse repertoire of functions. Neutrophils have the ability to respond to multiple environmental signals and to produce several effector molecules according to them. Neutrophils have been demonstrated to produce not only key pro-inflammatory mediators, such as cytokines IL-1β, TNF-α and IL-12 (41–43), but also anti-inflammatory molecules, as TGF-β (44), that help in the resolution of inflammation. Hence, neutrophils are able to modulate inflammation and to interact with innate immune cells, including macrophages and dendritic cells (DCs), and also adaptive immune cells, namely T and B lymphocytes, influencing their activity (34,35). Moreover, neutrophils may also have an active role in several diseases, including chronic inflammation and cancer (45–47). This multitude of neutrophils' features is possible since these cells are transcriptionally active, being able to induce changes in the expression of effector molecules and undergo polarization towards distinct phenotypes, as a consequence of exposure to specific environmental signals (34,35).

Thus, in the past few years, neutrophils have emerged as crucial effectors and regulators of both innate and adaptive immune systems and appear also as important participants in a variety of diseases, becoming a focus of growing interest. Recent studies have indicated neutrophils as important players in tumor biology and cancer development and so their particular role in cancer will be further explored in the next subsection.

### *3.2 Neutrophils in cancer*

The presence of the immune infiltrate, and particularly the presence of neutrophils, was once considered an attempt of the immune system to eradicate cancer. However, in the past few years,

this has become a considerably more complex topic, since cancer-associated inflammation has been proved to play a crucial role in tumor initiation and progression (48). Indeed, patients with advanced stages of cancer present high levels of neutrophils in their blood. Neutrophilia and the neutrophil-to-lymphocyte ratio (NLR) have been associated with poorer prognosis in several types of cancer, namely melanoma, breast, gastric, and non-small cell lung cancer (49–52). The mechanisms by which tumors induce neutrophilia and further recruit neutrophils to the TIME are yet to be completely unravelled. However, the production of granulocyte macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), IL-6 and IL-8 in the TIME, have been indicated as possible mechanisms (53,54).

Several researchers are now focused on trying to uncover the distinct functions of neutrophils throughout the process of carcinogenesis, from tumor initiation to growth and metastasis. As previously mentioned, neutrophils are heterogeneous and both tumor-associated and circulating neutrophils are capable of presenting phenotypical and functional plasticity when exposed to specific TIME-derived cues (55).

Thereby, several subpopulations have emerged for both tumor-associated and circulating neutrophils, based on their phenotype and function (55). These distinct neutrophil subsets will be further explored in the following subsections.

#### *3.2.1 Tumor-associated neutrophils*

Tumor-associated neutrophils (TANs) represent a significant part of the immune cell infiltrate of solid tumors. After being traditionally viewed as bystanders, it is now established that, once in the TIME, neutrophils become influenced by tumor-secreted factors, experiencing possible changes in their phenotype and taking on different functions, depending on the environmental cues.

This modulation leads to the arising of distinct subpopulations and is frequently referred to as neutrophil polarization. In 2009, Fridlender *et al*., were the first to suggest that TANs may undergo polarization into N1, with a proinflammatory and antitumor phenotype, or N2, with a protumor phenotype, mirroring the existent nomenclature for T-helper cells and macrophages, Th1/Th2 and M1/M2, respectively (20). It has been demonstrated that TGF-β, an immunosuppressive cytokine, is one of the main modulators of neutrophil polarization (20). TGF-β is both produced by cancer cells and immune cells present in the TIME and it has been shown to promote the polarization towards a protumor phenotype (N2), as the blockade of this TGF-β signalling, in tumor-bearing mice, favoured the accumulation of N1 (20). TGF-β blockade also enhanced antitumor immunity, as N1 neutrophils become cytotoxic and activate CTLs, helping to demonstrate that opposingly to N1, the presence of N2 TANs benefits tumor progression (20,56). Additionally, IFN-β has also been shown to be involved in neutrophil polarization. IFN-β has the opposite effect of TGF-β, stimulating N1 polarization (Figure I.3) (57).

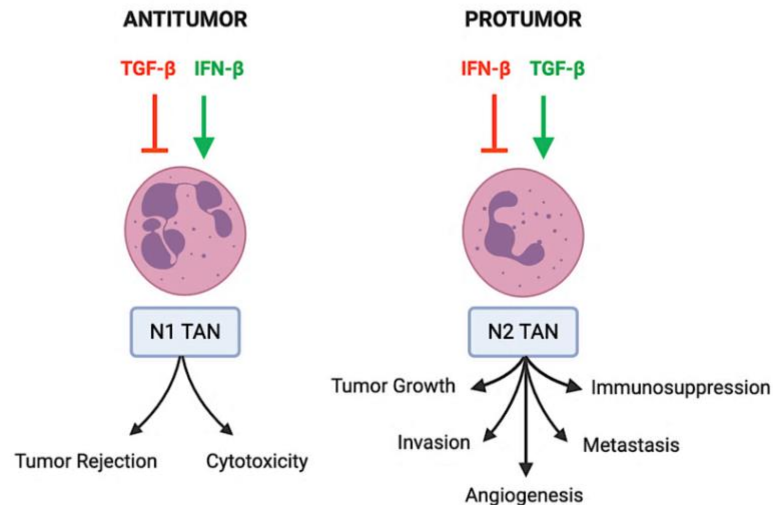



**Figure I. 3 – N1/N2 polarization of TANs and respective activity.** Adapted from Masucci, Minopoli and Carriero, Frontiers in Oncology 2019. Figure elaborated in Biorender.

As mentioned, the N1 and N2 subsets represent anti- and pro-tumorigenic populations, respectively, which differ both morphologically and functionally. Antitumor N1 TANs act in order to prevent tumor growth and progression. They have hyper-segmented nuclei, a short lifespan and a mature phenotype (58). While protumor N2 TANs act in order to support tumor development. These cells have band-shaped nuclei, a longer lifespan and tend to present a mixture of mature and immature phenotypes (58).

On a functional level, N1 neutrophils can display their antitumor activity through numerous mechanisms, some of them not completely understood, but many involving the normal antimicrobial and immune regulatory functions of neutrophils. These mechanisms include cytotoxicity through the secretion of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), through the release of high levels of TNF-$\alpha$, nitric oxide (NO), $H_2O_2$ and hypochlorous acid (HOCl), which are all mediators of cytotoxicity, leading to damage and eventual death of tumor cells (59–61), or through antibody dependent cell-mediated cytotoxicity. Additionally, proinflammatory N1 TANs are also capable of stimulating antitumor immune responses, recruiting and activating T cells, DCs and macrophages (59). This subset expresses high levels of chemokines (e.g. CCL3, CXCL9 and CXCL10) and proinflammatory cytokines (e.g. IL-12, TNF-$\alpha$, GM-CSF and VEGF) that attract and stimulate CTLs, enhancing the adaptive antitumor immune responses (20). For instance, N1 TANs have been shown to promote T cell proliferation and lead to IFN-$\gamma$ increased production, limiting tumor development (59,60).

On the contrary, N2 neutrophils have low cytotoxicity and have been shown to promote tumorigenesis through a variety of mechanisms, most of them are yet to be completely explored. These mechanisms include: i) promotion of genetic instability through the release of ROS (62); ii) support of tumor growth through the production of growth factors and NE, which stimulate tumor

cell proliferation (63); iii) involvement in the remodelling of the extracellular matrix, supporting tumor cell invasion and dissemination, by secretion of metalloproteinases (e.g. matrix metalloproteinase (MMP-) 9) (64); iv) stimulation of angiogenic pathways through the release VEGF (65) and v) suppression of T cell antitumor responses (66,67) (Figure I.3).

N2 TANs do not produce high levels of proinflammatory cytokines as do N1 TANs. Instead, N2 have high expression of CCL17, CCL2 and arginase 1 (ARG1), indicating an immunosuppressive phenotype (68). The chemokine CCL17 encoding gene was found to be strongly upregulated in TANs with N2 phenotype, in comparison to the N1 TANs (69). CCL17 is a ligand for the CCR4 receptor expressed in Tregs (70), and neutrophils have been shown to attract Tregs into the TIME via CCL17 secretion, which in its turn may promote a suppressive environment, inhibiting the antitumor activity of CD4+ and CD8+ T cells (31). N2 neutrophils may also limit CTLs' functions through the release of ARG1, which is a potent inhibitor of T cells' proper activation (54). Additionally, it has been demonstrated that high expression of the programmed death ligand 1 (PD-L1) in neutrophils has a profound suppressive role on T cells that express its receptor, the programmed death protein 1 (PD-1) (71). All of these neutrophil-mediated suppression pathways of T cell cytotoxicity enables tumor immune evasion, which is crucial for tumor survival and progression. It is clear that neutrophils have a dual role in cancer, supporting or limiting tumor progression through a large variety of mechanisms. Nonetheless, it is important to mention that the paradigm of N1/N2 TANs has been described mainly based on murine models and has yet to be replicated with human TANs, with the risk of representing an oversimplification of what actually happens with tumor infiltrated neutrophils. In fact, the nature and function of human neutrophils in the TIME remains largely unknown and it is likely that N1 and N2 represent only the two extremes of a biological spectrum of neutrophil plasticity dependent on environmental modulation (34,58).

#### *3.2.2 Circulating neutrophils*

To the resemblance of what happens with TANs, circulating neutrophils are also a heterogeneous population that can be divided into different subsets, distinguished based on density variation. In normal health conditions, neutrophils can be isolated from whole blood using a density based gradient, as they sediment above red blood cells in the normal density granulocytes fraction, being known as high density neutrophils (HDN) (61). In contrast, during inflammatory and/or pathological conditions, neutrophils can also emerge in the mononuclear fraction, being considered low density neutrophils (LDN) (61) (Figure I.4).

Several studies have suggested LDN as a distinct subpopulation of neutrophils that appear transiently during self-resolving inflammations and that tend to accumulate in several pathologies including cancer, sepsis and autoimmune diseases (72–75). Recently, LDN has become an object of considerable clinical interest, as their frequency often appears to be correlated with disease aggressiveness and/or response to treatment (76,77).

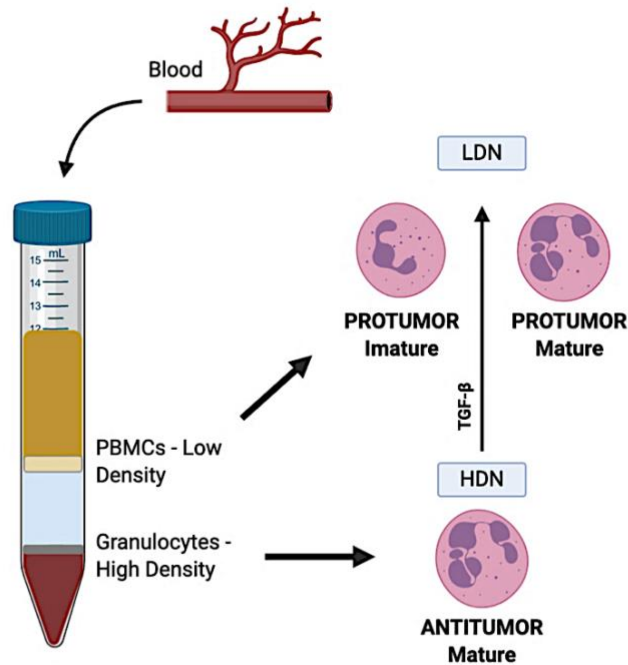



**Figure I. 4 – Subsets of circulating neutrophils, LDN and HDN, and respective phenotype.** Adapted from Grecian, Whyte and Walmsley, British Medical Bulletin 2018. Figure performed in Biorender.

Morphologically, HDN comprise a homogenous population of mature cells, whereas LDN represent a mixed population composed of mature neutrophils and immature neutrophils (75) (Figure I.4). Phenotypically, it is still difficult to distinguish LDN from HDN, since both subsets express the same cluster of differentiation (CD) molecules. However, the expression levels of CD11b and CD66b are higher in LDN (75,78). CD11b and CD66b are adhesion molecules expressed by granulocytes, which are considered activation markers since they are highly expressed by more activated cells (79). Therefore, LDN are frequently suggested as a subpopulation of activated/degranulated neutrophils with impaired function (75,76). Regarding their functions, LDN comprise a subset with immunosuppressive properties, although their exact mechanisms of action are still not clearly elucidated, while HDN play the conventional role of neutrophils, described previously in subsection 3.1 (75).

In the particular case of cancer, HDN have been shown to have the capability to kill tumor cells and to promote antitumor immune responses, whereas LDN are not cytotoxic and display properties that support tumor progression (77). Similarly to what happens in the tumor, it has been shown, in tumor-bearing mice models, that HDN are capable of switching towards the LDN' phenotype in a TGF-β-triggered way, losing cytotoxicity against tumor cells and gaining immunosuppressive properties (77). However, this transition may occur spontaneously, as the tumor progresses to later stages (77). Hence, HDN and LDN are believed to mirror the phenotypes and properties of TANs N1 and N2, respectively, presumably sharing similar mechanisms of action as the ones described in the previous subsection. Additionally, LDN can help in the metastatic process through several

mechanisms, though the main one involves NETs. NETs have been shown to play a protumor role during tumor progression. By entrapping malignant cells, NETs can either promote their proliferation through the NE present on the structure or support the early adhesion of circulating tumor cells in distant organ sites (80–82). Moreover, MPO present in the NETs has been shown to impair T cell activity, sustaining the protumor role of NETs (83).

Although it is well established that neutrophils feature heterogeneity in their morphology, phenotype and function, a clearer and more precise division is yet to be achieved. Indeed, so far there are no surface markers that allow a clear distinction into the different subsets. In fact, there is still an ongoing debate on whether LDN and MDSCs are separate populations or not. As previously described, MDSCs are immature myeloid cells that exhibit immunosuppressive properties. The appearance of MDSCs is normally associated with pathological conditions like cancer or chronic inflammation and are rarely present in homeostatic conditions (84). When activated these cells release ROS, NO and arginase, inhibiting T cells' activity (84). As no clear membrane marker has been identified to differentiate neutrophils from MDSCs and the later and LDN have similar properties, some authors suggest that MDSCs may be, in fact, a part of the neutrophil subsets with a different level of activation (55).

Thus, there is still a lack of knowledge, especially in humans, when it comes to the full characterization of each neutrophil subset and also clear comparison between the characteristics of circulating neutrophils and TANs, in order to fully comprehend these topics.

In this thesis, the role of neutrophils in cancer, especially the LDN, was characterized, with a focus on breast cancer.

## 4. Immunotherapy

As mentioned, there is accumulating evidence from mice models and human cancer patients that emphasize the importance of the immune system in identifying and eliminating malignant cells. However, on the other hand, the immune system can also play a key role in supporting tumor progression. Considering the pivotal role that the immune system has in the regulation of either tumor elimination or progression, clinicians have started to take advantage of the knowledge gathered about immune processes in order to improve cancer treatment. Indeed, immunotherapy has been gaining considerable interest, particularly in the past decade (85). Immunotherapeutic approaches have the advantage of using and potentiating the components of the patient's own immune system to specifically target cancer cells, therefore having the potential to become more precise, personalized and effective and also to mitigate some of the side effects, when opposed to the conventional treatments (86).

Many strategies to enhance the antitumor immune responses have been and continue to be tested in clinical trials and some of them have even been already approved to be used in the clinic. These

approaches include, for instance, targeted antibodies, cancer vaccines, dendritic cell-based vaccines, checkpoint inhibitors and adoptive T cell therapy. One of the immunotherapies that has been particularly successful so far is the immune checkpoint blockade.

Therapies using immune checkpoint inhibitors are based on the capacity that tumor cells have to activate inhibitory immune checkpoint pathways that suppress the function of T cells. Thus, the blockade of this immune checkpoints reinvigorates antitumor immune responses by interrupting the inhibitory signaling pathways and ultimately promoting immune-mediated elimination of tumor-cells (87). There are several immune checkpoints that have been studied to be used in these blockade therapies, some of the most promising include the use of antibodies against the PD-1, PD-L1 and CTLA-4 checkpoints.

Tumor cells are able to increase the expression of PD-L1 to interact with PD-1 expressed in CTLs, inhibiting their cytotoxic activity (88). As such, antibodies against either PD-1 or PD-L1 have been developed and some have already been approved to be used in the clinic in different cancer types, for instance, nivolumab (anti-PD-1) has been approved for the treatment of melanoma, lung, kidney, head and neck, colorectal and liver cancers (89) and atezolizumab (anti-PD-L1) for the treatment of metastatic triple negative breast cancer, bladder and lung cancer (89).

CTLA-4 is another immune checkpoint and it is commonly expressed on the surface of T cells, particularly on the surface of Tregs (90). The interaction between CTLA-4 and its ligands, CD80 and CD86, present on the surface of antigen-presenting cells competes with the co-stimulatory signal between CD28 and CD80/86, leading to the downregulation of T cells' activity. Anti-CTLA-4 antibodies potentiate antitumor responses by blocking the inhibitory CTLA-4 receptors and obstructing the suppressive function exerted in T cells, facilitating T cell activation (90). Ipilimumab is one the antibodies that target CTLA-4 and it has been approved for the treatment of melanoma, kidney and lung cancer (89).

Although immune checkpoint blockade therapies have opened new avenues in cancer treatment, the patients' response to these treatments is still very variable and only a minority of patients have durable benefits and do not relapse (91). However, even if immunotherapy doesn't work for every patient it still represents a promising option for cancer treatment, either to be administrated by itself or to be combined with conventional treatments, such as chemotherapy, to improve their effectiveness. Therefore, a deeper analysis of the interplay between the tumor and the immune system is still needed in order to continue to improve the existent therapies, as well as to reveal new possible targets that can be used to modulate the host immune features as novel cancer treatment options.

## 5. Breast Cancer

### *5.1 Breast cancer features*

Breast cancer (BC) is the most common malignancy in women worldwide, accounting for up to 2 million new cases per year and being estimated that 1 in every 8 women is going to be diagnosed with this disease during their lifetime (92,93). Although male BC is rare, contributing to only 0.5-1% of all diagnoses, when considering both females and males, BC is the second most frequent type of cancer (92,94). As to the mortality, BC is estimated to kill more than 600 000 women per year worldwide, representing 15% of all cancer-related deaths in women (Figure I.5) (92). Thus, BC remains the main cause of cancer-related death in women despite the advances in early diagnosis and treatment.

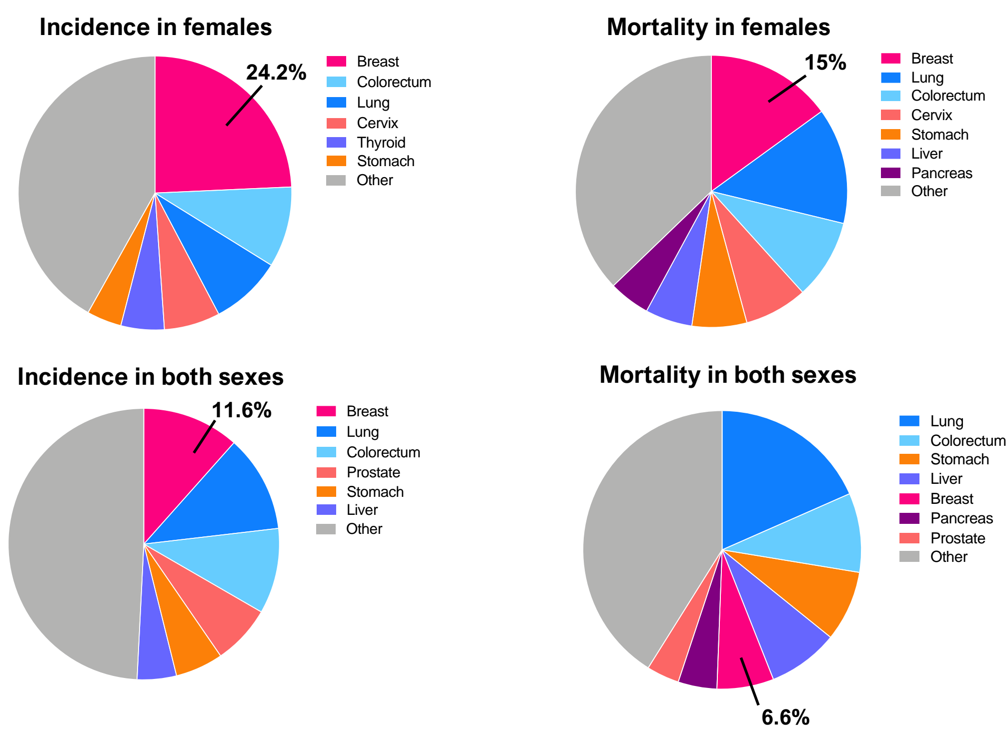
There are numerous risk factors that may be associated with BC development. These risk factors can be divided into two different groups, the first including inherent factors, such as sex, age, race, family and reproductive history, and the second including extrinsic factors, such as an unhealthy lifestyle or diet (95). BC incidence is highly correlated with age and the majority of patients are menopausal women with more than 50 years (95). Another important intrinsic factor is the family history, namely the inheritance of mutations in the *BRCA1* and *BRCA2* genes, with about 20-25% of hereditary breast cancers and 5-10% of all breast cancers being caused by mutations in these genes (96). Reproductive factors such as early menarche, late menopause, or late age of first pregnancy may also increase breast cancer risk (96). Moreover, unhealthy lifestyles, based on a high-fat diet, excessive alcohol consumption and smoking, can also contribute to an increased risk to develop BC (95,96).

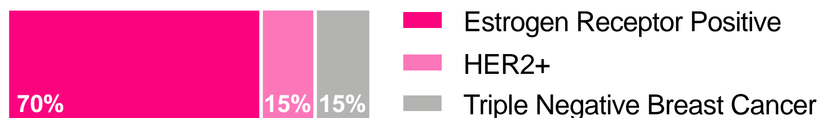
BC is a heterogeneous disease that can be divided into three different subtypes based on different molecular markers (Figure I.5). The most frequent subtype is the one that has an overexpression of the estrogen receptor (ER) and that may also have an upregulation of the progesterone receptor (PR). Abnormal expression and regulation of these receptors lead to uncontrolled cell division and activation of oncogenic growth pathways in breast cancer cells (97,98). This subtype is referred to as hormone positive or ER+ and represents around 70% of all cases. Tumors with an expression of either of the mentioned receptors on at least 1% of tumor cells are included in the hormone positive subtype (97). The second main molecular subtype is characterized by an amplification of the *HER2* gene (human epidermal growth factor receptor 2), which encodes for a transmembrane tyrosine kinase receptor. Tumors with amplification or overexpression of this gene are classified as HER2+ and represent approximately 15% of breast cancers (97). Patients with HER2+ breast cancer may also present upregulation of the hormone receptors (97). The third subtype is referred to as triple negative breast cancer (TNBC), characterized by the lack of expression of the three molecular markers, ER, PR and HER2, and represents approximately 15% of the breast cancer patients (97).

Besides the molecular-based categorization, BC can also be classified histopathologically according to the localization of the tumor. It can be either ductal or lobular, depending if the tumor mass starts to develop in the milk ducts or the milk-producing lobules (99). Additionally, BC can be invasive, when it has spread from the milk duct or lobules to other tissues in the breast, or non-invasive, referred to as "*in situ*" (99).

BC can be further classified according to the TNM system, in which T refers to the tumor size, N to the presence of cancer cells in the axillary lymph nodes and M to the presence of distant metastases (100) (Figure I.5). The T category can be divided into T1 – tumor size less than 2 cm, T2 – tumor size less than 5 cm, T3 – tumors bigger than 5 cm and T4 – tumors that spread into the skin and/or chest wall or it can also represent inflammatory tumors (T4d) (101). N describes the lymph node status: N0 – absence of affected lymph nodes, N1 – palpable mobile axillary lymph nodes involved, N2 – fixed axillary lymph nodes involved and N3 – infraclavicular, internal mammary or supraclavicular lymph nodes involved (101). Finally, M reveals if cancer has spread to other parts of the body or not, M0 and M1 indicate the absence and presence of distant metastasis, respectively (101).

BC survival varies according to the features of the tumor and it is different for each of the molecular subtypes. In fact, patients with breast tumor smaller than 2 cm and without lymph node involvement can have a survival of 99% for ER+ tumors, at least 94% for HER2+ cases and at least 85% for TNBC (97). Whereas in more aggressive cases, when the disease has spread to other organs, patients have a median overall survival of 5 years for ER+ and HER2+ subtypes and only 1 year for TNBC (97).



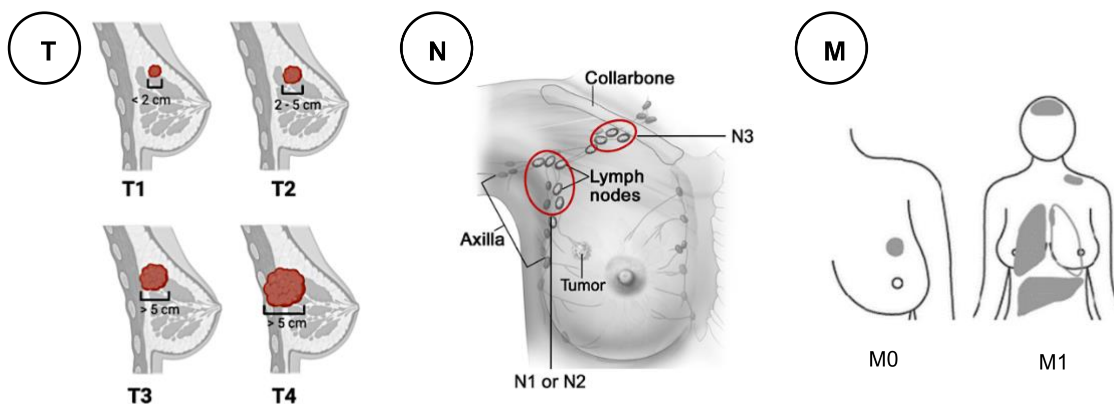




**Figure I. 5 – Breast Cancer – Incidence and mortality; Molecular Subtypes and TNM Staging.** Breast cancer accounts for approximately 25% of the cancers in women and around 12% in both sexes, worldwide; representing about 15% of cancer-related deaths in the female population and approximately 7% in both sexes. There are three molecular subtypes of breast cancer with different representativeness in the population. TNM staging is used to classify breast tumor according to their size (T), the presence of affected lymph nodes (N) and the presence of distant metastasis (M). Figure elaborated with Biorender.

### *5.2 Breast cancer treatment*

There are several treatment strategies for BC, depending on the molecular subtype and whether the tumor has already metastasized or not.

For non-metastatic BC the main goal is to eliminate the tumor from the breast and from the regional nodes when affected. Local therapy consists of surgical removal of the tumor and the compromised axillary lymph nodes and, frequently, the surgery is followed by a radiotherapy regime (97). As for systemic therapy, it may consist of preoperative chemotherapy (neoadjuvant chemotherapy) or postoperative chemotherapy (adjuvant chemotherapy). In some cases, both pre- and postoperative chemotherapy may be necessary. Neoadjuvant chemotherapy (NACT) was initially prescribed only for patients with tumors larger than 2 cm and/or with extension to the axillary lymph nodes or with inoperable and inflammatory tumors, with the purpose of shrinking the tumor before surgery (102). However, NACT is starting to be used more widely, including in patients with operable early-stage BC, due to its potential to achieve higher frequencies of complete or partial clinical response and to allow more conservative interventions (103,104).

As mentioned above, there are several treatment approaches and chemotherapeutic regimens available that are adjusted to each patient according to their molecular BC subtype. All patients with ER+ tumors are submitted to endocrine therapy with antiestrogen medication (generally tamoxifen) and some might also require chemotherapy (97). For HER2+ BC patients, the standard treatment is the combination of chemotherapy with targeted therapy that consists of an antibody against HER2 (trastuzumab). The targeted therapy can be administrated during either neoadjuvant or adjuvant chemotherapy regimens. For the HER2+ cases that are simultaneously positive for ER, endocrine therapy is given in addition (97). In the case of TNBC, there are no specific therapies, hence the treatment relies on chemotherapy alone, which can also be pre- or postoperative (97).

For metastatic BC patients, the therapeutic goals are to prolong life and alleviate symptoms, since the disease remains incurable upon metastization. The strategies for systemic treatment used in metastatic patients are fairly the same as the ones summarized above. Nevertheless, for metastatic TNBC, a combination of chemotherapy with the immunotherapy anti-PD-L1 (Atezolizumab) was approved (105).

### *5.3 Predictive biomarkers*

As mentioned in the previous subsection there are several therapeutic approaches for BC and the choice for the best treatment for each patient can be difficult for the clinicians. Furthermore, focusing on the patients who are indicated for NACT, there are more than 50% of patients who do not respond to this treatment, undergoing six months of chemotherapy without taking benefits and having delayed an effective treatment and/or surgery (104,106). Also, by allowing a more breast-conserving surgery, NACT could be associated with an increased local recurrence risk (104). This

enlightens the unmeet clinical need to find predictive markers of response to treatment, in particular to neoadjuvant regimens.

Since tumor development and response to treatment are highly dependent on the interplays that take place in the TIME, two immune-related markers have been indicated as possible predictive markers of breast cancer patients' response to NACT: the tumor infiltrating lymphocytes (TILs) and the neutrophil-to-lymphocyte ratio (NLR).

TILs have been shown to have predictive value for pathological complete response (pCR) in breast cancer patients. Several clinical trials have supported the idea that BC patients with higher levels of TILs have higher chances to achieve pCR. For instance, in the GeparDuo and GeparTrio (validation study) trials, about 40% of the BC patients with biopsies presenting more than 60% of TILs achieved pCR, while tumors without TILs only achieved 10% of pCR rates (107). Moreover, a recent study from our group reported that activated CTLs expressing HLA-DR (an activation marker) were mainly present in the biopsies of BC patients with a good response to NACT and that this biomarker could efficiently predict BC response to NACT (108). This might indicate that the infiltration of activated CTLs is a more meaningful predictive biomarker than TILs themselves, since the latest are functionally heterogeneous and some of the populations, such as Tregs, have even been associated with bad prognosis in BC (109).

Contrarily to TILs that are assessed in tumor tissue samples, the NLR is assessed in the peripheral blood and has also been associated with patient prognosis in cancer (110). In breast cancer, most studies suggest higher NLR values to be associated with poor prognosis. An NLR higher than 3 was indicated as a pointer of poor prognosis in TNBC (111). Similarly, other authors suggested a cut-off value for NLR of 2.05 in a cohort of ER+ breast cancer patients (112). Other studies in cohorts of patients with the 3 different breast cancer subtypes calculated cut-off values for NLR of 4 (49), 3.33 (113) and 2.1 (114). Nevertheless, data is still conflicting regarding a threshold value for the NLR that could make the distinction between good and bad prognosis and there are even other authors that state there is no predictive or prognostic value for NLR in early-stage breast cancer patients treated with NACT (115). This implies that to use the NLR in a clinic setting, further studies are needed.

Hence, the reason why these markers of BC response to treatment are still scarcely used by clinicians may be explained by the fact that tumors have complex mechanisms to escape immune surveillance and that both lymphocytes and neutrophils are composed of different cell types with opposite functions (anti- and protumor).

## 6. Rationale and Specific Aims

The dependency of cancer cells on their microenvironment suggests that targeting the non-cancer-cell component of the tumour might form a basis for the development of novel therapeutic anti-

cancer approaches. It is now well accepted that tumor infiltrated immune cells modulate the tumor microenvironment to promote “immunotolerance” and support tumor growth (116). Neutrophils represent a significant portion of the tumor microenvironment and impact tumor development at multiple levels, from the remodelling of the extracellular matrix to malignant transformation, angiogenesis and modulation of other tumor-infiltrating cells (117,118). Recently, there has been a growing interest in characterizing neutrophils in cancer and in understanding how these cells might impact tumor progression. It has been established that neutrophils are more heterogeneous than initially thought and different subpopulations with distinct activities in diseases have been suggested, based on the production of different effector molecules, under different conditions. In cancer, tumor-associated neutrophils (TANs) can be divided into anti-tumor (N1) or pro-tumor (N2) subsets (20). More recently, it was suggested that circulating neutrophils can also be classified into high density neutrophils (HDN) and low density neutrophils (LDN), which functionally mirror N1 and N2 neutrophils, respectively (77). However, there is still much uncertainty around neutrophils’ populations and their respective features. Moreover, although many research groups have been studying the role of neutrophils in cancer and their prognostic value, the majority, especially regarding the LDN, have been described in murine models. Thus, further investigation and validation still need to be accomplished using human patients’ samples.

Breast cancer is the most frequent type of cancer in women worldwide (92) and, even though there have been advances in early diagnosis and treatment, breast cancer remains the main cause of cancer-related deaths in women. For patients who have tumors larger than 2 cm and/or with extension to the axillary lymph node or inflammatory/inoperable tumors, the treatment of choice is neoadjuvant chemotherapy (NACT). NACT is prescribed before surgery in order to reduce the tumor size and allow a more conservative procedure. However, more than half of the patients submitted to this treatment do not achieve pCR (106). Hence there is an urgent clinical need to find new biomarkers to predict the response to NACT.

Due to the roles suggested for LDN in tumor progression and metastization and the lack of studies in this topic using human patients, we evaluated, in BC, the clinical significance of LDN, particularly in predicting response to NACT. Specifically, we intended to:

1. Investigate if LDN influence the clinical outcome in breast cancer patients – patient response to NACT and tumor progression

In order to investigate if the LDN subpopulation may, in fact, influence the clinical outcome of breast cancer patients, we intended to establish associations between the presence of LDN in the blood and the patients’ clinical data, including NACT response, stage of the disease (non-metastatic/metastatic) and breast cancer subtype.

2. Characterize systemic neutrophils (both high and low density neutrophils) of breast cancer patients

We performed a thorough phenotypic and functional characterization of patient derived LDN and compared it to HDN. The characterization of both subsets included the assessment of the expression of several markers, by flow cytometry, and the implementation of functional assays to evaluate neutrophils' functions, such as the production of reactive oxygen species (ROS), phagocytic capacity and formation of neutrophil extracellular traps (NETs).

To further clarify the immunosuppressive features of LDN we also performed correlations between the frequency of LDN and Tregs or CTLs present in the whole blood of these patients, as well as with some relevant cytokines in patients' plasma. Additionally, we performed *in vitro* co-cultures of peripheral blood mononuclear cells (PBMCs) and LDN to determine if these BC patients derived-LDN can indeed influence the activation of T cells.

To develop this work, we established a collaboration with 4 hospitals in the Lisbon area (Hospital de Vila Franca de Xira, Hospital Santa Maria, Hospital Professor Doutor Fernando Fonseca and Hospital CUF Descobertas) in order to obtain blood samples from breast cancer patients, who were mainly going to be submitted to NACT.

# Chapter II – Materials and Methods

## 1. Patients' Samples

Blood samples from 60 breast cancer patients (48 non-metastatic and 12 metastatic) were provided by Hospital de Vila Franca de Xira, Hospital Santa Maria, Hospital CUF Descobertas and Hospital Professor Doutor Fernando Fonseca. Approximately 10 mL of whole blood was collected in Vacutainer tubes with EDTA (BD Biosciences) and handled within one day post collection. An aliquot was promptly prepared for immunophenotyping by flow cytometry (see section 5) and neutrophils were isolated as described in section 4.

### *1.1 Patient characteristics*

A total of 60 breast cancer patients were included in this study. The inclusion criteria defined that patients should have an invasive breast tumor, more than 18 years, understand the study in which they will be involved and give informed consent for sample collection.

The 48 non-metastatic breast cancer patients included in the study followed the same therapeutic regime, having been submitted to neoadjuvant chemotherapy (NACT). This treatment is mainly administered in patients with tumor size larger than 2 cm and/or disease extension to the axillary lymph node, or inflammatory/inoperable breast cancer and aims to reduce the size of the primary tumor before surgery, allowing a more conservative intervention. In general, NATC includes several cycles of treatment with chemotherapeutic drugs (doxorubicin, cyclophosphamide and paclitaxel), during 6 months.

The patients' clinical characteristics are summarized in Table II.1 below.

**Table II. 1 - Characteristics of non-metastatic patients enrolled in the study** (age and body mass index). Clinical data, such as subtype of breast cancer, tumor grade, tumor dimension, Ki67 (related to the tumor proliferation rate), node status and response to treatment are also summarized.

| | |
|---:|---|
| Age | Median – 57 (range: 31 - 80) |
| Body Mass Index (BMI) | Median – 26.09 (range: 19.36 – 46.68) |
| ER+ (PR -/+) | 31.43% |
| HER2+ including triple positive breast cancer | 48.57% |
| TNBC | 20% |
| Dimension (mm) | Median – 38 (range: 6 – 110) |
| Ki67 | Median – 35% (range: 5%– 95%) |
| Axillary lymph node invasion status | Positive – 61.76% |
| | Negative – 38.24% |

| | |
|---|---|
| NACT response | Response - 50% |
| | Non-response - 50% |

## 2. Healthy Donors' Samples

Approximately 10 mL of whole blood from 8 healthy donors were also collected in Vacutainer tubes with EDTA. Plasma, high and low density cell fractions were obtained and processed as described below (section 4) and used as controls for comparison with the breast cancer patients' samples.

## 3. Ethics

This study was accepted by the Ethical committees of Hospital de Vila Franca de Xira, Hospital Santa Maria, Hospital CUF Descobertas, Hospital Professor Doutor Fernando Fonseca and NOVA Medical School, Faculdade de Ciências Médicas da Universidade Nova de Lisboa.

Participants were recruited voluntarily and written informed consent was obtained (see appendix). Blood samples were collected during the patients' clinical routine and this collection did not influence the patients' treatment or diagnosis.

Sample processing was only performed at CEDOC/NOVA Medical School, according to the Declaration of Helsinki.

## 4. Neutrophil Isolation

Neutrophils from the high and low density fractions were isolated from whole blood through Histopaque-based density gradient centrifugation for further quantification and characterization. Whole blood was carefully layered on top of a solution of equal volumes of Histopaque-1077 and Histopaque-1119 (Sigma-Aldrich) in a 1:1 ratio and centrifuged at 2000 rpm for 20 min, without break. After the centrifugation, the blood components were separated into different layers according to density. From lower to higher density: plasma; peripheral blood mononuclear cells (PBMCs), which are composed of lymphocytes, monocytes and, in some cases, low density neutrophils (LDN); Histopaque solution; granulocytes, including high density neutrophils (HDN) and red blood cells (Figure II.1). The plasma fraction was collected and frozen at -80ºC for ELISA (see section 10). Both cellular fractions were transferred to microtubes and aliquoted for further characterization (see sections 5, 6, 7, 8 and 9).

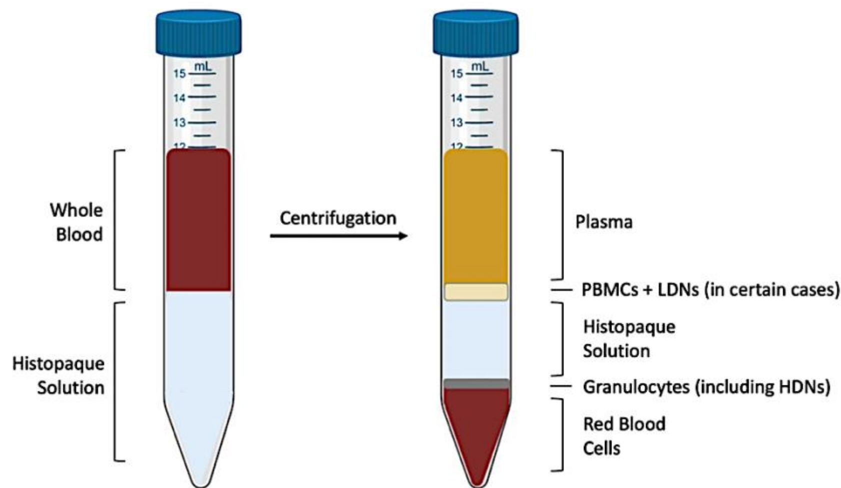



**Figure II. 1 – Separation of whole blood components by density gradient centrifugation.** Figure elaborated with BioRender.

## 5. Immunophenotyping by Flow Cytometry

Flow cytometry is a widely used technology to perform a multi-parametric analysis of single cells in suspension, allowing the assessment of the expression of cell surface and intracellular molecules. In the cytometer, a continuous flow is formed and the cells in suspension pass through the laser beam, one cell at a time. As each cell passes through the beam, the light is scattered in two different directions. A detector in front of the light beam measures the forward scatter (FSC) and a detector on the side of the beam measures the side scatter (SSC). The FCS gives information about the relative size of the cells and the SSC indicates the internal complexity or granularity of the cell. Additionally, cells can be labeled with fluorescent markers that bind to specific molecules and the fluorescence emitted from positively stained cells is measured by fluorescence sensors. As such, distinct cell populations can be distinguished based on differences in their size and granularity alone, as well as based on the expression of particular molecules (119).

### *5.1 Antibody staining*

Antibody staining for flow cytometry was performed in whole blood, as well as in the high and low density cell fractions obtained from the whole blood. After processing the blood samples, a cocktail of mouse anti-human monoclonal fluorescent antibodies (mAbs) was added to the samples and the mixtures were kept in the dark for 15 min at room temperature. The mixtures consisted of 2 μL of each mAb and 100 μL of the sample. For both whole blood and high density fraction, the staining was followed by a step of red blood cell lysis with 2 mL of RBC lysis buffer (Biolegend), for 20 min at 4ºC. A wash step was performed by adding 1 mL of PBS 1X and centrifuged at 300 g for 5 min. The samples were then fixed with 200 μL of FlowFix (Polysciences) and resuspended in 200 μL of

PBS 1X before analyzing. Data were acquired in a BD FACS Canto II with FACSDiva software v8.0.1 (BD Biosciences) and the results were analyzed using FlowJo software v10.

The mAbs used for the staining were: anti-CD3-APC (clone UCHT1), anti-CD4-FITC (OKT4), anti-CD8-PE (HIT8a), anti-CD11b-FITC (ICRF44), anti-CD15-PE (HI98), anti-CD25-PE (BC96), anti-CD33-APC-Cy7 (P67.6), anti-CD66b-APC (G10F5), anti-CD69-PercP (FN50), anti-CD127-PE-Cy7 (A019D5), anti-CCR4-BV421 (L291H4), anti-HLA-DR-APC (L243) and anti-PD-L1-APC (29E.2A3), all from Biolegend.

### *5.2 Gating Strategy*

Using the mAbs mentioned above, the immune populations were defined as follows: neutrophils as $CD15^+$, cytotoxic T lymphocytes as $CD3^+$/$CD8^+$, helper T lymphocytes as $CD3^+$/$CD4^+$ and regulatory T lymphocytes as $CD4^+$/$CD25^{high}$/ $CD127^{low}$.

The gating strategy to identify the different cell populations is represented in Figures II.2-4. The immune populations are represented as a percentage of single cells. In the case of the immune markers CCR4, CD11b, CD66b, CD33, CD69 and PD-L1 we wanted to evaluate their level of expression and for this, the median fluorescent intensity (MFI) was determined. The ratio between the MFI of the positive population and the MFI of the negative population was then calculated in order to minimize and discard the influence of potential fluctuations in the auto-fluorescence of the samples as well as the voltages of the flow cytometer. The negative population was established taking into account the unstained controls.

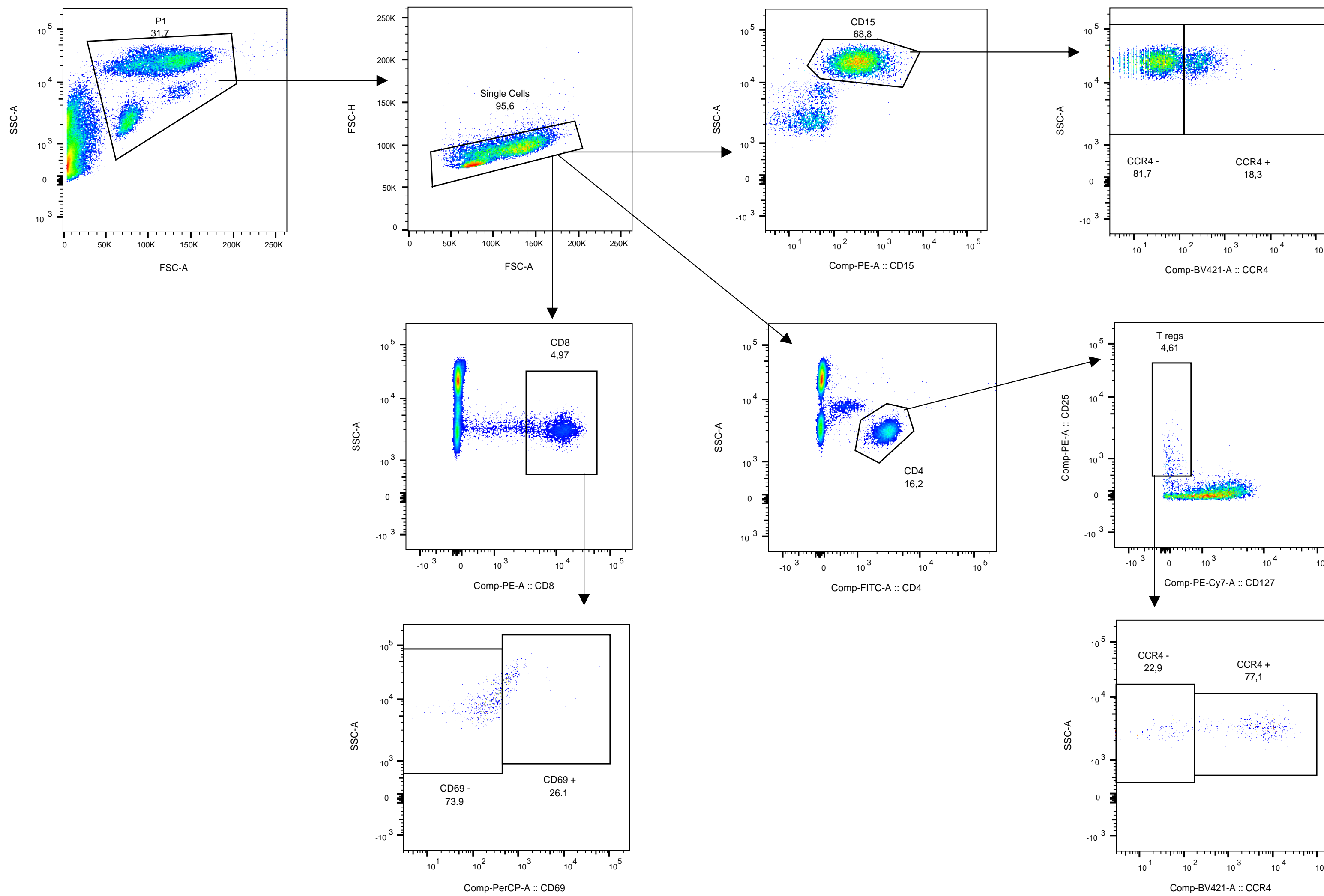



**Figure II. 2 – Gating strategy used to select different immune populations in the whole blood and to analyze the expression of specific markers within the CD15+, regulatory T cells (Tregs) and CD8 + populations.**

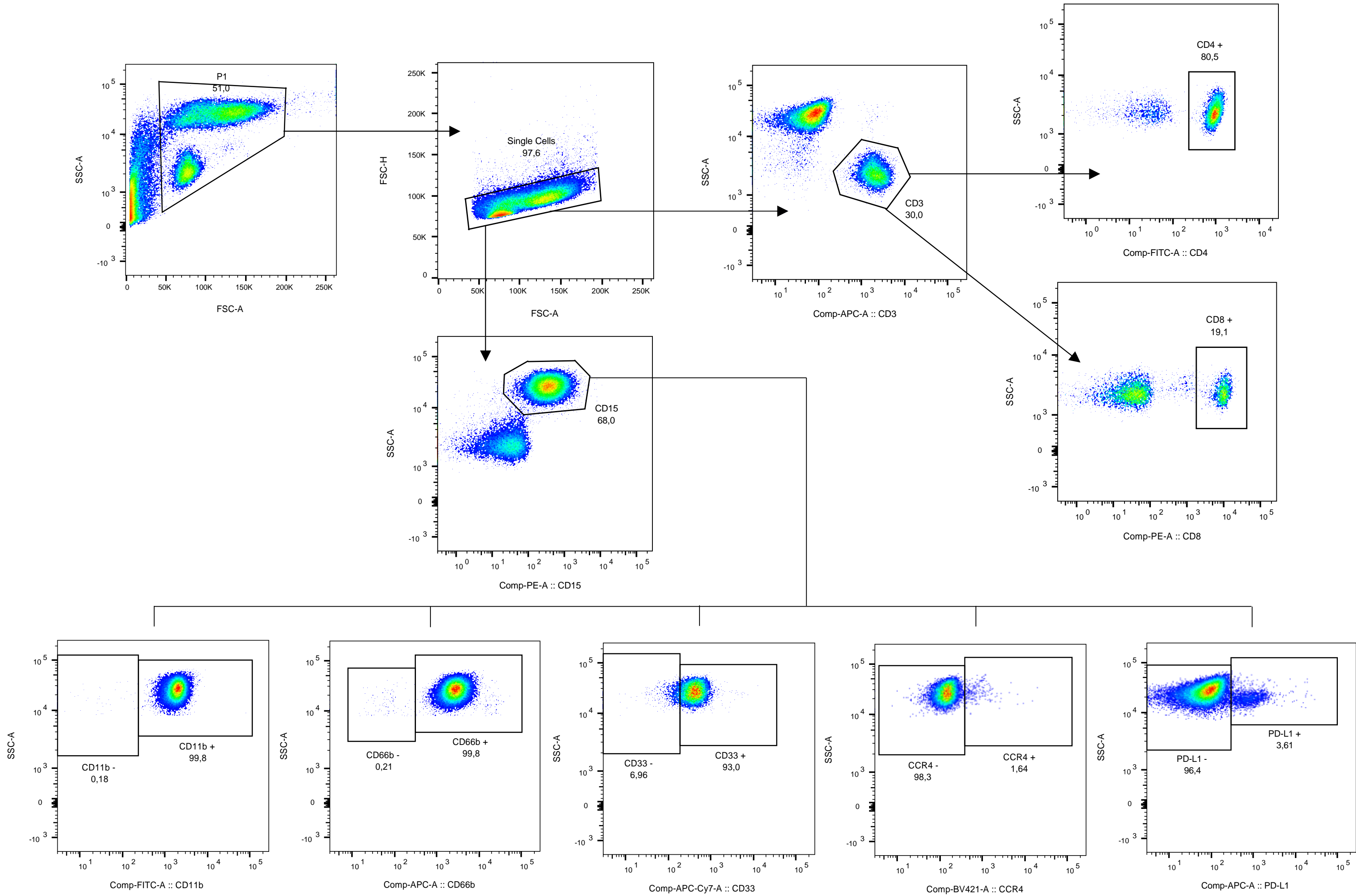



**Figure II. 3 – Gating strategy used to select different immune populations in the high density fraction obtained from the whole blood and to analyze the expression of different markers within the CD15+ population.**

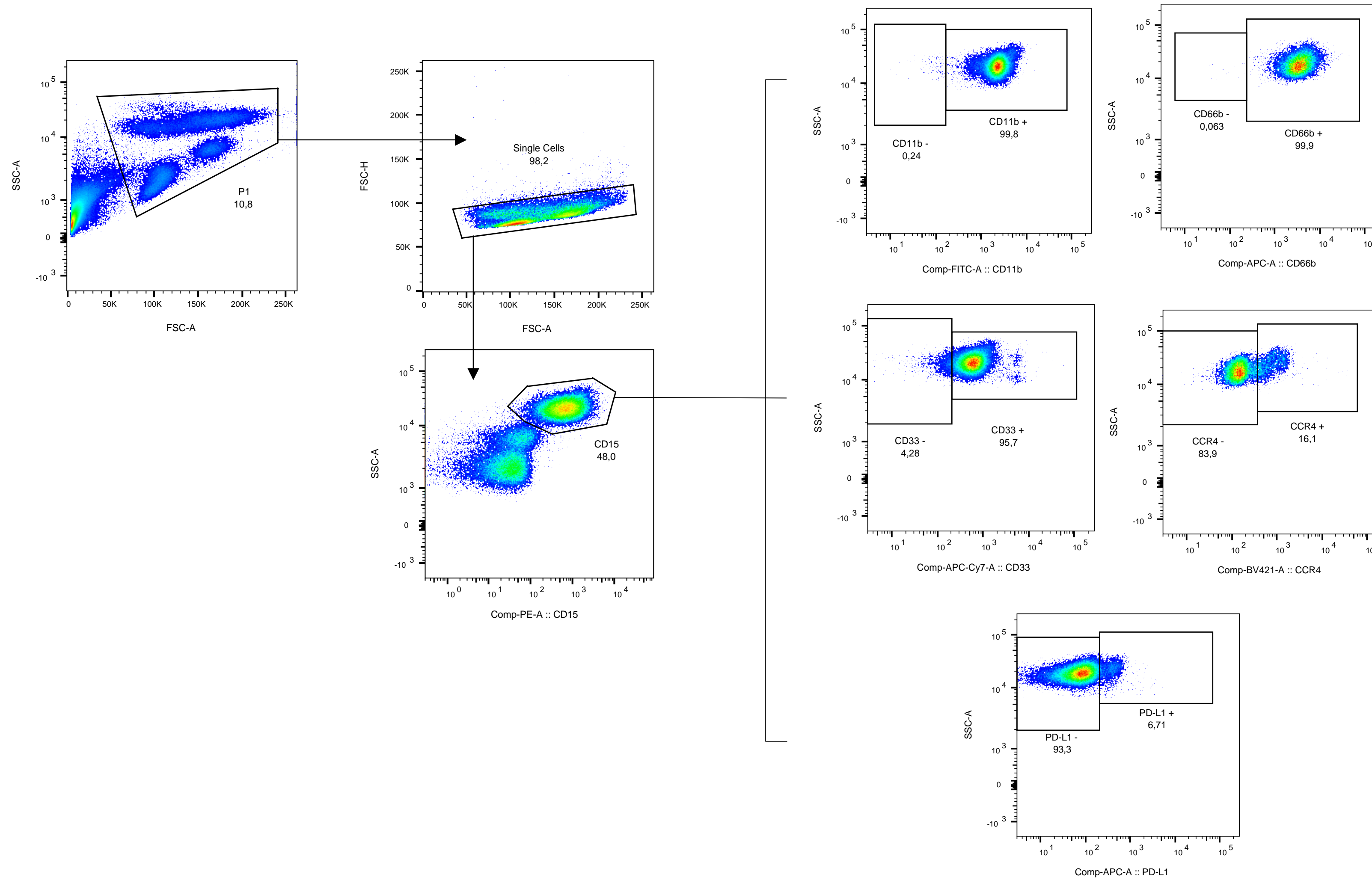



**Figure II. 4 – Gating strategy used to select different immune populations in the low density fraction obtained from the whole blood and to analyze the expression of different markers within the CD15+ population.**

## 6. Evaluation of the phagocytic capacity

The phagocytic capacity of neutrophils from each subset (HDN and LDN) was assessed by using *Escherichia coli* (*E. coli*) labeled with fluorescein isothiocyanate (FITC, Sigma).

### *6.1 E. coli labelling with FITC*

*E. coli* was grown overnight in Lysogeny broth (LB) in a shaker at 37ºC. The cultures were centrifuged at 12 000 g and the pellet was resuspended in PBS 1X. Bacteria were heat-killed at 95ºC for 1h and centrifuged at 12 000 g for 10 min. The bacterial pellet was resuspended in 1 mL of 0.1 M sodium carbonate buffer (pH = 9) and incubated with 0.1 mg/mL of FITC (from a stock of 10 mg/mL in DMSO) for 1h in the dark with shaking at room temperature. In order to remove the unbound dye, cells were rinsed 3 times with PBS 1X and centrifuged as described above. The pellet was resuspended in 1 mL PBS 1X and stored at -80ºC until further use.

### *6.2 Phagocytosis Assay*

Neutrophils obtained (as described in section 4) from the high and low density fractions, were split into 100 µL aliquots and incubated with 10 µL of FITC-labelled *E. coli* suspension, for 30 min at 37ºC or 4ºC. The cells incubated at 4ºC were used as negative controls since phagocytosis does not occur at this temperature. After incubation, 100 µL of trypan-blue solution (1:50 in PBS) was added to each tube to quench FITC fluorescence of bacteria possibly attached to the neutrophils' membrane. Cells were then washed two times with 3 mL of Hank's Balanced Salt Solution (HBSS, HyClone) and centrifuged at 250 g for 5 min, at 8ºC. The cells from the high density fraction were submitted to an extra step of red blood cell lysis with 2 mL of RBC lysis buffer, for 20 min at room temperature. A second wash step was performed by adding 3 mL of HBSS and centrifuged at 250 g for 5 min. The cells (HDN and LDN) were fixed with FlowFix and resuspended in 200 µL of PBS 1X before analyzing.

The phagocytic capacity was evaluated by flow cytometry and the gating strategy is represented in Figure II.5. The internalized bacteria were estimated by measuring the MFI of the cells. More specifically, by calculating the ratio between the MFI of the positive population at 37ºC and the MFI of the positive population at 4ºC, in order to discount the influence of the surface attached bacteria. Higher phagocytic capacity is proportional to a higher value of internalized bacteria. The populations were established taking into account a control without *E. coli*.

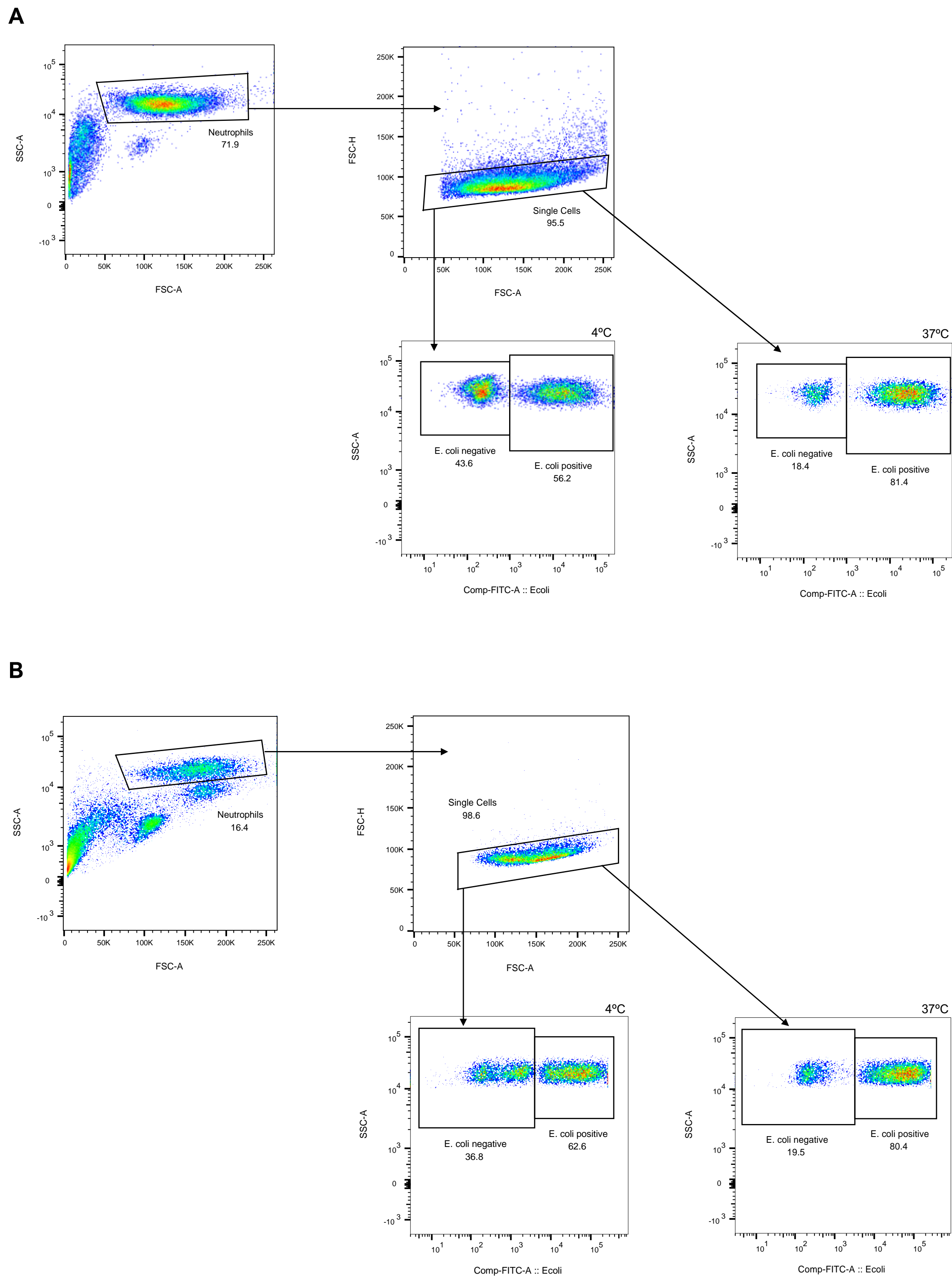



**Figure II. 5 – Gating strategy used to assess the phagocytic capacity.** Phagocytic ability was assessed in both **(A)** high density neutrophils and **(B)** low density neutrophils. The internalized bacteria were estimated by calculating the ratio between the MFI of the positive population at 37ºC and the MFI of the positive population at 4ºC. Higher phagocytic capacity corresponds to higher levels of internalized bacteria.

## 7. Evaluation of the oxidative burst

The oxidative burst of the high and low density neutrophils upon stimulation with phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) was assessed using the 2′,7′-Dichlorofluorescin diacetate (DCFH2-DA, Invitrogen) probe.

After isolating the HDN and LDN as described above (section 4), the cell suspensions were divided into aliquots of 100 μL. For the HDN, red blood cell lysis was performed by adding 2 mL of RBC lysis buffer and incubating for 20 min at 4ºC. The cells were rinsed 2 times with PBS 1X and centrifuged at 300 g for 5 min. After the lysis step, both HDN and LDN tubes were incubated with DCFH2-DA probe diluted in HBSS, in a final concentration of 5 μM (stock 1 mg/mL in DMSO), for 15 min, at 37°C protected from light. Following this incubation period, neutrophil stimulation was performed by adding 200 ng/mL of PMA for 30 min. At the end of the incubation, the tubes were immediately transferred to ice to stop the stimulation and consequent release of reactive oxygen species (ROS). One last wash was performed as described above, before analyzing the ROS production levels. Two types of controls were used in this assay: a tube containing only cells (HDN or LDN) was used as negative control and another tube with cells (HDN or LDN) containing the probe but not the PMA stimulus to access the basal level of ROS production.

The oxidative burst upon stimulation was accessed by flow cytometry, using the gating strategy represented in Figure II.6. The populations were established considering the negative control. The level of ROS released was estimated by measuring the MFI of the stimulated neutrophils; the MFI of the non-stimulated neutrophils was also measured to assess the basal level of ROS production.

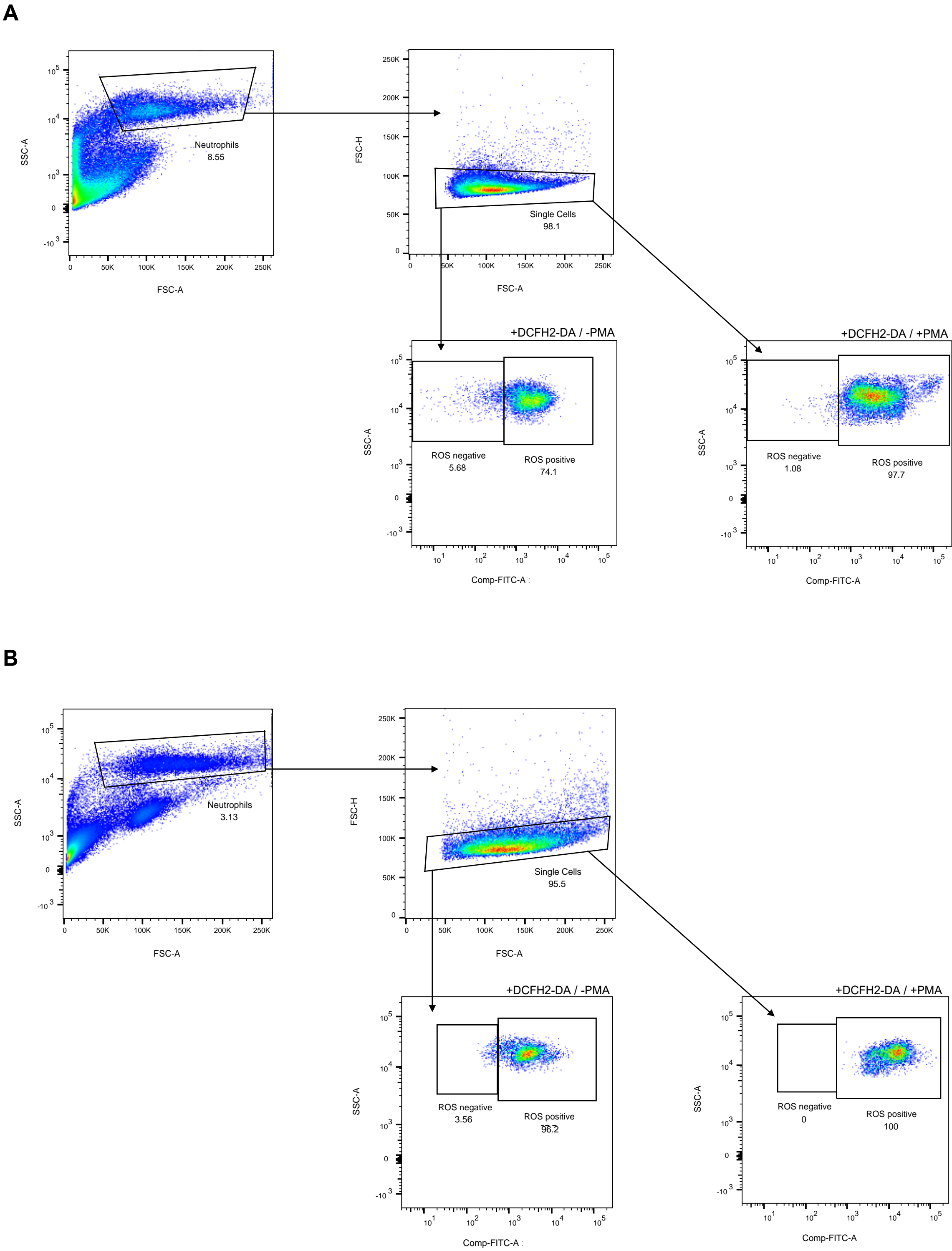



**Figure II. 6 – Gating strategy used to assess the oxidative burst upon PMA stimulation.** The oxidative burst was assessed in both **(A)** high density neutrophils and **(B)** low density neutrophils. The level of ROS produced was estimated by measuring the MFI of the stimulated neutrophils (+DCFH2-DA / +PMA). The MFI of the non-stimulated neutrophils (+DCFH2-DA / -PMA) was measured to assess the basal level of ROS production.

## 8. Evaluation of neutrophil extracellular traps (NETs) formation

In order to evaluate and compare the ability to form neutrophil extracellular traps (NETs), neutrophils from each subset (HDN and LDN) were stimulated in culture and then stained to visualize NETs formation through confocal microscopy.

### *8.1 Stimulation for NETs formation*

HDN and LDN were isolated from the whole blood as described above (section 4) and each fraction was transferred to a new 15 mL falcon. Immediately after the separation process, red blood cell lysis was performed with 10 mL of RBC lysis buffer, for 10 min at room temperature, followed by centrifugation at 1100 rpm for 5 min. After washing with 5 mL of HBSS and centrifugation in the same conditions, the cells were resuspended in 5 mL of RPMI-1640 medium (Gibco) supplemented with 1% of autologous plasma. Following this, HDN and LDN were seeded on top of coverslips (13 mm), in a 12-well plate (VWR). Stimulation with 100 ng/mL of PMA was performed during 3h, at 37ºC.

### *8.2 NETs visualization and quantification*

After the incubation time, the plate was centrifuged at 1100 rpm for 5 min and the supernatant was collected, centrifuged at 2000 rpm for 10 min to exclude all cellular debris and stored at -20ºC for ELISA. The cell culture medium was removed, and the wells were washed with PBS 1X. The cells were fixed with 4% paraformaldehyde (PFA) for 10 min at room temperature. The wells were rinsed two times with PBT (PBS 1X + 0.1% Triton X-100, ACROS Organics) and then blocked with PBT + 1% bovine serum albumin (BSA, Sigma-Aldrich) for 10 min. After removing the blocking solution, the primary antibody (at a concentration of 1:100 in blocking solution) was added to each well and left to incubate for 1h at room temperature. The primary antibody used was a mouse monoclonal anti-Myeloperoxidase (MPO, clone 266-6K1, Santa Cruz Biotechnology). Following this, two rounds of washing were performed with PBT. The secondary antibody goat anti-mouse Alexa 568 (Invitrogen) was added at a concentration of 1:500 in blocking solution and incubated for 45 min in the dark at room temperature. Again, the plate was washed as described above. Counterstaining was performed with 4′,6-diamidino-2-phenylindole (DAPI) solution (0.001 mg/mL in PBS 1X), for 10 min protected from light. Washing was performed 3 times with PBS 1X with shaking and waiting 5 min between washes. Finally, the coverslips were mounted with Fluorescent Mounting Media (DAKO) into microscopy slides. Images were acquired in a confocal microscope (LSM710, Zeiss) and analysed with Fiji software (version: 2.0.0-rc-69/1.52p).

NETs formation was quantified by assessing the NETs area and the MPO median fluorescence intensity. For the NETs area quantification, an automatic threshold was applied in the DAPI channel, to remove the fluorescence related to the nuclear staining. Normalization was performed by doing

a ratio with the nuclei number. Nuclei area was also calculated in the DAPI channel by applying an automatic threshold and measuring the area in the "analyse particles" menu. In all quantifications, 3 different images per patient were analysed and the mean value was obtained.

## 9. Co-culture of LDN with PBMCs

Whole blood from 13 breast cancer patients was collected and the PBMCs fraction was isolated as described in section 4. PBMCs were stained with anti-CD15 for 15 min in the dark on ice, followed by a washing step with PBS 1X and centrifugation at 300 g for 5 min. Cells were then resuspended in PBS 1X supplemented with 2% FBS and 10% Penicillin/Streptomycin (GE Healthcare) and filtered. Cells were sorted into two populations: CD15- (PBMCs) and CD15+ (LDN). Cell sorting was performed in BD FACS Aria III and cells were collected in RPMI-1640 supplemented with 10% FBS and 10% Penicillin/Streptomycin. Following this, PBMCs depleted from CD15+ cells were cultured alone or in a 1:1 ratio with the LDN population (CD15+ cells) in a 96-well plate (Orange Scientific). Stimulation was performed with 35 ng/mL of PMA and 1 μg/mL of ionomycin (Merk Millipore) for 24h. After the incubation, the supernatants were collected as described in section 8.2 and stored at -20ºC for ELISA. The cells were stained with anti-CD3-PerCP (clone HIT3a), CD4, anti-CD8-PacificBlue (SK1), anti-CD69-APC-Cy7 (FN50), CD25, HLA-DR and anti-Ki-67-PE (Ki-67). The staining was performed as described above (section 5.1), except for the intracellular marker Ki67. After the cell surface staining, cells were fixed and permeabilized with the Fix/Perm kit (Invitrogen) for 30 min at room temperature, in the dark. Ki67 was added for 30 min, followed by a washing step with 1 mL of PBS 1X and centrifugation at 300 g for 5 min. The samples were then fixed with 200 μL of FlowFix and resuspended in 200 μL of PBS 1X before analyzing. Data were acquired in BD FACS Canto II with FACSDiva software and the results were analyzed using FlowJo software.

## 10. ELISA

Enzyme-Linked Immunosorbent Assay (ELISA) was performed for the quantification of secreted cytokines in patients' plasma and healthy donors' plasma for comparison, as well as in the supernatants of cell cultures (described in sections 8 and 9). For the first case, plasma was obtained from whole blood as described above (section 4) and the levels of IL-10, IFN-γ, CCL17, TGF-β and IL-17 were measured using commercial ELISA kits. In the second case, the supernatants were harvested and stored as described above. For the supernatants from the cultures with HDN and LDN (section 8) only CCL17 was quantified; while for the supernatants from the co-cultures with PBMCs and LDN (section 9) only the level of secreted IFN-γ was assessed. The IL-10, IFN-γ, TGF-β and IL-17 kits, all from Biolegend, and the CCL17 kit, from R&D Systems, were used according to the manufacturer's instructions.

The ELISA kits for each of the chosen cytokines were based on the sandwich method (Figure II.7). The general steps of this assay are next described. Briefly, a 96-well plate was coated with the specific capture antibody in coating buffer and left to incubate overnight at 4ºC. In the next morning, the plate was washed with PBS 1X + 0.05% Tween-20 (Sigma) and then blocked with PBS 1X + 1% BSA for 1h with shaking at room temperature. After a washing step, the standards and the samples were added to the respective wells and incubated for 2h with shaking. Another round of washes was performed, the detection antibody was added to each well and incubated for 1h with shaking. Another cycle of washing was performed before adding the Avidin-HRP (horseradish peroxidase), followed by an incubation of 30 min with shaking. A final washing step was performed and the 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate (Biolegend) was added to the plate and incubated in the dark for approximately 30 min. Lastly, in order to finish the reaction, stop solution (Biolegend) was added to the plate before reading the absorbance. The absorbances (450 and 570 nm) for each well were read in a plate reader (Synergy HT Multi-Detection Microplate Reader, BioTek) and used to calculate the concentration of the cytokines in the samples, taking into consideration the standard curve values.

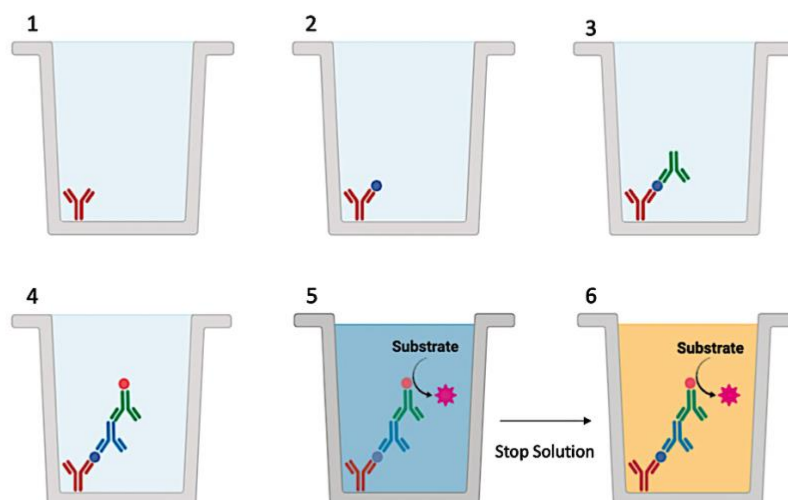



**Figure II. 7 – General Sandwich ELISA Steps.** 1 – Coating with capture antibody; 2 – Incubation with the specific antigen; 3 – Incubation with the detection antibody; 4 – Addition of Avidin-HRP; 5 – Addition of the substrate; 6 – Addition of the stop solution. Figure elaborated with BioRender.

## 11. Statistical analysis

Statistical analysis was performed in GraphPad Prism v8.2.1. Statistical significance was considered for $p < 0.05$. Comparison between samples was performed by a nonparametric Mann-Whitney test and correlations were calculated with Spearman r test. For the comparison of the immune markers in HDN and LDN and for the comparison of the immunological parameters regarding patient's age,

tumor dimension, breast cancer subtype and body mass index, a two-way ANOVA, either with Sidak's multiple comparisons (2 groups) or Tukey's multiple comparisons (3 groups), was performed. T-test was used to compare samples in a stimulated *vs* unstimulated condition.

# Chapter III – Results

## 1. LDN are associated with a worse prognosis in breast cancer patients

Breast cancer (BC) remains the main leading cause of cancer-related deaths among women worldwide (92). There is evidence suggesting that tumors can be heavily infiltrated by immune cells and that the efficacy of anti-cancer chemotherapy can be influenced by the immune system (116). Recently, neutrophils and their role in cancer have become a topic of interest. Several groups are interested in characterizing these cells in a context of cancer and in investigating how neutrophils might impact tumor progression. It has been established that neutrophils are a heterogeneous population composed by different subsets with distinct activities. In fact, tumor-associated neutrophils (TANs) can be divided into antitumor (N1) or protumor (N2) subsets (20). Moreover, it was proposed that circulating neutrophils can also be divided into high density neutrophils (HDN) and low density neutrophils (LDN), which functionally mirror N1 and N2 neutrophils, respectively (77). However, most of the knowledge regarding neutrophils' populations and their respective features, as well as their roles in cancer, comes from studies using murine models. Thus, further investigation and validation of these insights still need to be accomplished using human patients' samples. In order to tackle this question, we intended to evaluate if circulating LDN have clinical relevance in a cohort of BC patients.

### 1.1. Higher levels of LDN in the blood are associated with a worse prognosis and a poor response to neoadjuvant chemotherapy in breast cancer patients

To pursue the goal of investigating if LDN have clinical relevance in BC, we assessed the presence of LDN in the blood of three groups of individuals: healthy donors, non-metastatic BC patients and metastatic BC patients. After a density gradient centrifugation, we determined, by flow cytometry, the frequency of LDN and HDN present in the blood of these individuals. As expected, LDN were almost absent in the blood of healthy individuals when comparing to non-metastatic and metastatic BC patients ($p<0.01$, Figure III.1A). Indeed, LDN are known to arise only in inflammatory or pathological conditions (120). Furthermore, within BC patients, we observed that the percentage of LDN present in metastatic patients was significantly higher than the one present in non-metastatic patients ($p<0.05$, Figure III.1A). These results sustain the idea that LDN are more frequent in more advanced stages of cancer, corroborating that their presence is possibly correlated with metastization and aggressiveness, as it happened in mice (82,121).

As mentioned in Chapter I, neoadjuvant chemotherapy (NACT) is the conventional treatment for BC patients with tumors larger than 2 cm and/or disease extension to the axillary lymph nodes, or with inoperable and inflammatory tumors. However, less than 50% of patients have a good response

to this treatment and a poor response to NACT has been indicated as a prognostic factor for recurrence and disease progression (106,122). As such, it is of major importance to find new biomarkers that are able to detect, prior to treatment, which patients will not respond to NACT, in order to promptly direct them to alternative treatments. Considering that LDN frequency in the blood is associated with BC aggressiveness, we decided to further investigate the role of LDN in non-metastatic patients, particularly regarding response to NACT. We hypothesized that LDN should be more prevalent in patients who do not respond to treatment, being associated with consequent disease progression. To assess this hypothesis, we used the blood of a cohort of 48 non-metastatic BC patients who had been selected to perform NACT. However, only 22 patients had already finished the treatment; so, clinical information regarding response to NACT of 26 BC patients is still missing.

After 6 months of treatment, patients are subjected to the surgical removal of the remaining tumor and the response is assessed trough histopathological analysis in the surgical specimen. Patients were divided into responders and non-responders, following the criteria already established (108). NACT responders were classified as patients who achieved a pathological complete response (n=5) or patients who had a significant decrease in tumor size without axillary lymph node involvement after treatment (n=6). NACT non-responders were classified as patients who did not achieve a tumor down-staging and/or still had disease extension to the axillary lymph nodes after treatment (n=11). We observed that NACT non-responders had a significant higher percentage of LDN present in their blood before treatment, in comparison to NACT responders (p<0.05, Figure III.1B). Thus, these results demonstrate that LDN have a discriminating power regarding patient response to treatment, as higher levels of these neutrophils are present in patients who do not respond to NACT. As such, this immune trace could be potentially used as a predictive biomarker.

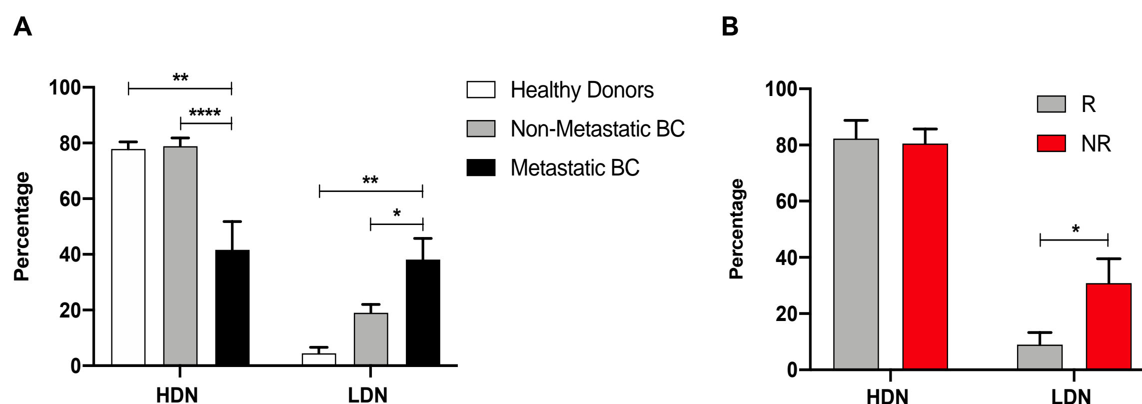



**Figure III. 1 – Low density neutrophils are associated with advanced stages of breast cancer and with poor response to neoadjuvant chemotherapy. (A)** Percentage of high density neutrophils (HDN) and low density neutrophils (LDN) in the whole blood of healthy donors (white bars, n=7), in non-metastatic breast cancer patients (grey bars, n=48) and in metastatic breast cancer patients (black bars, n=12). **(B)** Percentage of high density neutrophils (HDN) and low density neutrophils (LDN) in the whole blood of non-metastatic breast cancer patients with response to NACT (R, grey bars, n=11) and without response to NACT (NR, red bars, n=11). Data are represented as mean ± SEM. Statistical analysis: two-way ANOVA with Sidak's multiple comparisons, *p<0.05, **p<0.01, ****p<0.0001.

## 1.2. LDN are more prevalent in patients with the ER+ breast cancer subtype

Worse prognosis in BC is usually associated with younger age (pre-menopause), obesity, disease spread to the axillary lymph nodes and tumors larger than 20 mm (123,124). Thus, besides investigating if LDN are associated with breast cancer aggressiveness and patient response to NACT, we performed several comparisons between the two neutrophil subsets considering the patients' available clinical data (see Table II.1 of Chapter II). Namely, we took into consideration patients' breast cancer subtype (ER+, HER2+ or TNBC, Figure III.2A), axillary lymph node status (node positive (N+) or node negative (N-), Figure III.2B), tumor size (<20 mm or >20 mm, Figure III.2C), age (<50 years old and >50 years old, Figure III.2D) and body mass index (BMI, normal (18.5-24.9), overweight (25.0-29.9) or obese (>30), Figure III.2E). These comparisons were performed in order to evaluate if LDN are also associated with other factors that can be related to prognosis.

Since NACT is prescribed to BC patients regardless of their tumor subtype it was interesting to observe that, when segregating patients by their BC subtype, patients with the ER+ subtype had a significantly higher percentage of LDN when compared to patients with the HER2 subtype ($p=0.0003$, Figure III.2A) and patients with TNBC ($p=0.03$, Figure III.2A). This highlights that the assessment of LDN could be used as an important tool to determine response to NACT prior to treatment, especially for patients with ER+ BC.

Besides the association with the BC subtype, no other significant differences were observed between the evaluated groups, hence these results sustain the fact that this trace has the potential to be used to predict response to NACT independently of axillary lymph node involvement, tumor dimension, patients' age and body mass index.

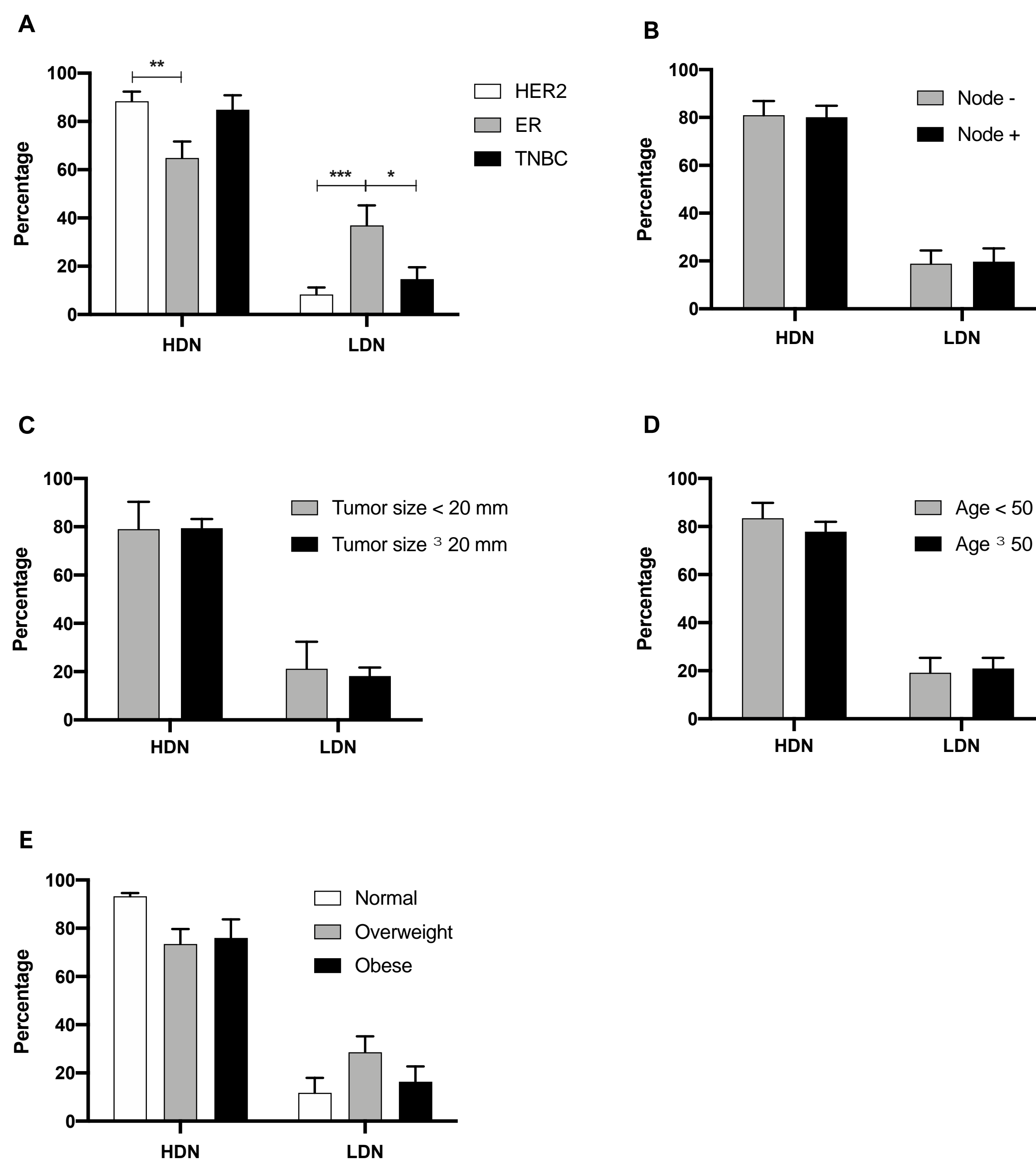



**Figure III. 2 – Low density neutrophils are more prevalent in the ER+ breast cancer subtype. (A)** Percentage of high density neutrophils (HDN) and low density neutrophils (LDN) in the whole blood of HER2 non-metastatic BC patients (white bars, n=17), of estrogen receptor (ER+) non-metastatic BC patients (grey bars, n=11) and of triple negative non-metastatic BC patients (TNBC, black bars, n=7). **(B)** Percentage of HDN and LDN in patients without (N-, grey bars, n=13) or with (N+, black bars, n=20) axillary lymph node metastases. **(C)** Percentage of HDN and LDN in patients with tumors smaller than 20 mm (grey bars, n=6) or tumors with at least 20 mm (black bars, n=33). **(D)** Percentage of HDN and LDN in BC patients younger (grey bars, n=13) or older than 50 years old (black bars, n=28). **(E)** Percentage of HDN and LDN in BC patients segregated by their body mass index: normal (white bars, n=11), overweight (grey bars, n=14) or obese (black bars, n=9). Data are represented as mean ± SEM. Statistical analysis: two-way ANOVA with Turkey's multiple comparisons (A and E) or with Sidak's multiple comparisons (B, C and D), *p<0.05, **p<0.01, ***p<0.001.

### 1.3. LDN are a more meaningful predictive biomarker than the NLR

As mentioned, the neutrophil-to-lymphocyte ratio (NLR) has already been implied as a possible predictive biomarker in BC response to NACT (114,125). However, data regarding which threshold value should be considered for NLR is still conflicting, as different authors suggest distinct values (110). Hence, we decided to perform a comparison of the performance as predictive biomarkers between LDN and NLR in this BC cohort. For this, we assessed the percentage of total neutrophils and lymphocytes present in the patients' whole blood, in order to calculate the NLR (Figure III.3A). After calculating the NLR the BC cohort was divided into NACT responders and non-responders, according to the patients' clinical information (Figure III.3B). The NLR was also assessed in the blood of healthy individuals as a comparative term (Figure III.3B).

We observed no significant differences in the NLR when comparing both groups of BC patients. This result combined with the observation that NACT non-responders have significantly higher levels of LDN present in their blood pre-treatment, endorses LDN as a greater predictive factor, than NLR.

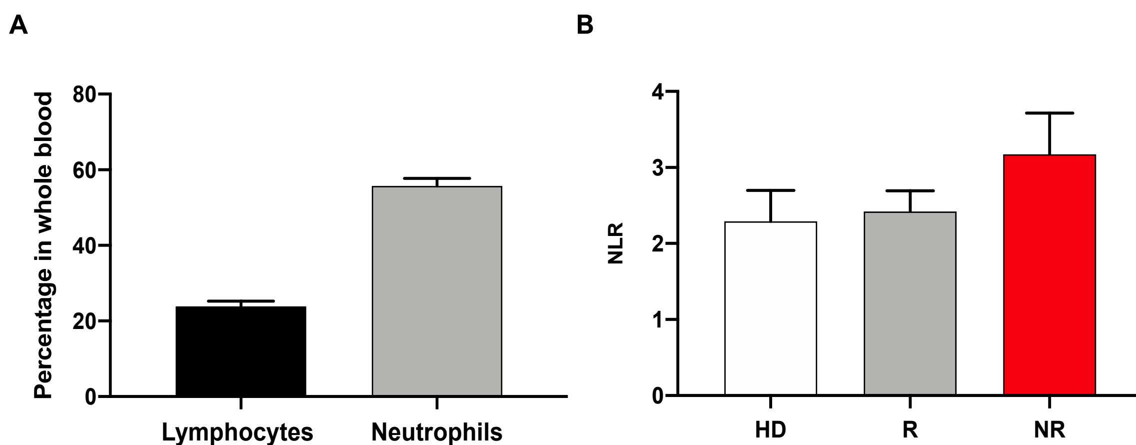



**Figure III. 3 – Neutrophil-to-lymphocyte ratio is not a predictive factor of breast cancer response to neoadjuvant chemotherapy**. **(A)** Quantification, by flow cytometry, of the total lymphocytes and the total neutrophils in the whole blood of breast cancer patients (n=52). **(B)** The quantification performed in (A) was used to calculate the neutrophil-to-lymphocyte ratio (NLR) in healthy donors (HD, white bar, n=6), in breast cancer patients with response to NACT (R, grey bar, n=11) and without response to NACT (NR, red bar, n=11). Data are represented as mean ± SEM.

## 2. LDN are a subset of highly activated cells with enhanced functions

We have seen that LDN are associated with BC aggressiveness, as more advanced, metastatic patients, as well as patients who do not respond to NACT, have increased levels of LDN in their blood (section 1).

The role of neutrophils and, particularly, of LDN in cancer have been intensively studied in the past few years. Nonetheless, studies regarding this topic using human samples are still scarce and so, the majority of the knowledge comes from studies with animal models. Considering that human and mice neutrophils exhibit significant biological differences, it is crucial to validate these insights in cancer patients, in order to fully comprehend their potential clinical utility. To assess this subject, we performed a detailed characterization of BC patients' neutrophils, both HDN and LDN, in terms of their phenotype and function.

## 2.1. LDN have increased expression of activation and immunosuppressive markers

In order to better understand why LDN are associated with worse BC prognosis and response to treatment, we decided to perform a thorough characterization of the LDN and HDN, obtained after density gradient centrifugation of the whole blood from the non-metastatic BC patients involved in this study.

To perform the immunophenotyping of the LDN and HDN subsets, we assessed a set of immune markers, by flow cytometry, regarding both the percentage of cells expressing a specific marker and the level of expression, given by the median fluorescence intensity (MFI), of said marker. The set of markers evaluated in this characterization was the following: CD11b, CD66b, CD33, CCR4 and PD-L1. We chose to assess these markers, based on their functions. CD11b is an adhesion molecule present in granulocytes that is expressed by activated cells (126). CD66b is also a molecule involved in the adhesion mechanisms, present in neutrophils and eosinophils, which is highly expressed in activated cells (79). CD33 is expressed by granulocytes precursors and its expression decreases as the cell matures, being considered a maturation marker (127). CCR4 is a chemokine receptor with affinity for CCL17 and CCL22, involved in immunosuppressive pathways. Lastly, PD-L1 is an immune checkpoint that when bound to its receptor PD-1 in T cells, inhibits their activity.

The percentage of cells expressing these markers was similar between LDN and HDN, except for CCR4 and PD-L1. The percentage of cells expressing CCR4 was significantly higher in the LDN subset ($p<0.0001$, Figure III.4A), when compared with HDN. This result is particularly interesting since the expression of CCR4 has not been previously described in neutrophils, hence this result suggests that CCR4 may represent a good marker to distinguish LDN from HDN. Moreover, the percentage of cells expressing PD-L1 was also higher in LDN than in HDN ($p=0.007$, Figure III.4A). The higher percentage of cells expressing CCR4 and PD-L1 in the LDN population implies that this subset of neutrophils has an immunosuppressive phenotype, being potentially involved in the impairment of antitumor T cells' responses.

When taking into account the level of expression of each marker, given by the MFI, both activation markers, CD11b and CD66b had a significantly higher MFI in LDN, when compared to HDN

(p=0.0018 and p<0.0001 respectively, Figure III.4B), highlighting that LDN are more activated than HDN.

Altogether, these results suggest that LDN are a subset of more activated cells, which have immunosuppressive action in the context of cancer.

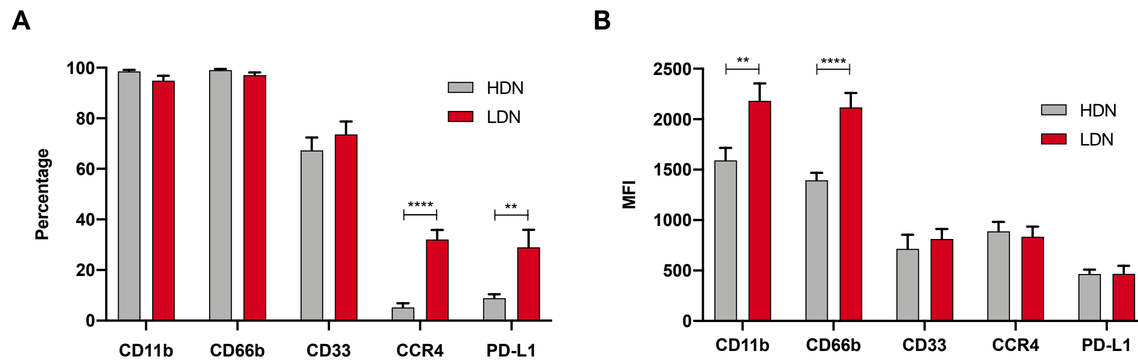



**Figure III. 4 – Low density neutrophils are more activated cells with immunosuppressive function. (A)** Percentage of high density neutrophils (HDN, grey bars) and low density neutrophils (LDN, red bars) expressing CD11b, CD66b, CD33, CCR4 and PD-L1 (assessed by flow cytometry). **(B)** Median fluorescence intensity (MFI) of CD66b, CD11b, CD33, CCR4 and PD-L1 in high density neutrophils (HDN, grey bars) and in low density neutrophils (LDN, red bars). Data corresponds to 48 non-metastatic breast cancer patients and is represented as mean ± SEM. Statistical analysis: two-way ANOVA with Sidak's multiple comparisons, **p<0.01, ****p<0.0001.

Moreover, we performed the same phenotypic characterization for HDN and LDN obtained from the blood of metastatic BC patients, in order to evaluate if neutrophils suffer alterations in their phenotype with cancer progression. For this, we performed a comparison between HDN and LDN of non-metastatic and metastatic BC patients, regarding the percentage of cells expressing the CD66b, CD11b, CD33, CCR4 and PD-L1 markers, as well as their respective MFI (Figure III.5).

When considering the comparison regarding these markers between HDN and LDN of metastatic BC patients, we observed that there is also significantly higher percentage of LDN expressing CCR4, in comparison to HDN (p<0.01, Figure III.5A). Additionally, although the difference is not significant, the tendency to have more LDN expressing PD-L1 than HDN (Figure III.5A) is maintained in metastatic BC patients. Thus, in both non-metastatic and metastatic BC patients the percentages of LDN CCR4+ and PD-L1+ are higher, when compared with HDN from their counterparts. Regarding the level of expression of each marker, no significant differences were found between LDN and HDN of metastatic BC patients, however it appears that a tendency for the activation markers, CD11b and CD66b, to have higher MIF in LDN when compared to HDN, is also maintained.

Interestingly, when comparing neutrophils from metastatic and non-metastatic BC patients, the percentage of cells expressing PD-L1 is increased in both HDN (p<0.05, Figure III.5C) and LDN (p<0.05, Figure III.5E) of metastatic BC patients. Also, there is a tendency in metastatic patients to

have increased percentages of LDN CCR4+, when compared to non-metastatic patients (Figure III.5E). On the other hand, the expression levels of CD11b were lower in HDN ($p=0.0003$, Figure III.5D) and in LDN ($p<0.01$, Figure III.5F) of metastatic patients, in comparison to non-metastatic.

Overall, these results imply that neutrophils, from both subsets, of metastatic patients have an enhanced immunosuppressive phenotype, suggesting that as cancer progresses the neutrophils tend to become more immunosuppressive, supporting an environment that is favourable to tumor development and metastization.

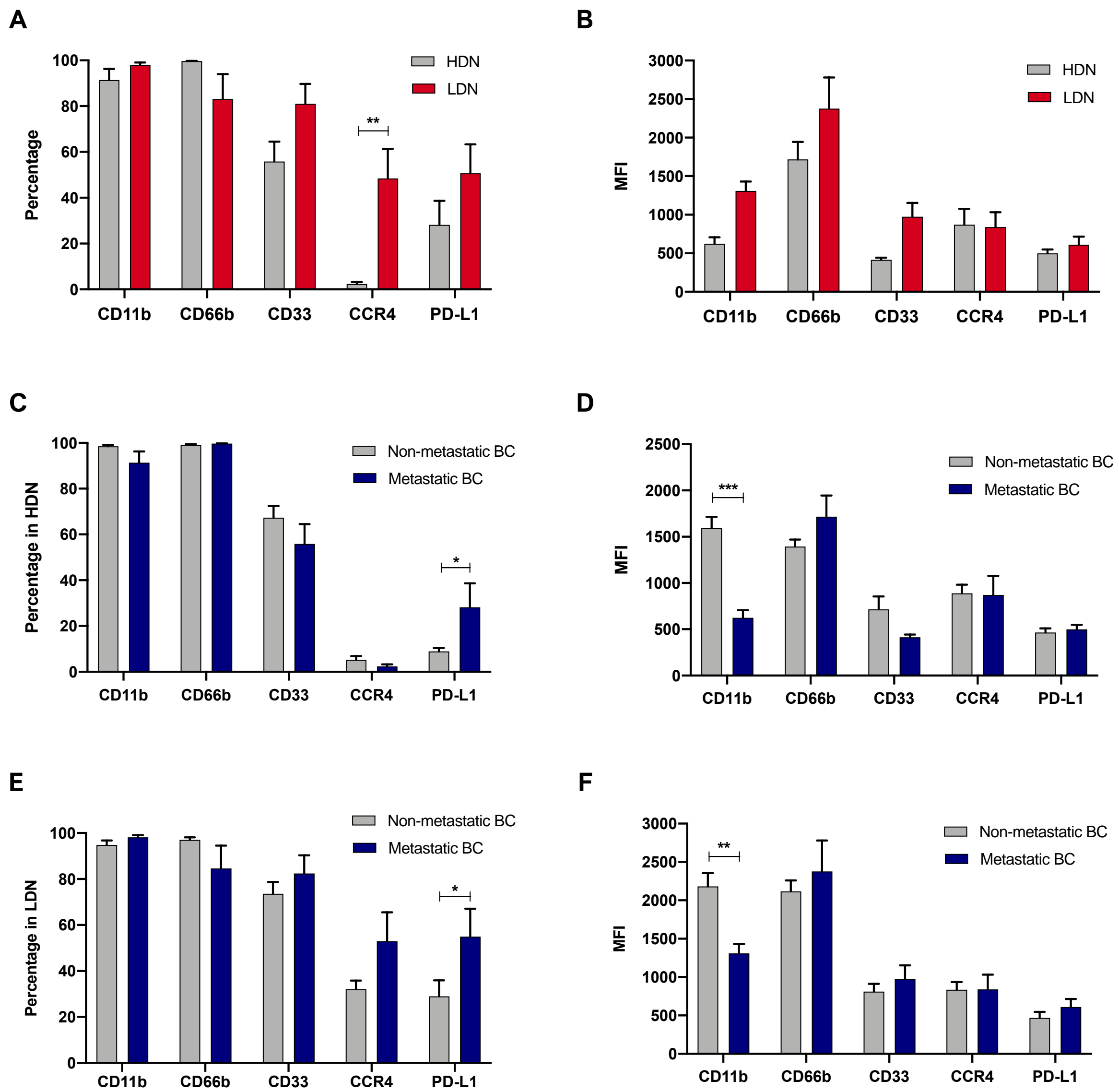



**Figure III. 5 – Neutrophils from metastatic breast cancer patients have an immunosuppressive phenotype even more pronounced than neutrophils from non-metastatic patients. (A)** Percentage of high density neutrophils (HDN, grey bars, n=12) and low density neutrophils (LDN, red bars, n=12) from metastatic breast cancer (BC) patients expressing CD11b, CD66b, CD33, CCR4 and PD-L1 (assessed by flow cytometry). **(B)** Median fluorescence intensity (MFI) of CD11b, CD66b, CD33, CCR4 and PD-L1 in high density neutrophils (HDN, grey bars, n=12) and in low density neutrophils (LDN, red bars, n=12) from metastatic BC patients. **(C)** Percentage of high density neutrophils (HDN) expressing CD11b, CD66b, CD33, CCR4 and PD-L1 in non-metastatic BC patients (grey bars, n=48) and in metastatic BC patients (blue bars, n=12). **(D)** Median fluorescence intensity (MFI) of CD11b, CD66b, CD33, CCR4 and PD-L1 in high density neutrophils (HDN) of non-metastatic BC patients (grey bars, n=48) and of metastatic BC patients (blue bars, n=12). **(E)** Percentage of low density neutrophils (LDN) expressing CD11b, CD66b, CD33, CCR4 and PD-L1 in non-metastatic BC patients (grey bars, n=48) and in metastatic BC patients (blue bars, n=12). **(F)** Median fluorescence intensity (MFI) of CD11b, CD66b, CD33, CCR4 and PD-L1 in high density neutrophils (LDN) of non-metastatic BC patients (grey bars, n=48) and of metastatic BC patients (blue bars, n=12). Data are represented as mean ± SEM. Statistical analysis: two-way ANOVA with Sidak's multiple comparisons, *p<0.05, **p<0.01, ***p<0.001.

## 2.2. LDN have enhanced phagocytic capacity, produce higher levels of reactive oxygen species and release more neutrophil extracellular traps

Besides performing the immunophenotyping of LDN and HDN, by assessing the expression of the mentioned markers, we decided to further characterize these cells in terms of their function. The neutrophils' function relies on three main distinct activities: the capacity to phagocyte pathogens, the ability to generate an oxidative burst and, finally, the capacity to release neutrophil extracellular traps (NETs) in a process designated as NETosis (33). We evaluated all these three activities in both HDN and LDN, in order to understand whether LDN have an altered function or not.

To evaluate the phagocytic capacity, we used FITC-labelled *E. coli* and incubated these bacteria with neutrophils from both subsets at 37ºC and 4ºC (as a negative control, since phagocytosis does not occur at this temperature). Then, by flow cytometry, we estimated the quantity of internalized bacteria by assessing the median fluorescence intensity (MFI) of FITC at 37ºC, normalized with the FITC MFI at 4ºC. We observed that FITC intensity, which is correlated with a higher amount of phagocytosed bacteria, was higher in LDN when compared to HDN ($p<0.0001$, Figure III.6A). This result indicates that LDN have a higher phagocytic capacity than HDN.

The oxidative burst was assessed by quantifying the levels of reactive oxygen species (ROS) produced upon neutrophil stimulation with PMA, using the DCFH2-DA probe. This probe becomes fluorescent after interacting with ROS, therefore the level of ROS production was estimated by quantifying, by flow cytometry, the median fluorescence intensity of the probe. When stimulated, both neutrophils' subpopulations produced similar levels of ROS (Figure III.6B). For both HDN and LDN the levels of ROS produced following stimulation were significantly higher than the ones produced by their unstimulated counterparts ($p=0.0005$ and $p=0.01$, respectively, Figure III.6B). Interestingly, LDN tended to have higher ROS production even without any stimulation when compared to HDN in the same condition (Figure III.6B), suggesting that LDN tend to have an enhanced ability to produce and release ROS, even at a basal level.

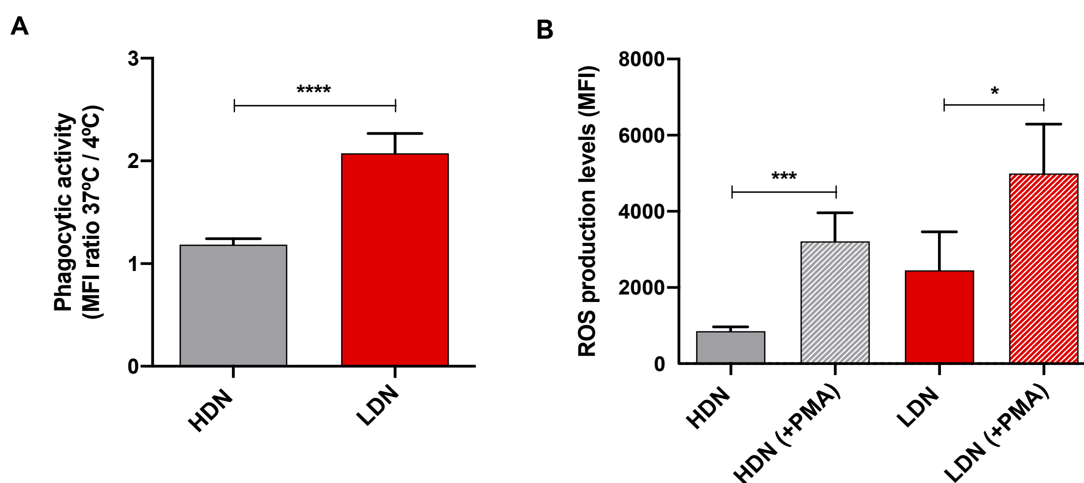



**Figure III. 6 – Low density neutrophils have higher phagocytic capacity and produce more reactive**

**oxygen species. (A)** Phagocytic capacity was quantified as the ratio between the median fluorescence intensity (MFI) of FITC-labelled *E. coli* incubated with high density neutrophils (HDN grey bar, n=11) and low density neutrophils (LDN, red bar, n=12) at 37ºC and 4ºC. (**B)** Levels of released reactive oxygen species (ROS) were assessed by flow cytometry in high density neutrophils without stimulation (HDN, grey bar, n=14) and with PMA stimulation (HDN (+PMA), grey bar with stripes, n=14), and in low density neutrophils without stimulation (LDN, red bar, n=14) and with PMA stimulation (LDN (+PMA), red bar with stripes, n=14). Data are represented as mean ± SEM. Statistical analysis: Mann-Whitney, *p<0.05, ***p<0.001, ****p<0.0001.

NETs are filamentous structures composed by decondensed chromatin and also by histones, cytoplasmatic proteins and granular enzymes, such as myeloperoxidase (MPO), that attach to the DNA (33). In cancer, NETs have been shown, mainly using animal models, to help in the metastization process through their ability to entrap malignant cells and support the early adhesion of circulating tumor cells in distant organ sites (81,128). We analyzed NETs formation in both HDN and LDN with or without PMA stimulation. After 3h of stimulation, the neutrophils were fixed and stained with anti-MPO followed by a counterstaining with DAPI, to visualize and quantify NETs formation. To fully analyze the NETs, we decided to quantify three distinct features of these structures: NETs area, MPO fluorescence intensity and nuclei enlargement. We observed that both subsets were able to release NETs (Figure III.7). Regarding NETs area, stimulated HDN released NETs that occupied a larger area, in comparison to stimulated LDN (p=0.002, Figure III.7B). Interestingly, unstimulated LDN tended to produce NETs that occupied a higher area, when compared to non-stimulated HDN (p=0.06, Figure III.7B). As for the MPO intensity, NETs produced by LDN had significantly higher levels of MPO in their structure when compared to HDN, both in the stimulated and unstimulated conditions (p=0.03 and p=0.04, respectively, Figure III.7C). Finally, since nuclear enlargement is an initial step of NETosis, we also quantified the nuclear area of both subsets. Again, unstimulated LDN have an increased nuclei area when compared to unstimulated HDN (p=0.002, Figure III.7D), even if this difference is abrogated when neutrophils were stimulated (Figure III.7D). Therefore, these results demonstrate that LDN have an enhanced capacity to form and release NETs with increased levels of MPO attached to their structure, at the basal level.

The increased capacity of LDN to phagocyte bacteria, release ROS and form NETs containing MPO, even in the absence of stimulation, appears to be correlated with the fact that this subset has a higher level of activation, demonstrated by the higher expression of CD11b and CD66b (section 2.1). In the context of cancer, there are growing evidence that an increase of these activities may be implicated in the acceleration of tumor progression (80), sustaining the hypothesis that LDN are a subset with protumor functions.

A

DAPI MPO Merge

HDN

Control

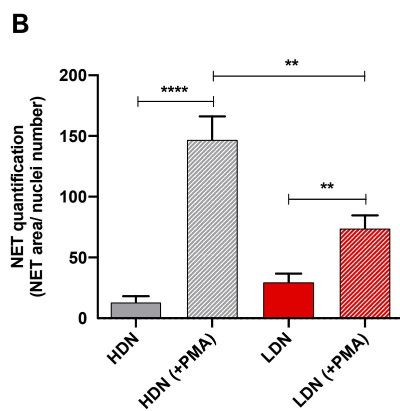
PMA Stimulation

LDN

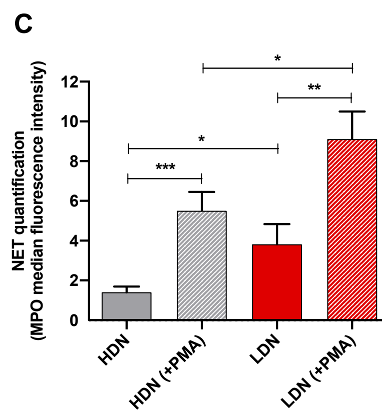
Control

PMA Stimulation

B




C




D

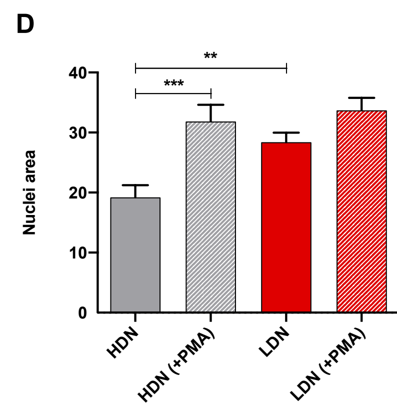


**Figure III. 7 – Low density neutrophils have an enhanced capacity to produce neutrophil extracellular traps. (A)** Representative images of neutrophil extracellular traps (NETs) in high and low density neutrophils (HDN and LDN, respectively) with and without PMA stimulation analyzed with DNA staining (DAPI, blue channel, left panel) and MPO staining in red (middle panel). Both channels were merged (right panel) and Z stacks (1 μm between each stack) were projected with the maximum fluorescence intensity. Scale bar (white line): 20 μm. **(B)** Neutrophils extracellular traps (NETs) quantification by assessing the NETs area and normalizing to the nuclei number in high density neutrophils without stimulation (HDN, grey bar, n=12) and with PMA stimulation (HDN (+PMA), grey bar with stripes, n=12), in low density neutrophils without stimulation (LDN, red bar, n=12) and with PMA stimulation (LDN (+PMA), red bar with stripes, n=12). **(C)** Neutrophil extracellular traps (NETs) quantification by the median fluorescence intensity of myeloperoxidase (MPO) in high density neutrophils without stimulation (HDN, grey bar, n=11) and with PMA stimulation (HDN (+PMA), grey bar with stripes, n=11), of low density neutrophils without stimulation (LDN, red bar, n=11) and with PMA stimulation (LDN (+PMA), red bar with stripes, n=11). **(D)** Quantification of the nuclei area in high density neutrophils without stimulation (HDN, grey bar, n=11) and with PMA stimulation (HDN (+PMA), grey bar with stripes, n=11), in low density neutrophils without stimulation (LDN, red bar, n=11) and with PMA stimulation (LDN (+PMA), red bar with stripes, n=11). Data are represented as mean ± SEM. Statistical analysis: Mann-Whitney, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

## 3. LDN are correlated with immunosuppressive molecules and regulatory T lymphocytes

We have demonstrated that LDN have an immunosuppressive phenotype, expressing higher levels of CCR4 and PD-L1, and also that these cells have enhanced functions that have been associated with tumor development, namely increased ROS production and NETs formation (section 2).

Considering this, we intended to investigate if LDN are also associated with other important features of cancer. Particularly, we decided to assess if these neutrophils were associated with cytokines with relevant roles in cancer and also with other immune cell populations, namely effector T lymphocytes.

### 3.1. LDN are associated with the levels of the immunosuppressive cytokines, CCL17 and TGF-β, present in the plasma of breast cancer patients

From the whole blood of BC patients, we also collected the plasma, in order to assess the concentration of several relevant circulating cytokines, namely IL-10, IFN-γ, TGF-β, IL-17 and CCL17.

The chemokine CCL17 is produced by neutrophils and previous studies have shown that its expression is increased in N2 tumor-associated neutrophils, characterized by their protumor function (69). Interestingly, we observed that there is a positive correlation between the percentage of LDN present in the blood and the concentration of CCL17 in BC patients' plasma (r=0.57, p=0.0007, Figure III.8A, Table III.1). Hence, resembling N2, LDN are also correlated with higher levels of secreted CCL17.

TGF-β has been demonstrated to have the capacity to induce the polarization of "normal" neutrophils towards a protumor phenotype (20); therefore, we hypothesized that the levels of this cytokine would be increased in the plasma of patients with higher levels of LDN in their blood. As expected, there was a positive correlation between the concentration of TGF-β in the plasma of BC patients and the percentage of LDN in the blood (r=0.45, p=0.025, Figure III.8B, Table III.1).

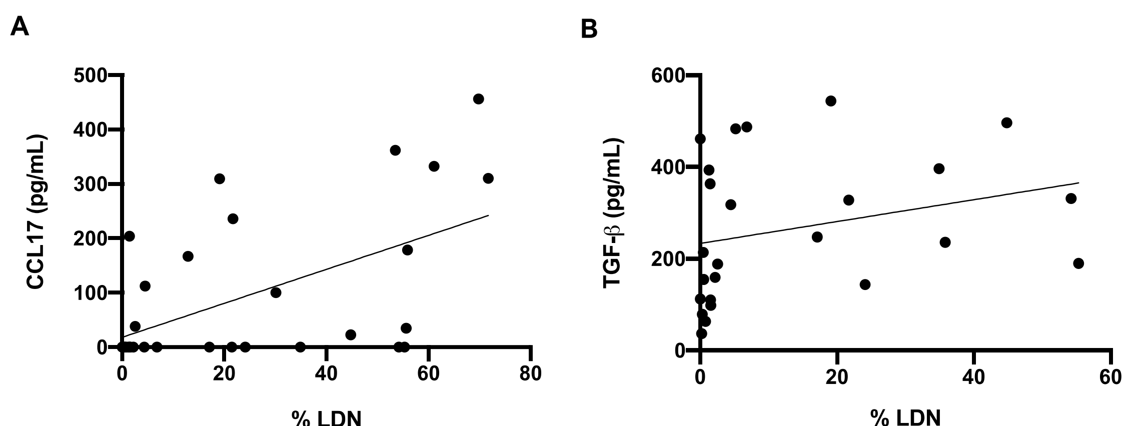



**Figure III. 8 – Low density neutrophils are positively correlated with the levels of immunosuppressive cytokines in the plasma of breast cancer patients. (A)** Correlation between the percentage of low density neutrophils (LDN) in the blood and the concentration of CCL17 in the plasma of breast cancer patients (Spearman r = 0.57, p=0.0007, n=32). **(B)** Correlation between the percentage of low density neutrophils (LDN) in the blood and the concentration of TGF-β in the plasma of breast cancer patients (Spearman r = 0.45, p=0.025, n=25).

As for the remaining cytokines, they were chosen because IFN-γ reflects the activation status of cytotoxic T lymphocytes (CTLs), whereas IL-10 reflects an anti-inflammatory environment and IL-17 is a key player in the promotion of neutrophil expansion and polarization towards an immunosuppressive phenotype. However, the concentration of IL-10 and IFN-γ in the plasma of BC patients did not show any correlation with the level of LDN in the blood (Table III.1) and IL-17 was not detected in the plasma of BC patients (Table III.1).

**Table III. 1 – Cytokine profile of breast cancer patients' plasma and their correlation with low density neutrophils (LDN).**

| Correlation with % LDN | Spearman *r* | p-value | Significance |
|---|---|---|---|
| **CCL17** | 0.5653 | 0.0007 | *** |
| **IFN-γ** | 0.3285 | 0.0879 | ns |
| **IL-10** | -0.2787 | 0.1592 | ns |
| **IL-17** | NA | NA | NA |
| **TGF-β** | 0.4459 | 0.0255 | * |

NA (non-applicable) – IL-17 was not detected in the patients' plasma.

## 3.2. Higher levels of LDN are positively correlated with CCR4+ T regulatory lymphocytes and negatively correlated with activated cytotoxic T cells

As mentioned in the results above, higher levels of LDN in the blood of BC patients are positively correlated with higher secretion of CCL17. This chemokine is a ligand of CCR4, expressed in T lymphocytes, especially in regulatory T cells (Tregs), acting as a chemoattractant of these cells (17,31). Considering this interaction, we decided to evaluate if there was a correlation between the presence of LDN and the presence of CCR4+ Tregs in the blood of BC patients. After the quantification of both cell populations obtained from whole blood, we observed, as expected, that CCR4+ Tregs were increased in the blood of patients that had higher levels of LDN, as a positive correlation was established between these two cell populations (r=0.34, p=0.03, Figure III.9A).

Tregs are key players in the regulation of the adaptive immune responses, being capable of inhibiting the activity of CTLs. Therefore, we decided to investigate if the mentioned interactions of LDN and T cells was also reflected in the activation of CTLs. It was observed that CTLs expressing the activation marker CD69 showed a tendency to be negatively correlated with the percentage of LDN (r=-0.38, p=0.053, Figure III.9B) present in the patients' blood.

Overall, it seems that LDN can also exert indirect immunosuppression towards T lymphocytes, *via* the release of CCL17, consequently recruiting CCR4+ Tregs, which in turn would contribute to inhibit the activity of CTLs and, therefore, the antitumor immune responses.

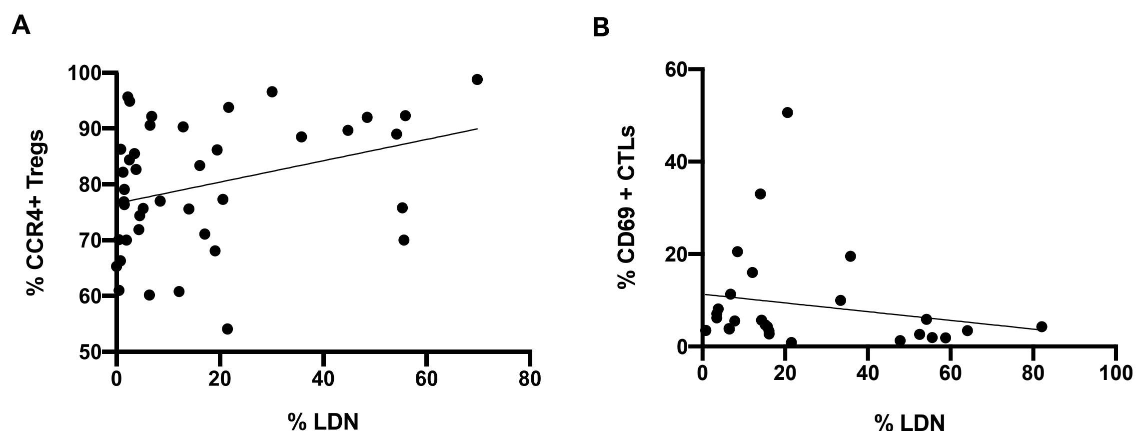



**Figure III. 9 – Low density neutrophils are positively correlated with CCR4+ regulatory T cells. (A)** Correlation between the percentage of low density neutrophils (LDN) and the percentage of circulating CCR4+ regulatory T cells (Tregs) in the blood of breast cancer patients (Spearman r = 0.34, p=0.03, n=41). **(B)** Correlation between the percentage of low density neutrophils (LDN) and the percentage of circulating CD69+ cytotoxic T cells (CTLs) in the blood of breast cancer patients (Spearman r = -0.38, p=0.053, n=27).

## 4. LDN can reduce the activation and proliferation of T lymphocytes

We have observed that LDN are increased in the blood of non-metastatic BC patients without response to NACT and of metastatic patients (section 1). Additionally, we have concluded that LDN are highly activated cells with immunosuppressive features (section 2), which are associated with higher levels of CCL17 in patients' plasma, higher percentage of CCR4+ Tregs present in the blood and also with a lower number of activated circulating CTLs (section 3).

To better understand the role of LDN in cancer and confirm our hypothesis that BC patient-derived LDN have the capacity to impair T lymphocytes' activity, we conducted *in vitro* experiments in order to further confirm the impact of these neutrophils in the activation and proliferation of T lymphocytes.

For these experiments, we isolated the peripheral blood mononuclear cells (PBMCs) fraction from patients' blood and sorted the cells in order to obtain two populations: LDN (CD15+) and PBMCs depleted of neutrophils (CD15-). Then, we performed a co-culture of LDN and PBMCs (depleted of neutrophils) derived from the same patient, with or without stimulation with PMA and ionomycin (Figure III.10). As a control, we used a monoculture of PBMCs also depleted of neutrophils. We then investigated, by flow cytometry, the activation and proliferation status of CD4+ T cells and CTLs, by assessing the expression of the activation markers CD25, CD69 and HLA-DR and the proliferation marker, Ki67. While no significant differences were found in the unstimulated condition, the stimulated PBMCs in the presence of LDN demonstrated an overall reduction of the activation and proliferation markers in both CD4+ T cells (Figure III.10A) and CTLs (Figure III.10B), when compared to stimulated PBMCs incubated without LDN.

This result corroborates the idea that LDN weakens the ability of effector T lymphocytes to become activated upon stimulation, which, in the context of cancer, is reflected in dampened antitumor responses.

Furthermore, since IFN-$\gamma$ is a key antitumor cytokine that reflects the activation status of lymphocytes, we also assessed the levels of this cytokine in the co-cultures' supernatants, as an additional readout to confirm the LDN-derived suppression of T cells (Figure III.10C). As expected, the IFN-$\gamma$ production was significantly reduced when T lymphocytes were stimulated in the presence of LDN ($p=0.007$, Figure III.10C). Hence, with these results, we corroborated that patient derived-LDN have indeed an immunosuppressive phenotype, with the capacity to reduce the activation and proliferation of effector T lymphocytes.

Moreover, in order to confirm the idea that LDN have immunosuppressive action towards T lymphocytes *via* the release of CCL17, we assessed the production of this chemokine by HDN and LDN. For this, we quantified the levels of CCL17 in the supernatants of cultures of HDN and LDN obtained from BC patients' blood, with or without 3h of stimulation with PMA (Figure III.10D). The levels of CCL17 produced by HDN were similar with or without stimulus (Figure III.10D). Whereas

LDN without stimulation showed a higher capacity to release this chemokine when compared to HDN (p=0.04, Figure III.11B). When stimulated, this difference was further enhanced (p=0.02, when compared to stimulated HDN, Figure III.10D). Therefore, this result highlights that LDN have a higher capacity to produce CCL17, which in turn, supports the idea, suggested by studies with mice (31), that CCL17 mediates, at least in part, the interaction between LDN and T lymphocytes, enabling a more immunosuppressive environment.

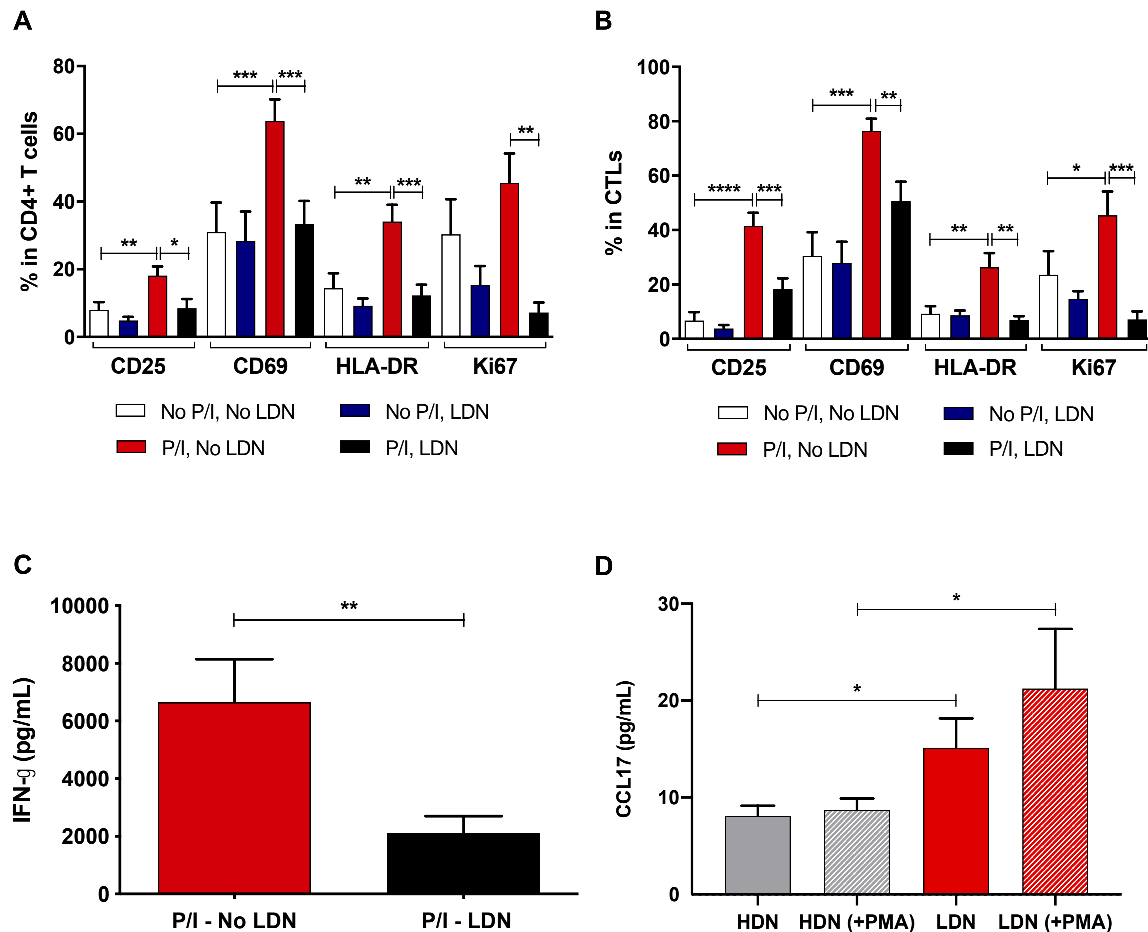



**Figure III. 10 – Low density neutrophils can reduce the activation level and the proliferation of effector T lymphocytes. (A)** Percentage of CD25, CD69, HLA-DR and Ki67 in cultured CD4+ T cells without stimulation and without the addition of LDN (No P/I, No LDN, white bars), without stimulation and with the addition of LDN (No P/I, LDN, blue bars), with PMA and ionomycin stimulation and without the addition of LDN (P/I, No LDN, red bars) and with both stimulation and the addition of LDN (P/I, LDN, black bars), n=10. **(B)** Percentage of CD25, CD69, HLA-DR and Ki67 in cultured cytotoxic T cells (CTLs) without stimulation and without the addition of LDN (No P/I, No LDN, white bars), without stimulation and with the addition of LDN (No P/I, LDN, blue bars), with PMA and ionomycin stimulation and without the addition of LDN (P/I, No LDN, red bars) and with both stimulation and the addition of LDN (P/I, LDN, black bars), n=10. **(C)** Concentration of IFN-γ produced in the PBMCs monoculture with PMA/ionomycin stimulation (P/I - No LDN, red bar, n=8) and in the PBMCs and LDN co-culture with PMA/ionomycin stimulation (P/I - LDN, black bar, n=8). **(D)** CCL17 produced by cultured high density neutrophils without stimulation (HDN, grey bar, n=6) and with PMA stimulation (HDN (+PMA), grey bar with stripes, n=6), low density neutrophils without stimulation (LDN, red bar, n=6) and with PMA stimulation (LDN (+PMA), red bar with stripes, n=6). Data are represented as mean ± SEM. Statistical analysis: unpaired t-test (A and B) and Mann Whitney (C and D), *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Overall, in this thesis, we have demonstrated that low density neutrophils have an important role in breast cancer progression and also the potential to be used as a predictive biomarker for patient response to neoadjuvant chemotherapy. We have also demonstrated that LDN are highly activated cells with immunosuppressive features, namely, a higher percentage of PD-L1 expression, enhanced capacity to produce ROS and to release NETs and increased production of CCL17. Additionally, we have observed that neutrophils express the CCR4 receptor and that this marker may be useful to distinguish LDN from HDN, since there is a higher percentage of neutrophils expressing CCR4 in the LDN subset. Finally, the findings of this study suggest that LDN have an immunosuppressive action in effector T lymphocytes, inhibiting their antitumor immune responses and promoting an environment favorable to tumor progression.

# Chapter IV – Discussion

Neutrophils, once seen as only the first responders of the innate immune system to infections, are now being considered a prominent part of the tumor immune microenvironment (TIME) and capable of influencing tumor development at multiple levels, becoming major players in cancer biology (55,61,77,117). The relevance of neutrophils in tumorigenesis was first implied with the observation that neutrophils accumulate in the peripheral blood of patients with advanced-stage cancer, which is reflected by an increased neutrophil-to-lymphocyte ratio (NLR) (110,114). Considering this, in the past few years, there has been a growing interest in characterizing tumor-associated neutrophils (TANs) and investigating their role in the tumorigenic process. Indeed, it has been demonstrated that TANs can either protect against the onset of cancer, or they can have a protumor activity and help in tumor progression and metastization (80,129). This dichotomy led to the establishment of two distinct TAN subsets – N1 (antitumor) and N2 (protumor) (20). These subsets are also reflected systemically and circulating neutrophils can be divided into high density neutrophils (HDN) and low density neutrophils (LDN), which functionally mirror N1 and N2, respectively (77).

Lately, LDN have been object of particular attention as they mainly appear in pathological contexts, such as autoimmune diseases and cancer (130). In the particular case of cancer, this subset of neutrophils has been shown to have immunosuppressive features and the ability to enhance tumor progression and metastization (82,121). However, the role of LDN in cancer has been predominantly studied using mice models and there are various dissimilar aspects in neutrophil biology between mice and humans (131). Hence, there is an unmet need for validation studies using human samples that support these findings and that better clarify the role played by LDN in cancer.

As such, in this thesis, we aimed to understand the clinical relevance of LDN in breast cancer (BC). For this, we established a cohort of non-metastatic and metastatic BC patients in order to investigate the role of LDN in the patients' outcome, particularly regarding their response to neoadjuvant chemotherapy (NACT, conventional treatment prescribed to BC patients with tumors larger than 2 cm or inflammatory/inoperable tumors). Additionally, we aimed to perform a thorough characterization of human LDN's phenotype and function and to investigate their impact in T lymphocytes' activity. Based on what was previously described, our main hypothesis was that LDN should be increased in BC patients with worse prognosis, as they have immunosuppressive and protumor characteristics.

First, we observed that, LDN were virtually absent in the blood of healthy individuals and that metastatic BC patients had an increased percentage of LDN in their blood, when compared to non-metastatic patients. This corroborates the previously suggested idea, based mainly on animal studies, that LDN accumulate continuously during cancer progression (77) and are involved in the metastization process (117,132). Additionally, when dividing the non-metastatic BC patients, selected for neoadjuvant chemotherapy (NACT), according to their response to treatment, we

observed that patients without response to NACT had a significantly higher percentage of LDN present in the blood before starting the treatment, when compared to non-metastatic patients who responded to NACT. Although a higher prevalence of neutrophils in the low density fraction had already been observed in cancer patients, namely with breast and lung cancers, the majority of these studies enrolled patients with advanced-stage disease and no correlation between the LDN population and response to treatment was established before (75,77). Since an absence of response to NACT is associated with a worse prognosis in BC (102), this suggests that LDN are associated with the aggressiveness of the disease.

As such, our observations indicate that the percentage of LDN has the potential to be a predictive biomarker, which could be useful to discriminate, prior to treatment, the patients who will truly take advantage from NACT, allowing to direct the non-responders to alternative therapies, avoiding 6 months of chemotherapy-associated toxicity without benefit.

Interestingly, the most studied biomarkers for the prediction of NACT response, tumor infiltrating lymphocytes (TILs) and NLR, have mainly been associated with triple negative breast cancer (TNBC) (133–135). However, in this study, we observed that the estrogen receptor (ER+) BC subtype was the one with higher percentages of LDN. Thus, it appears that LDN could be of particular importance to predict, in advance, the response to NACT of BC patients with ER+ tumors, which curiously are usually seen as the ones with better response to treatment and overall survival (136).

As mentioned, NLR is one of the possible biomarkers that has been extensively studied in cancer; however, we believe that the percentage of LDN could represent an interesting alternative with several advantages. Indeed, we did not observe any significant differences regarding the NLR between the NACT responders and non-responders from our cohort of BC patients. Besides this, it is important to notice that there is still a lack of consensus on the NLR threshold that divides patients regarding their response to treatment (110). Moreover, NLR represents the total lymphocytes and neutrophils in circulation, which, actually, contemplate different subsets of immune cells with divergent roles in cancer, such as cytotoxic T lymphocytes (CTLs), regulatory T cells (Tregs), HDN and LDN. Combining these opposite immune cells as single populations (either in the case of lymphocytes or neutrophils) is a very simplistic view that does not represent the complexity of the immunological status. Also, it is not clear yet whether NLR is truly representative of protumor neutrophils or simply reflects a tumor-associated inflammatory condition that leads to neutrophilia (54,137).

Furthermore, in order to better understand how LDN differ from normal neutrophils (HDN) and which of their features may help to explain their association with cancer progression, we performed a profound characterization of neutrophils from both subsets.

Phenotypically, we observed that LDN have a higher expression level, indicated by a higher median fluorescence intensity (MFI), of the activation markers CD11b and CD66b, when compared to HDN,

which was in accordance with previously published studies, involving HIV, asthma and cancer patients (75,138,139). Additionally, we saw that, when comparing to HDN, there was a higher percentage of LDN expressing the immunosuppressive molecules PD-L1 and CCR4. Interestingly, CCR4 expression has not been previously described in neutrophils, and here we observed that LDN, in particular, express this receptor and, thus, CCR4 could represent a helpful maker to distinguish LDN from HDN. Then, we functionally characterized HDN and LDN by assessing the typical neutrophils' functions: phagocytic capacity, ability to produce an oxidative burst and to release neutrophil extracellular traps (NETs). LDN had a higher capacity to phagocyte FITC-labelled *E. coli* and to release reactive oxygen species (ROS), which is in agreement with the highly activated phenotype (CD11b$^{high}$/CD66b$^{high}$) observed for this subset. Interestingly, it is known that the release of ROS can contribute to promote genetic instability, to the activation of cell survival signals and to the initiation of cancer-related angiogenesis and metastasis (62,140). Regarding the formation of NETs, we observed that, upon stimulation, neutrophils from both subsets were able to form these structures, although myeloperoxidase (MPO) fluorescence intensity was higher in LDN, whereas NETs area was increased in HDN. It has been shown that MPO only binds to the DNA traps in later stages of the process (39), which could mean that LDN are more prone to enter NETosis and form more mature NETs, although not as large as the ones produced by HDN. Mature NETs have been demonstrated to help in the establishment of tumor cells in niches distant from the primary tumor by attaching to them and carrying them throughout the circulation (81,82,121,128).

Overall, LDN presents, on one hand, a highly activated state and, on the other, a greater immunosuppressive status, when compared to HDN.

Concerning the LDN immunosuppression of effector T lymphocytes, it can be achieved through different mechanisms. Namely, PD-L1 (expressed in LDN), is a well-studied immune checkpoint inhibitor that dampens the antitumor responses of effector T lymphocytes, by binding to its receptor (PD-1) present in these cells (88,141). Actually, increased PD-L1 expression in neutrophils had already been demonstrated in pathologic contexts, for instance in HIV, using human samples, and in cancer, using mice models (142,143). Additionally, the release of ROS has been shown to reduce the activity of effector T lymphocytes (144). Moreover, the MPO present in the NETs has also been shown to limit T lymphocytes activity (145,146). As such, PD-L1 expression, release of ROS and NETs containing MPO may represent different ways by which LDN can impair T lymphocytes' antitumor action (Figure IV.1).

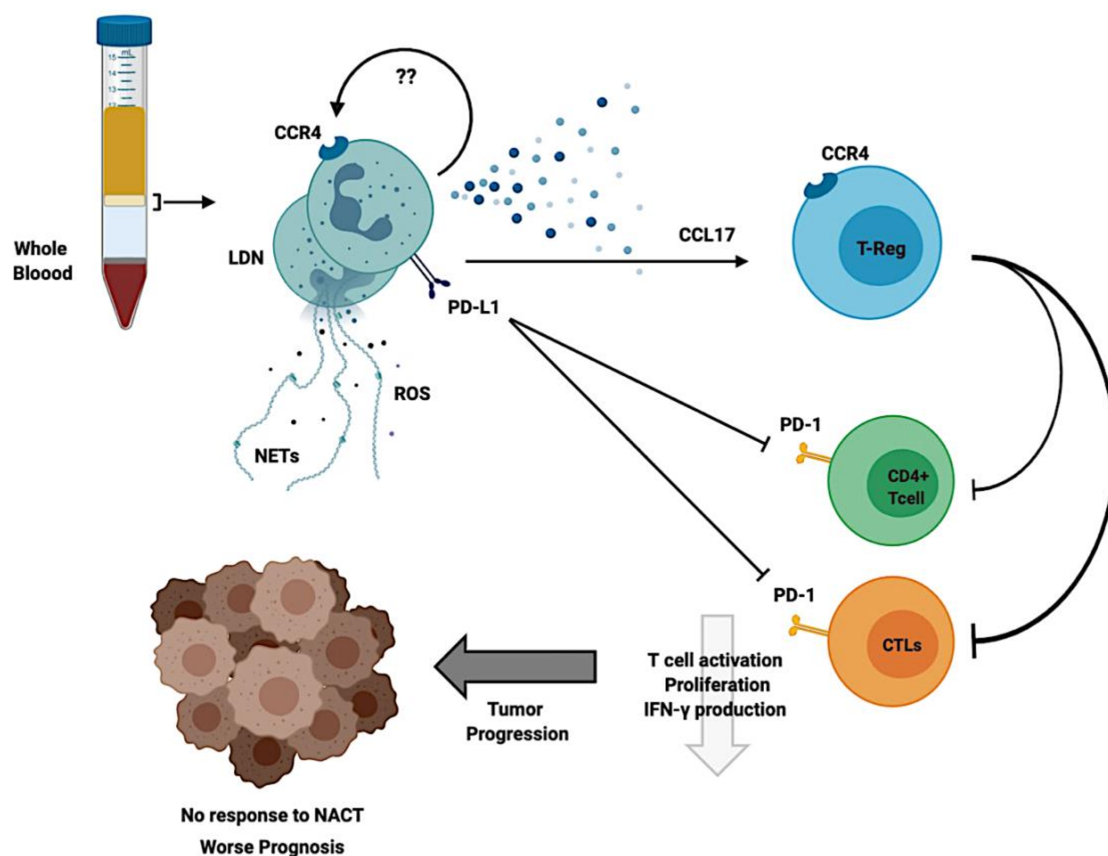



**Figure IV. 1 – The role of low density neutrophils in breast cancer prognosis and response to neoadjuvant chemotherapy.** Low density neutrophils (LDN), isolated from the peripheral blood mononuclear cell layer after whole blood density gradient centrifugation, are highly activated cells, which have a high capacity to release neutrophil extracellular traps (NETs) and reactive oxygen species (ROS), express PD-L1 and CCR4 and release CCL17. LDN can recruit CCR4+ regulatory T cells (Tregs) in a CCL17-dependent manner, which in turn will inhibit CD4+ T cells and cytotoxic CD8+ T lymphocytes (CTLs). CCL17 may also bind to CCR4+ present in the LDN, although its function in LDN is not clearly understood. The inhibitory effect that LDN have on T lymphocytes can also be achieved directly, through the PD-L1/PD-1 pathway. The inhibition will lead to lower T lymphocytes' activation, proliferation and IFN-γ production, leading to tumor progression, and consequently poor response to neoadjuvant chemotherapy (NACT) and a worse prognosis for breast cancer patients.

To further corroborate the immunosuppressive action of LDN, we investigated if their presence was associated with other immune players, particularly immunosuppressive cytokines and effector T lymphocytes. We observed that these neutrophils were positively correlated with the concentration of TGF-β and CCL17 in the plasma of BC patients. TGF-β is a known inducer of neutrophil polarization towards a protumor phenotype (20). Additionally, this cytokine is correlated with increased tumor growth (147) and also macrophage polarization towards the M2 phenotype (19), overall assisting in the maintenance of an immunosuppressive environment. As for CCL17, this chemokine is a ligand to the CCR4 receptor and works as a chemoattractant, capable of recruiting lymphocytes that express this receptor (Figure IV.1). Actually, we observed a positive correlation between LDN and CCR4+ Tregs, suggesting that CCL17-producing LDN can recruit immunosuppressive Tregs, which is in agreement with what had been previously reported in

animal models (31). Additionally, a previous transcriptomic analysis performed in TANs reported that N2 neutrophils have a significant upregulation of CCL17 expression (69). As such, to investigate if LDN maintain this trait, we quantified the concentration levels of CCL17 in the supernatants of HDN and LDN cultures after PMA stimulation. As expected, higher levels of CCL17 were produced by LDN, sustaining the idea that this subset of neutrophils is responsible for the recruitment and accumulation of activated Tregs in cancer patients. Moreover, since we have seen that LDN can also express the CCR4 receptor, we believe that CCL17 produced by these neutrophils may not only bind to CCR4+ Tregs, but also to the CCR4 expressed by the LDN themselves (Figure IV.1). This interaction may possibly originate a positive feed-back loop, although this hypothesis still needs to be further explored.

Tregs, on their hand, can inhibit the effector function of CTLs, impairing the antitumor immune response and, accordingly, we also observed a tendency for a negative correlation between LDN and activated CTLs (CD69+). To better elucidate this interaction and to validate this result with *ex vivo* experiments, we performed cultures with PBMCs (depleted of the neutrophils that can appear in this fraction) in the presence or absence of LDN obtained from the same patient. We observed that the T lymphocytes' activation, release of IFN-$\gamma$ and proliferation increased after PMA/ionomycin stimulation. However, activation, IFN-$\gamma$ production and proliferation of T lymphocytes were significantly reduced when the cells were stimulated in the presence of LDN (Figure IV.1). Once again, these results attest the immunosuppressive action of this neutrophil subset.

Our group had previously demonstrated that CTLs expressing the activation marker HLA-DR, are mainly present in biopsies of BC patients with good response to NACT, representing a biomarker that could predict efficiently BC patients' response to treatment (108). The combination of this information with the results of this thesis appears to further support the idea that the presence of higher levels of LDN will have an impact on effector T lymphocytes and that this impact is not only indirect, by the recruitment and stimulation of Tregs, but also direct, since LDN can decrease the activation and proliferation of CTLs, possibly through the release of ROS, expression of immunosuppressive markers (PD-L1 and CCR4) and release of NETs containing MPO (Figure IV.1). Thus, we believe that these results demonstrate the immunosuppressive features of LDN, reflect their protumor effect and support the idea that they can be used as a possible systemic predictive biomarker of poor response to NACT and a worse prognosis for BC patients (Figure IV.1).

The main limitations of this study were the low sample size comparing to other similar works and the patient follow up, as we have to wait 6 months (NACT duration) to obtain the response to treatment. Nevertheless, even with these limitations, mainly imposed by time restrictions, we have obtained remarkable results that better elucidate the role and characteristics of human LDN and how they may influence the outcome of BC patients.

Overall, with this thesis, we demonstrated that LDN have an important role in BC progression and also that they may have a higher predictive power than NLR to distinguish, *a priori*, patients who will

respond to NACT from the ones who will not. Additionally, we showed that human LDN are highly activated cells with immunosuppressive features and with enhanced capacity to produce ROS and to release NETs with increased levels of MPO attached to their structure. Moreover, our results also highlighted that LDN have an impact on effector T lymphocytes activation and proliferation.

Nonetheless, further studies still need to be conducted namely, to validate this new biomarker. Additionally, it is also necessary to investigate and clarify the role of CCR4 in neutrophils, as mentioned. To accomplish this, we could perform an experiment where we would block the CCR4 receptor in neutrophils, using, for instance, a specific blocking antibody, and then assess if the CCR4 blockade influences the secretome, the normal neutrophils' functions or even their immunosuppressive action on T lymphocytes.

Also, many other relevant aspects could be further explored, in the future, regarding LDN impact in cancer. For instance, it would be interesting to investigate if cancer cells are affected by LDN, namely in terms of their proliferation, migration and invasion abilities. For this, we could perform *in vitro* experiments using co-cultures between LDN and BC cell lines. To evaluate if tumor cells' migration capacity is affected by the presence of LDN we could perform wound healing assays (scratch assays) (148). Furthermore, we could also perform a transwell migration/invasion assay (148), where neutrophils would be in a well below a transwell containing the BC cells, in order to investigate if LDN can act as chemoattracts and help the migration of cancer cells. These assays would also be performed using HDN, as a comparison. Regarding the mentioned assays, the main limitation would be the fact that neutrophils have a short lifespan and, therefore, it is difficult to maintain these cells in culture for long periods of time.

Moreover, due to the immunosuppressive action of LDN in effector T lymphocytes, new targeted immunotherapies could emerge from a deeper understanding of these cells, particularly it could be interesting to explore possible targets to inhibit LDN activity and, consequently, release its inhibitory impact on effector T lymphocytes. So far, the manipulation of TGF-$\beta$ or the enhancement of IFN's activity, have shown to favor neutrophil antitumor functions (149); however, these therapies were proved to be toxic and not well tolerated. Therefore, there is a need to continue to investigate new immunotherapies targeting LDN that could improve treatment of breast cancer patients with poor response to NACT and, considering the results of this thesis, we suggest that CCL17 or CCR4 may represent potential novel therapeutic targets, although extensive studies on the topic are still necessary.

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# Appendix

1. Template of the informed consents: Hospital de Vila Franca de Xira, Hospital Professor Doutor Fernando Fonseca and Hospital CUF Descobertas

NOVA MEDICAL SCHOOL FACULDADE DE CIÊNCIAS MÉDICAS

UNIVERSIDADE NOVA DE LISBOA

CONFIDENCIAL

**Termo de Consentimento para Doação de Sangue e Tecido**

Tendo recebido convite para participar num Projeto de Investigação, a realizar no CEDOC, NOVA Medical School, Faculdade de Ciências Médicas da Universidade Novas de Lisboa, no Laboratório de Reparação de Tecidos e Inflamação (Investigador Principal Professor António Jacinto) vem o signatário subscrever o presente Termo de Consentimento para Doação de Sangue e/ou Tecido que serão utilizados para fins de investigação.

Previamente solicitei esclarecimentos e informações que me foram transmitidos e me permitiram, com conhecimento suficiente, optar por subscrever o presente Termo de Consentimento.

A minha decisão foi ainda suportada pela garantia de que:

**1. Todas as respostas às perguntas por mim formuladas tiveram pronta e clara resposta e o Técnico de Saúde que conduzirá o processo de recolha das amostras me informou sobre as matérias da sua área de intervenção;**

**2. A doação das minhas amostras serão 10 ml de sangue e/ou tecido, que serão utilizadas única e exclusivamente para os efeitos supra indicados;**

**3. Os meus dados pessoais (idade, sexo, etc.) serão anónimos e serão registados separadamente dos componentes do sangue isolados, impossibilitando o rastreio das amostras;**

**4. Não será possível relacionar a amostra por mim doada com a minha pessoa;**

**5. Não sou atualmente portador de nenhuma doença infectocontagiosa;**

**6. A minha participação voluntária no estudo não me conferirá direitos a qualquer indemnização ou outros lucros financeiros.**

**7. Posso terminar a minha participação no estudo assim que quiser, sem repercussão na minha prestação de cuidados de saúde**

Sexo: M F Idade: _________

Rubrica: ___________________________________

Nome do Médico Responsável: ______________________________________

Número da OM: ____________________________________________

Assinatura: _________________________________

Lisboa, ____ de _____________ de _______

CAMPO DOS MÁRTIRES DA PÁTRIA, 130 · 1169-056 LISBOA · PORTUGAL · T.+351 218 803 000 · F.+351 218 851 920 · WWW.FCM.UNL.PT

## 2. Template of the informed consents: Hospital Santa Maria

CENTRO HOSPITALAR
LISBOA NORTE, EPE

HOSPITAL DE
SANTAMARIA

Hospital
PulidoValente

CONSENTIMENTO INFORMADO, ESCLARECIDO E LIVRE
para Investigação Clínica

### Parte Informativa

1. Titulo do projecto
   Neutro-tumor

2. Descrição do projeto, sua natureza e objetivo
   Nos últimos anos, verificou-se que ao controlar o sistema imunitário presente nos doentes oncológicos, podemos melhorar o seu tratamento. Esta abordagem tem-se revelado muito promissora e com elevada eficácia. Alguns estudos revelam que os neutrófilos, as células mais abundantes do sistema imunitário, podem ajudar na progressão tumoral e levar a um pior prognóstico. Neste contexto, queremos estudar melhor estes neutrófilos e perceber o que produzem e como interagem com as células tumorais, de modo a que no futuro possamos inibir a sua função pro-tumoral.

   Para tal, pretendemos obter sangue de doentes oncológicos antes do tratamento e passados 6 meses após o início do tratamento, de modo a colher os neutrófilos e estudá-los no laboratório.

1

3. Benefícios

Sendo este um estudo observacional não existem benefícios para os dadores

4. Riscos graves e riscos frequentes

Sendo que este projeto consiste essencialmente na observação de células do sistema imunitário não existe nenhum risco associado para os dadores.

CENTRO HOSPITALAR LISBOA NORTE, EPE | HOSPITAL DE SANTA MARIA | Hospital Pulido Valente

Parte declarativa do profissional

**Confirmo que expliquei à pessoa abaixo indicada, de forma adequada e inteligível, os procedimentos necessários ao ato referido neste documento. Respondi a todas as questões que me foram colocadas e assegurei-me de que houve um período de reflexão suficiente para a tomada da decisão. Expliquei que pode pedir para interromper ou mesmo desistir, caso sinta necessidade ou vontade de o fazer, sem que daí advenha qualquer prejuízo no contexto da sua assistência clinica. Informei que para conforto e respeito da sua privacidade, os dados que me irá fornecer, permanecerão confidenciais e anónimos.**

Nome legível do profissional de saúde: |________________________________________|

Data … …/… …/… … … Assinatura, número de cédula profissional ou número mecanográfico **(se não aplicável a primeira disposição)**

Unidade de Saúde … … … … … … … … … … … … … … … … … … … … … … … … … …

Contato institucional do profissional de saúde … … … … … … … … … … … …

2

CENTRO HOSPITALAR
LISBOA NORTE, EPE

HOSPITAL DE SANTAMARIA

Hospital Pulido Valente

À Pessoa/representante

Por favor, leia com atenção todo o conteúdo deste documento. Não hesite em solicitar mais informações se não estiver completamente esclarecido/a. Verifique se todas as informações estão corretas. Se tudo estiver conforme, então assine este documento.

Parte declarativa da pessoa que consente

*Declaro ter compreendido os objetivos de quanto me foi proposto e explicado pelo profissional de saúde que assina este documento, ter-me sido dada oportunidade de fazer todas as perguntas sobre o assunto e para todas elas ter obtido resposta esclarecedora, ter-me sido garantido que não haverá prejuízo para os meus direitos assistenciais se eu recusar esta solicitação, e ter-me sido dado tempo suficiente para refletir sobre esta proposta. Autorizo/Não autorizo (riscar o que não interessa) o ato indicado.*

Nome: |_______________________________________________|

… …/… …/… … … (data) *Assinatura* … … … … … … … … … … … … … … … … … … … … … … … … … … …

3

SE NÃO FOR O PRÓPRIO A ASSINAR POR IDADE OU INCAPACIDADE **(se o menor tiver discernimento deve também assinar em cima)**

NOME: … … … … … … … … … … … … … … … … … … … … … … … … … … … … …

DOC. IDENTIFICAÇÃO N.º ......................................... DATA OU VALIDADE ….. /….. /……..

GRAU DE PARENTESCO OU TIPO DE REPRESENTAÇÃO: ..................................................

ASSINATURA … … … … … … … … … … … … … … … … … … … … … … … … … … … … … … … … … …

Nota: Este documento é feito em duas vias – uma para o processo/estudo e outra para ficar na posse de quem consente.

Adaptado da Norma nº 015/2013 de 03/10/2013 atualizada a 04/11/2015