# Systemic immune dynamics in cancer

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CHAPTER 1

# Introduction and outline of this thesis

Cancer presents us with a tremendous challenge that is crucial to address. Approximately one in five people will develop cancer in a lifetime, causing death in around one in nine men and one in 12 women[1]. In the Netherlands, the cancer incidence is even higher, with one in two people expected to develop the disease during their lifetime[2]. Breast cancer is the second most common form of cancer in the world, and among women it is the cancer type with the highest global incidence[1,3]. Additionally, breast cancer is the leading cause of cancer-related death in women globally[1], underscoring the urgent need for novel therapeutic possibilities.

## Histological subtypes of breast cancer

Breast cancer encompasses various histological subtypes (Figure 1), each with unique characteristics and clinical implications. The most prevalent subtype is Invasive Ductal Carcinoma (IDC), also known as "No Special Type"[4]. This histological subtype comprises about 70-80% of all breast cancer cases and is distinguished by the lack of specific features that would classify it into other special subtypes[5]. IDC begins in the milk ducts and invades surrounding breast tissue. It often presents as a palpable mass that can be detected during physical exams or imaging. While IDC lacks the unique histological traits of special subtypes, it can vary widely in its cellular appearance, tumor microenvironment and behavior, influencing its aggressiveness and response to treatment. The second most common histological subtype is Invasive Lobular Carcinoma (ILC), which makes up about 10-15% of breast cancers[4,6]. It begins in the milk-producing lobules and infiltrates nearby tissues, typically spreading in a single-file pattern that can make it more challenging to detect via physical examination or imaging. Other less common histological subtypes of breast cancer include, mucinous (colloid) carcinoma, tubular carcinoma, micropapillary carcinoma, metaplastic spindle cell carcinoma, lobular pleomorphic carcinoma, apocrine carcinoma, adenoid cystic carcinoma, which all exhibit distinct histological features and clinical behaviors[7].

## Molecular subtypes of breast cancer

In addition to the histological features that influence clinical behavior, the molecular subtype of the tumor is the main determinant for treatment and prognosis in the clinic. Using immunohistochemistry breast cancer can be categorized into three distinct molecular tumor subtypes (Figure 1).

The first subtype comprises tumors that exhibit positivity for the estrogen receptor (ER) which make up approximately 70-80% of invasive breast cancer cases[5,8,9]. Globally, the cut-off for ER-positivity used in the clinic is ≥1% of cancer cells expressing ER. However, some







countries including the Netherlands are using a cut-off of ≥10% ER-positive cancer cells, justified by endocrine treatment outcomes and TIL profiles[10-12]. The progesterone receptor (PR) is expressed in more than 50% of ER-positive tumors and is very rare in patients with ER-negative breast cancer because PR expression is regulated by ER[13]. Therefore, physiological PR levels provide information about the functional ER pathway. Absence of PR expression is a biomarker for poor prognosis in ER positive tumors[14,15]. Tumors with ER/PR expression are commonly referred to as hormone receptor-positive (HR+) tumors and represent both Luminal A and Luminal B tumors (Figure 1). Where Luminal A tumors always express PR, this may be absent in Luminal B tumors. Additionally, Luminal B tumors have a higher grade than Luminal A tumors, characterized by reduced tumor cell differentiation and increased Ki67 staining, and it is therefore not surprising that patients with a Luminal A HR+ tumor have the best prognosis[16]. The five-year relative survival rate for patients with HR+ breast cancer is 100% for localized disease and 90.5% for patients with regional disease. Patients with HR+ tumors with distant metastasis have a five-year survival rate of 35.4% (table 1)[3]. Unlike many other tumor types, HR+ breast cancer often recurs beyond 5 years, making a 10-year survival analysis more informative. The 10-year overall survival rate for patients with HR+ breast cancer in the non-metastatic setting is 87.8%[17].

The second breast cancer subtype includes tumors that overexpress the human epidermal growth factor receptor 2 (HER2+) or exhibit gene amplification of HER2, accounting for approximately 15-20% of breast cancer cases[18]. HER2 is an oncogene that encodes a receptor tyrosine kinase, which promotes cell growth by activating signaling pathways like PI3K/AKT and MAPK, driving proliferation and survival; its overexpression amplifies these signals, leading to tumor development. Tumors that are both HR+ and HER2+ are also classified within the HER2+ subtype. Patients with HR+HER2+ breast cancer have five-year survival rate of 99.3% when disease was localized, 90.4% when disease had spread regionally and dropped to 45.8% once the disease had spread to distant sites. Patients with HR-HER2+ breast cancer have a slightly worse prognosis, with a five-year survival of 97.3% when disease was localized, 84.2% when disease had spread regionally and dropped to 39.7% once the disease had spread to distant sites (Table 1)[3]. Notably, patients with HR+HER2+ tumors exhibit higher long-term survival rates than those with HR+HER2- tumors in the metastatic setting. This was historically not the case and can be attributed to the beneficial effects of HER2 targeted therapies[19]. The 10-year overall survival rate for patients with HER2+ breast cancer in the non-metastatic setting is reported to be 76.1%[17].

Lastly, triple-negative breast cancer (TNBC), characterized by the absence of hormone receptor expression and HER2 overexpression or amplification, comprises about 15% of all breast cancer cases[20]. Patients with TNBC have the worst prognosis of all breast cancer patients, especially in the metastatic setting. Their five-year survival rate with localized disease is 92.0%, with regional disease 66.8% and with distant metastatic spread the five-

year survival rate for patients with TNBC is only 14.3%[3] (Table 1). The 10-year overall survival rate for patients with triple negative breast cancer in the non-metastatic setting is 77.8%[17].

While histopathological testing methods provide valuable information about breast cancer subtypes, they are not the sole determinants. Molecular profiling techniques, such as gene expression platforms like Oncotype DX and MammaPrint, also offer comprehensive subtype information by analyzing expression patterns of multiple genes[21,22]. These assays

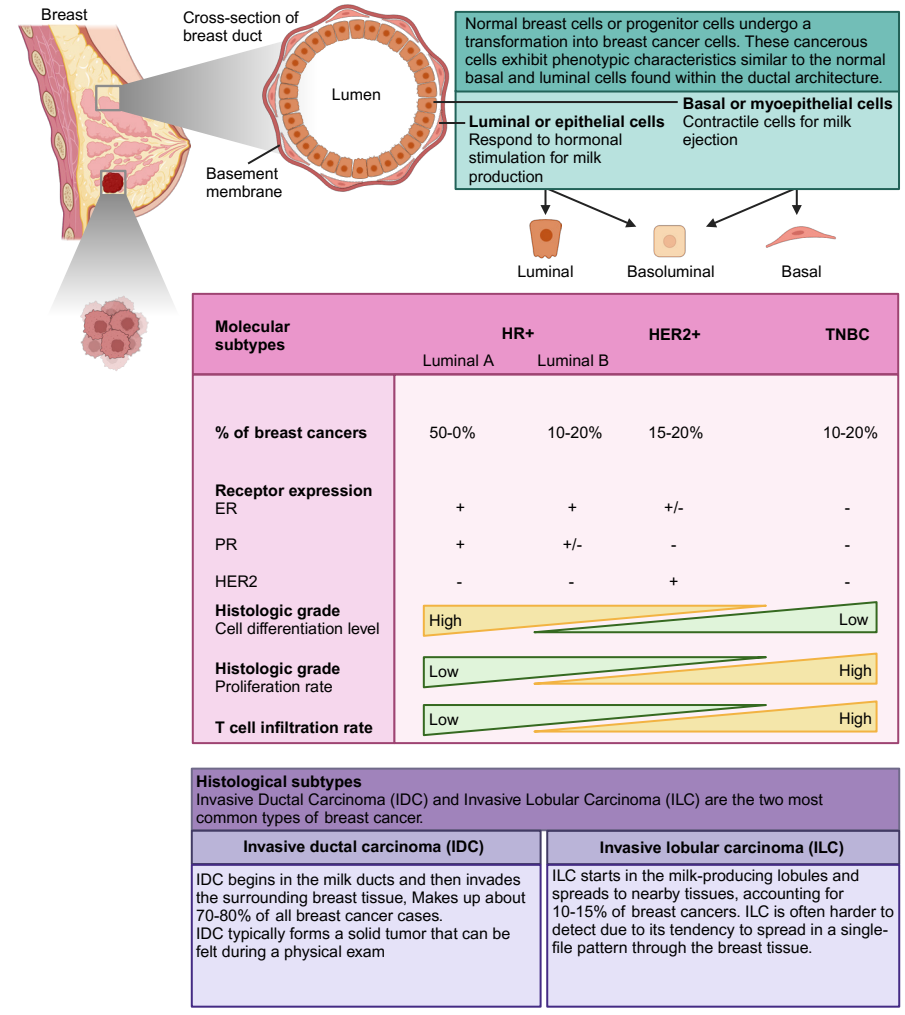


***Figure 1:*** *Overview of breast anatomy and the molecular and histological subtypes of breast cancer. Created in BioRender.com.*







***Table 1:*** *Five-year survival rate of female breast cancer patients by molecular subtype and stage at diagnosis. Based on 385841 women with HR+ breast cancer, 57990 women with HER2+ (HR+) breast cancer, 24028 women with HER2+ (HR-) breast cancer and 58438 women with TNBC. Five-year relative survival rates are calculated using monthly intervals and provided in combination with the upper and lower 95% confidence interval (CI). Data was obtained by the NIH National Cancer Institute SEER program, which was last updated in 2021[3].*

| | Local (CI) | Regional (CI) | Distant (CI) |
|---|---|---|---|
| HR+ | 100 (-) | 90.5 (90.2 – 90.8) | 35.5 (34.5 – 36.4) |
| HER2+ (HR+) | 99.3 (98.8 – 99.6) | 90.4 (89.8 – 91.0) | 45.8 (44.0 – 47.6) |
| HER2+ (HR-) | 97.3 (96.6 – 97.8) | 84.2 (83.2 – 85.2) | 39.7 (37.4 – 42.0) |
| TNBC | 92.0 (91.5 – 92.5) | 66.8 (65.9 – 67.6) | 14.3 (13.0 – 15.6) |

primarily enhance the molecular classification of breast cancer and provide prognostic information. Specifically, the Prediction Analysis of Microarray 50 (PAM50) was designed to classify breast cancer subtypes, assigning tumors to intrinsic molecular subtypes—Luminal A, Luminal B, HER2-enriched, and Basal-like—based on the expression of 50 key genes[23,24]. This classification highlights breast cancer heterogeneity and underscores the need for personalized treatment strategies tailored to specific molecular subtypes[25]. Throughout this thesis, breast cancer subtypes are based on histopathological feature of the tumor. Despite advances in targeted treatments, breast cancer remains the leading cause of cancer-related mortality among women, emphasizing the ongoing need for innovative therapeutic approaches.

## Breast cancer and the (systemic) immune landscape

The immune system is a highly complex assembly of cells that functions to protect the body against infections, eliminate damaged cells, and maintain overall homeostasis. The systemic immune composition refers to the diverse array of immune cells that circulate throughout the body and collectively contribute to the immune response. Understanding the systemic immune composition is particularly important in the context of diseases like cancer, where alterations can significantly impact disease progression and treatment efficacy. The immune system consists of two main branches: the innate immune system and the adaptive immune system, each with unique roles but deeply interconnected through various mechanisms of cross-talk.

### The Immune System in Homeostasis

In a state of homeostasis, the immune system maintains a delicate balance, effectively protecting the host from pathogens while avoiding excessive or inappropriate responses that could cause tissue damage or autoimmune diseases. This equilibrium is achieved

through a tightly regulated interplay of immune cells, cytokines, and regulatory mechanisms. Regulatory T cells (Tregs), for example, play a crucial role in maintaining this balance by suppressing potentially harmful immune responses and preventing autoimmunity[26,27]. These cells act as a safeguard, ensuring that the immune system does not overreact to benign stimuli, which could lead to tissue damage. The production of anti-inflammatory cytokines, such as interleukin-10 (IL-10) and transforming growth factor-beta (TGF-β), helps to mitigate inflammatory responses and promote tissue repair[28-31]. Without a proper functioning immune system, one would either be confined to life in a bubble, or succumb from otherwise harmless pathogenic infection.

**Innate and Adaptive Immunity**

Innate immune cells, such as neutrophils, eosinophils, basophils, monocytes, macrophages, dendritic cells (DCs), and natural killer (NK) cells, provide the first line of defense against pathogens. They are characterized by their ability to respond rapidly and non-specifically to invading microorganisms. For example, neutrophils are the most abundant type of white blood cells, making up ~70-80% of the circulating white blood cells (WBCs). Neutrophils are among the first responders to sites of infection, where they engulf and destroy pathogens through phagocytosis. Regarding the role of neutrophils in cancer, preclinical studies have shown that neutrophils promote metastasis through various mechanisms[32-37]. Tumor-induced systemic inflammation often leads to elevated neutrophil counts in the blood, commonly represented in clinical settings by the Neutrophil-to-Lymphocyte Ratio (NLR). Clinical studies indicate that a high NLR is associated with poor prognosis and reduced therapy response across several cancer types, including breast cancer[38-43]. Eosinophils are under homeostatic conditions primarily involved in combating parasitic infections and modulating allergic inflammatory responses by releasing toxic granules and cytokines. In the context of immunotherapy against cancer, eosinophils can enhance the response to immune checkpoint inhibition, and correlate with clinical response[44,45]. Basophils play a key role in allergic reactions by releasing histamine and other mediators that increase vascular permeability and attract other immune cells to sites of inflammation. Monocytes can differentiate into macrophages and dendritic cells upon entering tissues, where they play critical roles in both pathogen elimination and the initiation of adaptive immune responses. Macrophages are highly versatile phagocytic cells that engulf pathogens, clear dead cells, and release cytokines to regulate immune responses. Macrophages play a dual role in cancer, either supporting tumor progression by promoting immunosuppression, angiogenesis, and metastasis or—when properly activated—combating the tumor through immune stimulation, phagocytosis of cancer cells, and direct cytotoxic activity. Tumor-associated macrophages (TAMs) are often skewed toward a pro-tumoral phenotype, making them a crucial target for cancer immunotherapy. Innate immune cells recognize pathogens







through pattern recognition receptors (PRRs), which detect pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), such as Toll-like receptors (TLRs) and NOD-like Receptors[46,47]. TLRs are membrane-bound receptors that recognize bacterial lipopolysaccharides (LPS), lipoproteins, flagellin, and unmethylated CpG DNA from bacteria and viruses[48]. NOD-like receptors are cytoplasmic receptors that detect intracellular PAMPs such as bacterial peptidoglycans[49,50]. In addition to PRRs, also chemokines like IL-8 and LTB-4, and components of the complement system, such as C3a and C5a, can attract and activate innate immune cells; particularly neutrophils[51-55].

The adaptive immune system is characterized by its specificity and memory, primarily mediated by T cells and B cells. T cells play a crucial role in the adaptive immune response, and they can be primarily categorized into two subsets: CD8+ T cells and CD4+ T cells. Each subset has distinct functions, mechanisms of action, and roles in immune regulation.

CD8+ T cells, also known as cytotoxic T lymphocytes (CTLs), are primarily responsible for directly killing infected or cancerous cells. They recognize antigens presented by Major Histocompatibility Complex (MHC) class I molecules, which are found on nearly all nucleated cells. Upon recognizing a foreign antigen, CD8+ T cells become activated and undergo clonal expansion, differentiating into effector cells capable of performing cytotoxic functions. The primary mechanism through which CD8+ T cells exert their cytotoxic effects involves the release of perforin and granzymes. Perforin forms pores in the target cell membrane, allowing granzymes, which are serine proteases, to enter and induce apoptosis (programmed cell death) in the infected or tumor cells. This process is vital for eliminating cells harboring intracellular pathogens, such as viruses, as well as malignant cells that present tumor-specific antigens[56]. In addition to their direct cytotoxic activity, CD8+ T cells can also produce a range of cytokines, such as interferon-gamma (IFN-γ), which enhances the immune response by activating macrophages and promoting inflammation. The effectiveness of CD8+ T cells in tumor surveillance has made them a significant target for cancer immunotherapy strategies, including immune checkpoint inhibitors and adoptive cell transfer therapies, such as CAR-T cell therapy[57-59].

CD4+ T cells, commonly referred to as helper T cells, are essential for orchestrating the adaptive immune response. They recognize antigens presented by MHC class II molecules, which are primarily expressed on professional antigen-presenting cells (APCs) such as dendritic cells, macrophages, and B cells. Upon activation, CD4+ T cells differentiate into various subtypes, each with specialized functions, including Th1, Th2, Th17 cells and Tregs. In short, Th1 cells primarily produce IFN-γ, promoting the activation of macrophages and enhancing the ability of CD8+ T cells to kill infected cells. They are crucial for combating intracellular pathogens, such as viruses and some bacteria[60]. Th2 cells are involved in promoting antibody production by B cells and are essential in orchestrating responses against extracellular pathogens like helminths. They secrete cytokines such as IL-4, IL-5, and

IL-13, which facilitate B cell activation and class switching to produce IgE antibodies[61,62]. Th17 cells are characterized by the production of IL-17 and are particularly important in defending against fungal and extracellular bacterial infections. Th17 cells play a role in promoting inflammation and recruiting neutrophils to sites of infection[63,64]. Tregs are a subset of CD4+ T cells that play a crucial role in maintaining immune tolerance and preventing autoimmune responses. They help suppress excessive immune activation and are characterized by the expression of the transcription factor FoxP3[27].

Both CD8+ and CD4+ T cells are integral components of the systemic immune composition, each playing distinct yet interconnected roles in immune surveillance, pathogen elimination, and the orchestration of adaptive immune responses. The balance between these CD4+ T cell subsets is vital for an effective immune response[65]. Dysregulation of this balance can lead to inadequate immune responses against pathogens or contribute to autoimmune diseases and cancer progression.

A component of the adaptive immune system that bridges innate and adaptive immunity are the B cells. B cells are activated by helper T cells and by directly encountering antigens. Activated B cells differentiate into memory B cells, and plasma cells that produce antibodies specific to the antigens[66]. These antibodies neutralize pathogens and mark them for destruction by other immune cells, thereby integrating the adaptive response with innate effector mechanisms such as phagocytosis[67,68]. In the context of cancer, B cells play a dual role, functioning as contributors to tumor immunity and as facilitators of tumor progression. They can produce antibodies that target tumor antigens, enhancing immune recognition and destruction of cancer cells. Additionally, B cells are key in the formation of tertiary lymphoid structures (TLS) in the TME, which are associated with immunotherapy response[69,70]. However, in some contexts, B cells may also promote systemic immunosuppression through the expansion of regulatory B cells, which are characterized by production of the anti-inflammatory cytokine IL-10[71,72]. Chemotherapy has lasting effects on B cells, leading to impaired memory B cell generation and maintenance, altered antibody production, and shifts in isotype distribution, which persist for months post-treatment[73,74].

DCs also serve as a crucial bridge between innate and adaptive immunity[75]. Upon encountering a pathogen, dendritic cells undergo maturation and migrate to lymphoid tissues, where they present processed antigens to naive T cells, along with necessary co-stimulatory signals and cytokines, initiating the adaptive immune response. This activation leads to the proliferation and differentiation of naïve T cells into effector cells, that secrete cytokines to further stimulate immune responses[76-79]. DCs can be classified into a three major subsets; plasmacytoid DC (pDCs), conventional DC type 1 (cDC1) and conventional DC type 2 (cDC2), each driving distinct immune responses. pDCs are known for their ability to produce significant amounts of type I interferons, particularly IFN-α, in response to viral infections[80]. In the cancer setting, pDCs can either support tumor elimination or facilitate







tumor evasion[81-84], underscoring the necessity of understanding the context in which pDCs operate. cDC1 cells are characterized by CD8α positivity and IL-12 production, promoting robust CD8+ T cell activation and Th1 differentiation, crucial for antiviral and antitumor immunity[79,80]. In contrast, cDC2 cells, typically CD8α-negative, enhance CD4+ T cell responses and are associated with Th2 polarization, playing a vital role in responses to extracellular pathogens and also in antitumor immune responses[79,80].

The last cell type that I will describe that bridges the innate and adaptive immune responses are natural killer (NK) cells. NK cells recognize stressed or abnormal cells through a balance of activating and inhibitory receptors. Activating receptors (e.g., NKG2D) detect stress ligands on target cells, while inhibitory receptors (e.g., KIRs) recognize normal MHC molecules, allowing NK cells to distinguish healthy cells from those that are infected or transformed[85,86]. Upon activation, NK cells release cytotoxic granules containing perforin and granzymes[86]. NK cells also express the low-affinity Fc receptor CD16, which enables them to detect antibody-coated target cells and to exert antibody-dependent cell cytotoxicity (ADCC)[85]. Additionally, NK cells secrete pro-inflammatory cytokines, such as interferon-gamma (IFN-γ), which enhance the immune response by activating macrophages and promoting T cell proliferation[86]. These combined functions make NK cells essential for early defense against infections and tumors, as well as for maintaining immune homeostasis.

**Chronic Inflammation and Immunosuppression in Cancer**

Inflammation is a double-edged sword in cancer. While inflammation can destroy tumor cells, it can also promote tumorigenesis and metastasis. Persistent inflammatory conditions create a microenvironment rich in cytokines, growth factors, and reactive oxygen species (ROS) that can lead to DNA damage, promoting mutations and cancer progression[87,88]. For example, chronic inflammation caused by conditions like inflammatory bowel disease can lead to continuous cellular turnover and mutation accumulation, fostering colorectal cancer development[89,90]. Immunosuppression is a significant hurdle in cancer immunotherapy[91-93]. Tumors can create an immunosuppressive microenvironment by recruiting Tregs, immunosuppressive neutrophils (often referred to as polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs)), monocytes (often referred to as M-MDSCs), and by producing immunosuppressive cytokines like TGF-β and IL-10[92,94-99]. These cells and mediators inhibit the function of effector T cells and NK cells, allowing the tumor to evade immune detection and destruction. Inflammatory cells, such as tumor associated macrophages (TAMs) and tumor associated neutrophils (TANs), often support tumor growth by producing factors that promote angiogenesis, tissue remodeling, and suppression of adaptive immunity[100-104]. TAMs and TANs can create a pro-tumor environment by releasing cytokines that inhibit the activation and function of cytotoxic T cells[101,105]. Additionally, in some tumors, immune checkpoint molecules such as PD-L1 are expressed by cancer cells and TAMs. PD-L1 can bind to PD-1 on T cells thereby leading to reduced activity of T cells.

Immunotherapies like the antibodies directed against PD-1/anti-PD-L1 inhibit this suppressive mechanism and reinvigorating the T-cell immune response against cancer.

In addition to immunosuppressive mechanisms within the TME described above, also systemic inflammation influences cancer progression and poses a challenge in the treatment of cancer. Cancer cells can secrete factors that influence hematopoiesis, prompting the bone marrow to release immune cells that favor tumor progression[106,107]. Tumors, for instance, often produce granulocyte-colony stimulating factor (G-CSF) and granulocyte–macrophage colony-stimulating factor (GM-CSF), which enhances the production and mobilization of myeloid cells[100,108]. This tumor-induced inflammation skews hematopoiesis towards increased myeloid cell mobilization, creating a supportive environment for metastasis. Such systemic changes aid in establishing a metastatic niche, comprising of supportive, non-malignant stromal cells, soluble factors, vascular networks, essential nutrients and metabolic components, along with the structural architecture of the extracellular matrix[109]. Moreover, pre-clinical studies have shown that neutrophils promote metastasis formation through various mechanisms, such as inducing systemic immune suppression, aiding circulating cancer cells, fostering (pre-)metastatic niche formation, facilitating cancer cell infiltration into distant tissues, and reactivating dormant cancer cells[32-37,87,110,111].

In addition to tumor-induced expansion of the systemic myeloid compartment, tumors can induce immunosuppressive states in immune cells such as neutrophils and monocytes through reprogramming and polarization processes[107]. In tumor-associated macrophages and monocytes, this cancer-specific reprogramming alters their transcriptional profiles, enabling them to support tumor growth rather than initiating an immune response[112]. Similarly, findings on polarization highlight how tumors direct immune cells toward pro-tumor phenotypes that favor immune evasion and tumor growth, reinforcing the tumor's survival strategy and contributing to an immunosuppressive macro-environment[100].

The immune system's ability to maintain homeostasis, its robust innate and adaptive responses, and the critical crosstalk between these systems are essential for effective cancer defense[71]. In fact, systemic immunity is essential for successful cancer immunotherapy[113]. However, chronic inflammation and systemic immunosuppression disrupt the balance between adaptive and innate immunity, contribute significantly to cancer progression and pose a challenge to treatment. Understanding these dynamics is crucial for developing strategies that enhance anti-tumor immunity while minimizing immune-related adverse effects. As we continue to unravel the complexities of the systemic immune landscape in cancer, we pave the way for innovative therapeutic approaches that harness the power of the immune system to combat cancer effectively.







**Immunotherapy and Breast Cancer**

Immunotherapy has become an important pillar in the treatment of various cancer types. The development of immune checkpoint inhibitors, particularly anti-CTLA-4 and anti-PD-1 therapies, marked a revolutionary shift in cancer treatment. In the 1990s, James Allison's research demonstrated that CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) serves as an immune checkpoint that inhibits T-cell activation[114]. His subsequent work led to the development of anti-CTLA-4 antibodies, demonstrating improved survival for stage IV melanoma patients that were previously untreatable[115]. This led to the approval of ipilimumab for metastatic melanoma by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) in 2011, representing the first checkpoint inhibitor in clinical use. Around the same time, researchers, including Tasuku Honjo, were studying PD-1 (programmed cell death protein 1)[116], another inhibitory receptor on T cells. Blocking PD-1 with anti-PD-1 antibodies, such as nivolumab and pembrolizumab, showed remarkable clinical efficacy, particularly in cancers like melanoma, lung cancer, and renal cell carcinoma[117,118]. Pembrolizumab received FDA approval for melanoma in 2014 and EMA approval in 2015. These therapies unlocked the immune system's potential to attack tumors, initiating a new era of cancer immunotherapy. Allison and Honjo were awarded the 2018 Nobel Prize in Physiology or Medicine for their pioneering discoveries in immune checkpoint blockade.

After the introduction of immune checkpoint inhibitors like anti-CTLA-4 and anti-PD-1/PD-L1 antibodies, the application of immunotherapy in breast cancer has evolved significantly. Historically, breast cancer has been considered less immunogenic because of the limited mutational load and the typically lower levels of tumor-infiltrating lymphocytes (TILs) compared to other cancers such as melanoma, which resulted in a greater focus on traditional therapies[119,120]. However, we now know that breast cancer is heterogeneous in terms of TILs, and that the TME of breast tumors can be classified into three types: immune-desert ("cold") tumors, which lack lymphocytes; immune-excluded tumors, where lymphocytes are confined to the surrounding stroma; and immune-infiltrated ("hot") tumors, which are rich in TILs[121]. Recent advancements have revealed the potential of immunotherapy in breast cancer, particularly for specific subtypes and disease stages[122].

**Triple-Negative Breast Cancer**

Metastatic TNBC has emerged as a key area for immunotherapy research due to its aggressive nature and higher levels of immune infiltration compared to other subtypes. In the early days of immunotherapy for metastatic TNBC, the efficacy of PD-1 blockade was limited in the later lines of treatment for mTNBC[123-125], underscoring the need for novel approaches to enhance tumor microenvironment sensitivity to this therapy. Preclinical studies suggested that low-dose chemotherapy and irradiation could have beneficial

immunomodulatory effects. Irradiation has been shown to induce type I interferons via the stimulator of interferon genes (STING) pathway, thereby enhancing T cell priming[126,127]. Additionally, cyclophosphamide has been reported to deplete regulatory T cells, potentially restoring effector functions of both T cells and natural killer cells[128]. Cisplatin can upregulate major histocompatibility complex class I expression and directly stimulate T cell function[129,130], while doxorubicin has been associated with depletion of immunosuppressive myeloid cells, increased type I interferon levels, and induction of immunogenic cell death[131-133]. To investigate the immunomodulatory effects of irradiation and low-dose chemotherapy, the TONIC trial was designed[134]. In this trial, patients with metastatic TNBC were randomized into five different 2-week induction arms: 1) control arm without an induction treatment, 2) irradiation, 3) cyclophosphamide, 4) cisplatin, and 5) doxorubicin, with all five groups subsequently receiving nivolumab. Based on clinical and translational findings, both doxorubicin and cyclophosphamide induction prior to nivolumab treatment appear to create a more favorable tumor microenvironment, potentially increasing response rates to PD-1 blockade in metastatic TNBC. As a result, both induction treatments are currently being further studied in larger comparative cohorts of unselected patients with mTNBC.

The IMpassion130 trial (NCT02425891) was pivotal in demonstrating the efficacy of atezolizumab, an anti-PD-L1 antibody, combined with nab-paclitaxel for patients with metastatic PD-L1-positive TNBC[135]. This study showed a significant improvement in progression-free survival (PFS) and overall survival (OS), leading to the FDA and EMA approvals of atezolizumab for this indication. In addition, the KEYNOTE-355 trial (NCT02819518) explored the use of pembrolizumab, an anti-PD-1 antibody, in combination with chemotherapy for metastatic PD-L1-positive TNBC. Results from this trial indicated that pembrolizumab significantly improved PFS and OS, solidifying its role in treating this aggressive breast cancer subtype[136].

The potential of immunotherapy extends to early-stage TNBC as well. The KEYNOTE-522 trial (NCT03036488) evaluated pembrolizumab as neoadjuvant therapy combined with chemotherapy. This study demonstrated that adding pembrolizumab to standard-of-care chemotherapy significantly increased the rate of pathological complete response (pCR) and overall survival (OS) in high-risk early-stage TNBC[137], resulting in its recent approval for this treatment setting. Consequently, immunotherapy combined with chemotherapy has become the standard of care for stage II–III TNBC. However, it remains unknown which patients might benefit from immune checkpoint inhibitors alone, without chemotherapy, and what the potential advantages of combining immune checkpoint inhibitors (ICIs) could be in this patient population.

**Hormone Receptor-Positive Breast Cancer**

Hormone receptor-positive (HR+) breast cancer, has been less responsive to immunotherapy, primarily due to lower levels of immune infiltration. However, there is ongoing research to evaluate the efficacy of immune checkpoint inhibitors in combination with hormonal







therapies or other agents. Preliminary studies have explored the use of anti-PD-1/PD-L1 therapies in combination with endocrine therapy for advanced HR+ breast cancer, with mixed results[138,139]. However, two positive phase III studies have demonstrated preliminary evidence of the potential for combining immune checkpoint inhibitors with therapies such as chemotherapy and endocrine therapy for HR+ breast cancer[140,141]. Additionally, data from the I-SPY2 trial, which investigated pembrolizumab in combination with chemotherapy, showed promising outcomes in HR+ breast cancer[142]. These advancements highlight the importance of integrating immunotherapy strategies into HR+ treatment regimens while continuing research to refine patient selection and identify predictive biomarkers.

**HER2-Positive Breast Cancer**

For HER2-positive breast cancer, the incorporation of immunotherapy is also under investigation. Studies are exploring combinations of anti-HER2 therapies (like trastuzumab) with immune checkpoint inhibitors to evaluate whether this approach can enhance the immune response against HER2-expressing tumors, generating conflicting results[143-145]. Recent trials, such as those investigating the use of pembrolizumab in combination with trastuzumab and chemotherapy for advanced HER2-positive breast cancer are ongoing. The aim is to determine whether this combination can improve outcomes in a population that historically has benefitted from targeted therapies.

**Challenges and Future Directions**

While immunotherapy has made significant strides in TNBC, challenges remain in effectively treating other breast cancer subtypes, particularly HR+ and HER2-positive cancers. Ongoing clinical trials are focusing on combinations of immune checkpoint inhibitors with targeted therapies, chemotherapy, or radiation to overcome resistance and enhance therapeutic efficacy. Research is also focused on identifying predictive biomarkers, such as stromal TILs and immune gene signatures, to more effectively select patients likely to benefit from immunotherapy. Furthermore, efforts are directed at unraveling the mechanisms by which myeloid cells contribute to the immune response, aiming to understand their dual roles in promoting or suppressing immune activity within the tumor microenvironment.

Moreover, monitoring the systemic immune landscape in cancer patients could provide valuable insights into how immune suppression beyond the tumor microenvironment influences disease progression and response to therapy. Systemic immunosuppression may compromise the body's ability to mount an effective anti-tumor immune response. By assessing immune markers in peripheral blood, we could identify broader immune dysfunctions that contribute to tumor growth and resistance to treatments like immunotherapy. Understanding this relationship could guide new strategies to counteract immune suppression systemically, potentially improving therapeutic outcomes across cancer types.

## Vulvar intraepithelial neoplasia (VIN)

While breast cancer develops as a result of mutations in the DNA, there are also virus induced cancers. Viruses can cause cancer by inserting their genetic material into host cells, which can disrupt normal cellular functions and lead to uncontrolled cell growth. Some viruses, like human papillomavirus (HPV) and Epstein-Barr virus (EBV), produce proteins that interfere with tumor-suppressor genes or activate oncogenes, driving the development of cancerous cells over time[146]. The most common transforming virus is HPV. HPV can cause several types of cancer, including cervical, uterus, head and neck, anal, penile, vaginal and vulvar cancers. Though not all cases of the carcinomas listed above are a result of an HPV infection, the vast majority are, and some of these cancer types remain the main cause of cancer-related death in women in certain areas of the world[1,3]. HPV causes cancer by producing two key oncoproteins, E6 and E7, which interfere with the cell's tumor-suppressor mechanisms. E6 binds to and degrades p53, a protein that regulates cell death, while E7 inactivates the retinoblastoma protein (pRb), disrupting cell cycle control and promoting unregulated cell division, leading to cancer development[146]. HPV-induced tumors might theoretically be more straightforward to be detected and eradicated by the immune system because they express viral epitopes. These epitopes can make tumors appear more foreign to the immune system, potentially enhancing the body's capacity to recognize and eliminate cancer cells. Despite this, spontaneous regression of tumors remains rare in clinical practice.

One of the cancer types that is often HPV-induced in vulvar cancer[147]. The pre-malignant stage of vulvar cancer is termed vulvar intraepithelial neoplasia (VIN). Though VIN lesions are not yet cancerous, they cause considerable discomfort to the women affected. Spontaneous regression of the condition is uncommon, occurring in only 1%–2% of women, while progression to vulvar cancer is observed in approximately 2%–8% of cases[148-151]. The current treatment options for VIN, which include surgical excision, laser therapy, and topical medications, often lead to mutilating or otherwise uncomfortable side effects. These treatments can severely impact a woman's sexual health and overall quality of life. Additionally, the responses to these treatments are frequently not durable, leading to high rates of recurrence.

Given these challenges, the development of novel immunotherapeutic approaches holds great promise. Immunotherapy could target the viral components of HPV-induced lesions, providing a more effective and less invasive treatment option and hopefully leading to more durable responses.

## Scope of this Thesis

In this thesis, two parts can be distinguished. The first part consists of **chapters 2, 3, 4** and **5** and has a rather fundamental-translational character. In these chapters, the focus lies on







assessing the influence of breast cancer subtype and disease stage on the systemic immune landscape of breast cancer patients, and what the impact is of (triple negative) breast cancer on functional aspects of circulating immune cells, especially neutrophils. The aim of the studies described in the first part of this thesis was to unravel the influence of breast cancer subtypes and disease stages on the composition and function of the systemic immune system. We hypothesized that comprehensive immune profiling of breast cancer patients, compared to healthy donor reference profiles, would provide critical insights into systemic inflammatory states and immunosuppressive mechanisms.

The second part of this thesis, encompassing **chapters 6, 7,** and **8**, presents clinical trials in which I contributed as a member of the translational research team. The commonality of this part lies in investigating treatment effects, covering a range of approaches. These include examining treatment-induced changes in circulating immune cells in relation to specific clinical outcomes, as well as immunomonitoring of specific T cells that recognize antigens introduced by therapeutic vaccines. Regarding this second part of this thesis, it was my aim to monitor overall changes in the immune profiles of patients with breast cancer or VIN lesions during immunotherapy. This research explored varying response dynamics and immunosuppressive mechanisms, which may guide more effective immunotherapeutic interventions and lead to novel strategies to improve immunotherapy responses.

**Chapter 2** provides a comprehensive method to assess the direct *ex vivo* motility and migration of freshly isolated human neutrophils, offering valuable insights into their behavior. Understanding neutrophil motility and migration is critical for comprehending immune responses and inflammatory processes, as it sheds light on their substantial contribution to cancer progression. Neutrophils possess a remarkable migratory capacity, enabling them to extravasate and infiltrate tissues but also tumors, where they carry out essential or detrimental effector functions, depending on the context. This ability contributes to their pivotal role in orchestrating tumor-induced systemic inflammation and their growing recognition as key players in both the initiation and progression of cancer.

The dysregulating properties of cancer reaches beyond the local tumor microenvironment, however, it remains largely unknown how the systemic immune landscape is modified during breast cancer progression and whether this is breast cancer subtype dependent. In **chapter 3**, I describe a comprehensive analysis of the systemic immune landscape in a large cohort of breast cancer patients, covering different tumor molecular subtypes and disease stages, alongside a control group of healthy donors. Employing multi-parameter flow cytometry, we assessed the abundance, phenotype, and activation status of various innate and adaptive immune cell populations across 420 peripheral blood samples. Because all blood samples were analyzed immediately after collection, we were able to include the often overlooked granulocyte populations, including neutrophils and eosinophils, in our analysis. Our data indicate that the immune landscape

is more markedly altered in metastatic breast cancer compared to non-metastatic cases, with the most significant changes observed in the triple-negative subtype.

In **chapter 4**, we comprehensively profiled the systemic immune landscape in patients with TNBC at distinct disease stages, to understand how cancer progression and treatment history shape the systemic immune landscape. We performed multi-parameter flow cytometry analysis to assess the global systemic immune landscape, including often overlooked granulocytes. We demonstrated that the systemic immune landscape of TNBC patients differs from that of healthy donors in a stage-dependent manner, with some—but not all—of these alterations attributable to prior chemotherapy treatment.

In **chapter 5**, I describe a research project in which we conducted single-cell RNA sequencing on fresh blood samples from patients with mTNBC and HDs, without any prior immune cell type enrichments. While this project did not reveal clear differences between HDs and mTNBC patients, the results were inconclusive due to the small sample size and the considerable heterogeneity observed. As such, we do not conclude that no differences exist between the two groups. Alongside detailing the methods and results, I also offer recommendations for future studies to help others avoid the challenges we encountered. These insights aim to improve experimental design and optimize the likelihood of generating more definitive results.

**Chapter 6** describes the results of the adaptive phase II BELLINI trial, which explored the potential of short-term immune checkpoint inhibition (ICI) to induce immune activation in patients with non-metastatic TNBC. The aim was to explore the potential of treating non-metastatic TNBC patients with neoadjuvant ICI in the absence of chemotherapy. This window-of-opportunity trial describes three cohorts, each showing a response rate of 50-60%. In cohorts 1 and 2, the response was measured as a biological endpoint, while in cohort 3, pathological complete response (pCR) was used as the measure of response. Flow cytometry of fresh blood samples showed an increase in Ki-67+ cells within the PD-1+ conventional CD4+ T cell population in responders, with a similar trend observed for CD8+ T cells. The findings of the BELLINI-trial demonstrate that neoadjuvant immunotherapy, administered without chemotherapy, shows promising efficacy and warrants further investigation in patients with early-stage TNBC.

The translational research project described in **chapter 7** aims to identify factors associated with the response of mTNBC patients to PD-1 blockade (TONIC trial). Comprehensive immune profiling of fresh blood samples and paired tumor biopsies revealed that both systemic and intratumoral eosinophils increased in responders following PD-1 blockade, a pattern not observed in non-responders. *In vivo* experiments using spontaneous mouse models of primary and metastatic breast cancer further demonstrated the critical role of eosinophils in mediating the response to immune checkpoint blockade. These findings highlight that therapeutic engagement of eosinophils could enhance responses to immune checkpoint inhibitors in breast cancer.







**Chapter 8** presents the results of a phase I/II clinical trial in which patients with usual vulvar intraepithelial neoplasia (uVIN) received a genetically optimized HPV-16 E6/E7 DNA tattoo vaccination. The primary endpoint of the trial was the induction of an immunological response. To assess this, I conducted ex vivo functional assays using patients' peripheral blood mononuclear cells to monitor systemic HPV-specific T cell responses before and after treatment. In addition to immune monitoring, the chapter also reports on the safety and clinical outcomes of the vaccination.

Finally, the results described in this thesis are summarized and discussed in **chapter 9**. In this chapter I highlight future perspectives of the work presented and focus on potential new research avenues.

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