UNIVERSITÀ DEGLI STUDI DEL PIEMONTE ORIENTALE “AMEDEO AVOGADRO”

School of medicine - Department of Health Sciences

Master’s Degree in Medical Biotechnology

Circulating B and NK cell profiling as a predictor of response to neoadjuvant chemotherapy in patients with breast cancer

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Academic year 2025/2026

List of abbreviations

BC = Breast Cancer
ARG = Arginase

US = United States
NOS = Nitric Oxide Synthase

CDK = Cyclin-dependent Kinase
ROS = Reactive Oxygen Species

DCIS = Ductal Carcinoma in Situ
RNS = Reactive Nitrogen Species

LCIS = Lobular Carcinoma in situ
DFS = Disease free survival

IDC = Invasive Ductal Carcinoma
OS = Overall Survival

ILC = Invasive Lobular Carcinoma
TAN = Tumor-Associated Neutrophil

ER = Estrogen Receptor
MS = Mass-Spectrometry

PgR = Progesterone Receptor
PRS = Polygenic risk score

HER2 = Human Epidermal growth factor Receptor 2

TNBC = Triple Negative Breast Cancer
SOC = Standard of Care

MHC II = major histocompatibility complex class II

PDGF = Platelet-derived Growth Factor
TMS = Trimethylsilyl derivatives

NAC = Neoadjuvant Chemotherapy
ECM = Extracellular Matrix

EDTA = Ethylenediaminetetraacetic Acid
TLS = Tertiary Lymphoid Structures

ADCC = antibody-dependent cellular cytotoxicity

pCR = pathological Complete Response

MMPs = Matrix Metalloproteinases

SASP = Senescence-associated Secretory Phenotype

PBMCs = Peripheral Blood Mononuclear cells

VEGF = Vascular Endothelial Growth Factor

FACS = Fluorescence-Activated Cell Sorter

NK = Natural killer cells

RD = Residual Disease

Abstract

Background: Breast cancer is a biologically heterogeneous disease and remains the most frequently diagnosed cancer among women worldwide. Neoadjuvant chemotherapy is the preferred standard of care for many patients with HER2+ and triple-negative breast cancer, with the achievement of pathological complete response (pCR) representing its principal therapeutic goal. Increasing evidence suggests that treatment-induced immune modulation may influence therapeutic efficacy; however, the contribution of specific circulating immune cell populations remains incompletely understood. The aim of this study was to characterize circulating B-cell and natural killer cell subsets in breast cancer patients undergoing neoadjuvant chemotherapy and to evaluate their association with pCR.

Methods: Thirty-nine patients with operable breast cancer eligible for neoadjuvant chemotherapy were enrolled. Peripheral blood samples were collected before treatment initiation and after completion of chemotherapy. Flow cytometry was used to characterize class-switched memory B cells (CD27+IgD-), regulatory B cells (CD24+CD27+), and cytotoxic natural killer cells (CD56dimCD16+). An effector-to-regulatory immune ratio was also calculated. Immune cell frequencies at baseline and after treatment were compared and their association with pathological complete response was evaluated using non-parametric statistical analyses.

Results: All enrolled patients completed neoadjuvant chemotherapy and underwent surgery. The overall pCR rate was 46,15%, with the highest frequency observed in HER2+ tumors, followed by TNBC and ER+ disease. No statistically significant differences were observed between pre-treatment and post-treatment frequencies of class-switched memory B cells, regulatory B cells, or cytotoxic natural killer cells. In contrast, the effector-to-regulatory immune ratio showed a significant variation following treatment. Baseline immune cell frequencies and therapy-induced changes were not significantly associated with pCR. Furthermore, no significant correlations were identified between immune cell dynamics and patient age, and no significant differences in baseline immune profiles or treatment-induced changes were observed between TNBC and HER2+ patients.

Conclusions: Our findings suggest that while individual circulating immune cell frequencies are stable across molecular subtypes and resistant to direct chemotherapy-induced depletion, neoadjuvant treatment significantly shifts the systemic effector-to-regulatory equilibrium. The lack of direct association between peripheral cell dynamics and pCR underscores the complexity of the tumor microenvironment interactions. Limited by our cohort size, these promising results provide a strong foundation for future investigation of circulating immune biomarkers in larger breast cancer cohorts.

INTRODUCTION

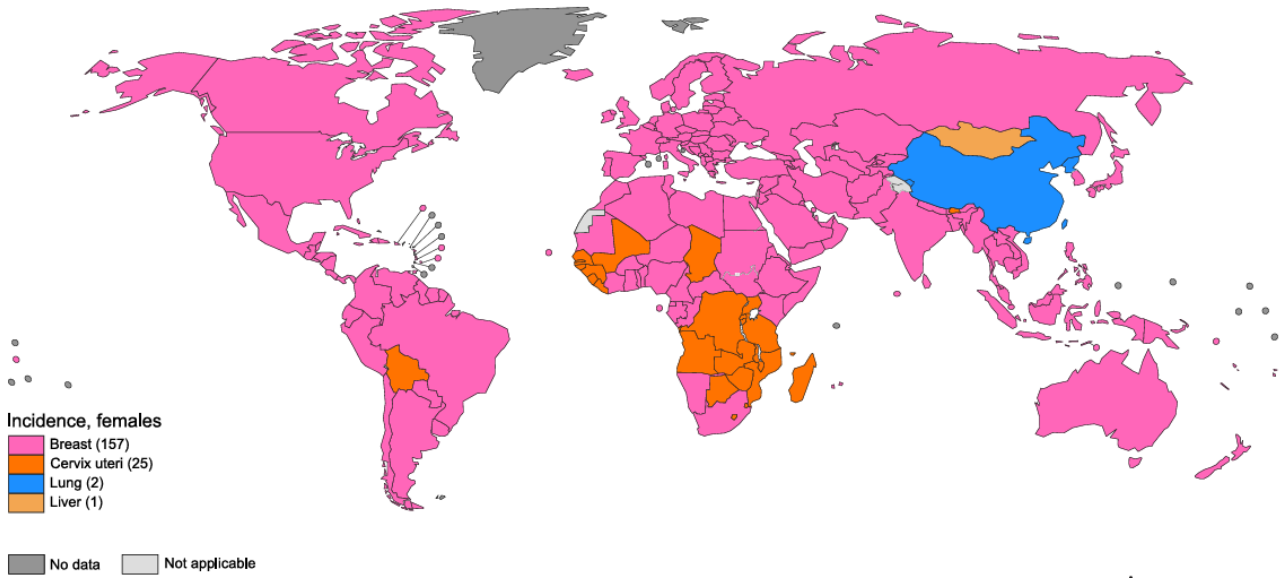
1.1 Breast cancer

Breast cancer (BC) remains a major global public health challenge and is the most frequently diagnosed cancer among women worldwide, with approximately 2.3 million new cases and about 670 000 deaths reported globally in 2022 [1].

In the United States, breast cancer accounts for nearly 30 % of all newly diagnosed cancers in women, with current estimates indicating that approximately 1 in 8 women will develop invasive breast cancer over their lifetime, compared with about 1 in 1 000 men [1,2].

The incidence of breast cancer increases markedly with age, with the majority of new diagnoses occurring in women aged 40 years and older, and the highest rates observed in women over 70 years of age [1].

Despite its high incidence, breast cancer mortality has declined substantially over recent decades in the United States, with death rates decreasing by more than 40 % since the late 1980s, largely attributable to improvements in early detection, screening practices, and systemic therapies [2,3] (Figure 1).



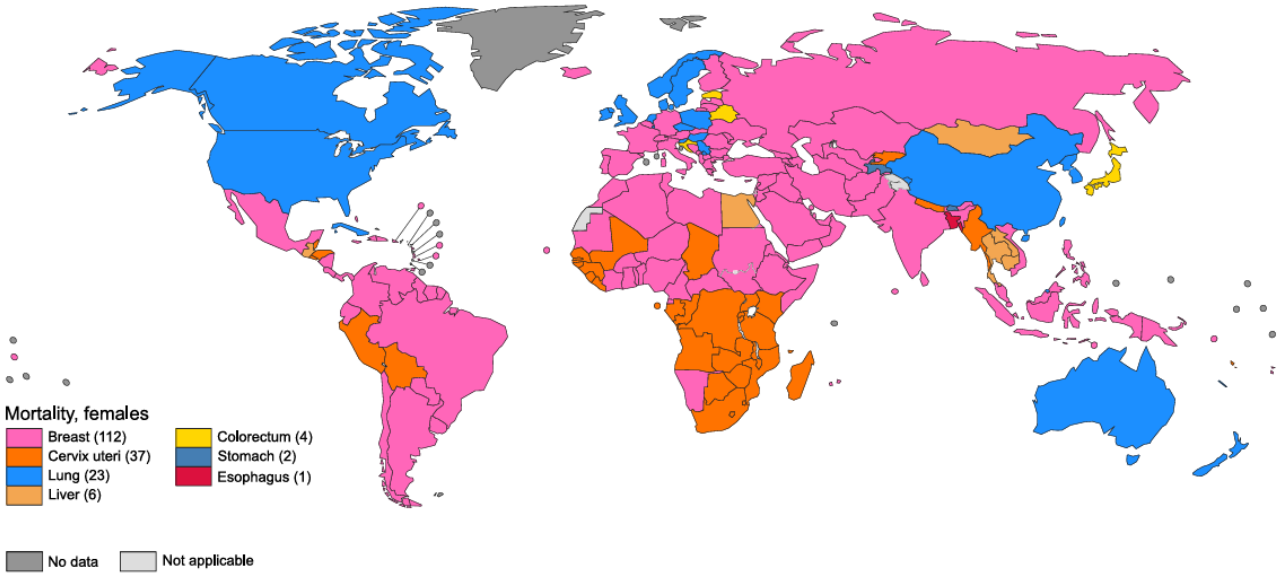


Figure 1: Most common cancer types by country in women for incidence (A) and mortality (B) in 2022, based on national cancer registry and statistical data. Each color represents the cancer type with the highest incidence or highest death count in that country. *Adapted from Ferlay et al., 2024 (GLOBOCAN 2022 estimates) [31].*

1.2 Pathogenesis and clinical classification of breast cancer

The precise mechanisms underlying breast cancer (BC) initiation are not yet fully elucidated; however, current evidence supports a multistep process involving the progressive accumulation of genetic and epigenetic alterations in normal or precursor mammary cells [4]. These molecular changes can disrupt key signaling networks that regulate cell proliferation, survival, differentiation, and migration, thereby promoting oncogenic transformation. Among the pathways most frequently implicated in breast tumorigenesis are Wnt/β-catenin, cyclin-dependent kinase (CDK) signaling, NOTCH, and the PI3K/AKT/mTOR axis, all of which are commonly altered across different breast cancer subtypes [4].

Breast cancer can arise from distinct anatomical and cellular compartments within the breast, including the ductal epithelium, lobular epithelium, or the surrounding stromal tissue. Accordingly, breast malignancies are broadly classified into carcinomas and sarcomas based on their cellular origin [5]. Carcinomas, which originate from the epithelial cells lining the ducts and lobules, represent the overwhelming majority of breast cancers, whereas sarcomas, derived from the mesenchymal stromal compartment, account for less than 1% of cases [5].

From a pathological and clinical perspective, breast cancer is further categorized according to invasiveness into non-invasive (in situ), invasive, and metastatic disease (Figure 2) [6].

Carcinoma in situ is characterized by malignant epithelial cells confined to the ducts or lobules without invasion through the basement membrane. These lesions are classified as ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS) [6].

In contrast, invasive breast cancer is defined by basement membrane disruption, allowing malignant cells to infiltrate surrounding tissue, most commonly presenting as invasive ductal carcinoma (IDC) or invasive lobular carcinoma (ILC) [6].

Metastatic breast cancer is distinguished by dissemination to regional lymph nodes, particularly the axillary nodes, or distant organs such as bone, liver, lung, and brain, typically arising from progression of untreated or treatment-resistant invasive disease [6].

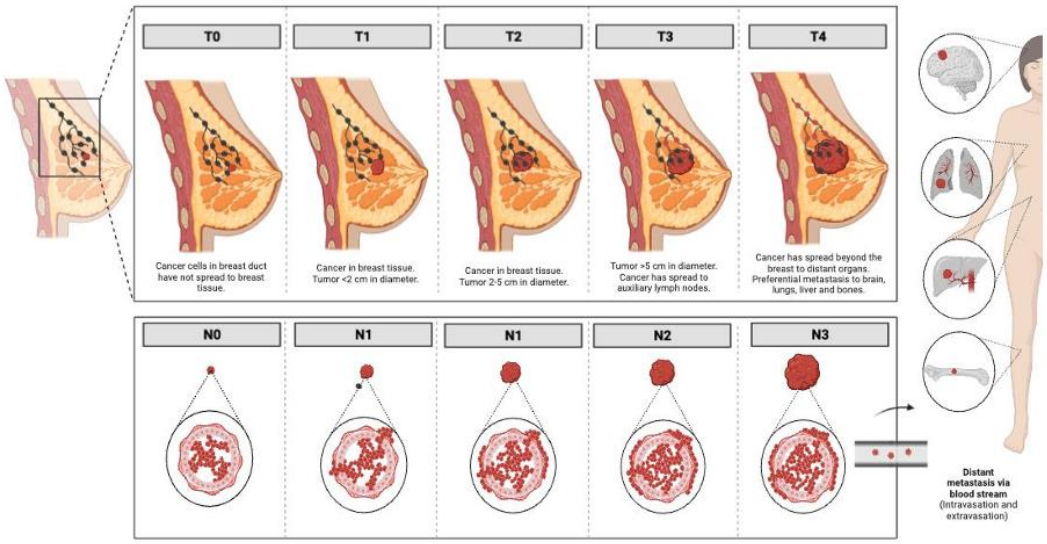


Figure 2: Schematic representation of breast cancer progression from non-invasive (stage 0) through invasive stages (I–III) to metastatic disease (stage IV). *Adapted from* Martin-García D, Téllez T, Redondo M, García-Aranda M. *Calcium Homeostasis in the Development of Resistant Breast Tumors.* Cancers. 2023 [32]. Figure created with *BioRender.com*.

Clinically, the anatomical extent of breast cancer is described using the Tumor-Node-Metastasis (TNM) staging system, which classifies disease from stage 0 to stage IV based on tumor size, lymph node involvement, and presence of distant metastases [6,7]. Briefly, stage 0 corresponds to non-invasive disease; stage I describes early invasive cancer with limited spread; stage II includes invasive tumors with involvement of one to three axillary lymph nodes; stage III encompasses locally advanced disease with more extensive nodal involvement; and stage IV denotes metastatic breast cancer [7]. The TNM classification provides both prognostic and predictive information and remains fundamental for treatment planning [6,7].

Beyond anatomical staging, contemporary breast cancer classification integrates additional clinicopathological and molecular features, including histological grade, hormone receptor status (estrogen receptor [ER], progesterone receptor [PR]), HER2 expression, and results from validated multigene expression assays [7]. Patient-related factors such as age, menopausal status, family history, breast density, race, and ethnicity are also incorporated into clinical decision-making. Together, these variables define an individualized clinical prognostic stage, which serves as the basis for guiding initial therapeutic strategies and estimating outcomes [6,7].

In clinical practice, the anatomical extent of breast cancer is most commonly described using the Tumor-Node-Metastasis (TNM) classification system, which categorizes disease from stage 0 to stage IV based on primary tumor characteristics, regional lymph node involvement, and the presence of distant metastases [7].

- Stage 0 corresponds to non-invasive disease confined to the ducts or lobules, encompassing in situ lesions.
- Stage I defines early invasive breast cancer with limited tumor size and no or minimal nodal involvement.
- Stage II includes invasive tumors associated with regional spread to one to three axillary lymph nodes.
- Stage III represents locally advanced disease, characterized by more extensive nodal involvement, typically four or more axillary lymph nodes or spread to internal mammary nodes, without evidence of distant metastasis.
- Stage IV denotes metastatic breast cancer, defined by dissemination to distant organs and corresponding to an M1 designation in the TNM system (Table 1) [5-7].

Overall Stage	T category	N category	M category
Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage IIA	T0	N1	M0
	T1	N1	M0
	T2	N0	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T0	N2	M0
	T1	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
Stage IIIB	T4	Any N	M0
Stage IIIC	Any T	N3	M0
Stage IV	Any T	Any N	M1

Table 1: Anatomical staging of breast cancer according to the TNM classification system. It shows the correspondence between overall stage (0-IV) and tumor size (T), regional lymph node involvement (N), and distant metastasis (M).

Beyond its role in anatomical description, the TNM staging system provides critical prognostic and predictive information and remains a cornerstone for treatment planning and outcome estimation in breast cancer.

1.3 Molecular subtypes of breast cancer

Breast cancer is a biologically, pathologically, and clinically heterogeneous disease, requiring an integrated classification approach that combines molecular, pathological, and patient-related characteristics. Contemporary clinical characterization incorporates tumor-specific features such as histological grade, mitotic activity, necrosis, hormone receptor expression (estrogen receptor [ER] and progesterone receptor [PR]), HER2 status, Ki-67 proliferation index, and the results of validated multigene expression assays, together with patient-related factors including age, menopausal status, family history, mammographic breast density, and race or ethnicity [7-9].

From a molecular pathology perspective, breast cancer is currently categorized into five major intrinsic subtypes: luminal A, luminal B/HER2-negative, luminal B/HER2-positive, HER2-enriched, and triple-negative breast cancer (TNBC). They are based primarily on ER, PR, HER2, and Ki-67 expression, as defined by the St. Gallen International Consensus and incorporated into European and national clinical guidelines [8,9] (Figure 3).

Luminal A tumors, characterized by strong ER and PR expression, HER2 negativity, and low proliferative activity, represent the most prevalent subtype and are associated with indolent behavior and favorable prognosis.

Luminal B tumors, which account for approximately 20-30% of cases, display higher proliferation rates and may be HER2-negative or HER2-positive, conferring an increased risk of recurrence compared with luminal A disease [8,9].

The HER2-enriched subtype, defined by HER2 overexpression in the absence of hormone receptors, comprises approximately 10-15% of breast cancers and is associated with aggressive tumor biology, although outcomes have markedly improved with the introduction of HER2-targeted therapies [8].

Triple-negative breast cancer, characterized by the lack of ER, PR, and HER2 expression and typically high Ki-67 levels, accounts for roughly 15% of cases and is associated with younger age at diagnosis, genetic predisposition, early metastatic spread, and higher rates of relapse; importantly, TNBC represents a biologically diverse entity encompassing multiple molecular subgroups beyond the absence of classical biomarkers [8-10].

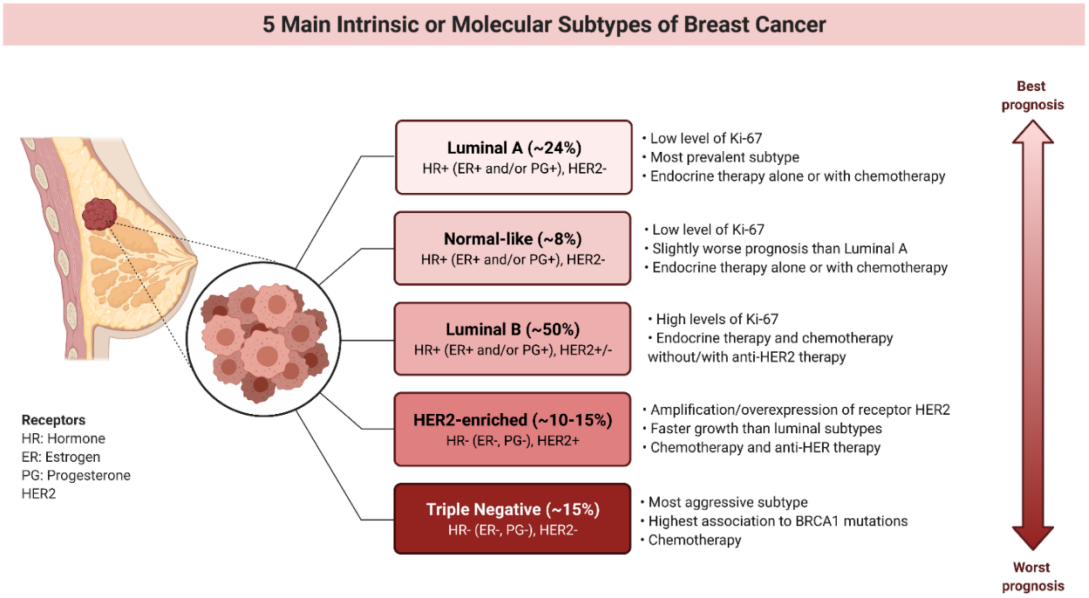


Figure 3: Schematic overview of the five main intrinsic (molecular) subtypes of breast cancer. In particular, luminal A, normal-like, luminal B, HER2-enriched, and triple-negative breast cancer. *Adapted from* Wawruszak A, Halasa M, Okoń E, Kukula-Koch W, Stępulak A. *Valproic Acid and Breast Cancer: State of the Art in 2021.[33]*

Collectively, the integration of anatomical stage, pathological characteristics, molecular subtype, multigene profiling, and patient-specific factors enables assignment of a refined clinical and

prognostic stage, which serves as a baseline for individualized risk stratification and guides initial therapeutic decision-making [10].

1.4 Breast cancer risk factor

Breast cancer risk results from a multifactorial interplay between genetic susceptibility and non-genetic factors. A minority of cases, approximately 5-10%, are attributable to inherited predisposition, most notably pathogenic variants in the high-penetrance tumor suppressor genes BRCA1 and BRCA2, whose carriers face a markedly increased lifetime risk of developing the disease.

In addition, mutations in other high- and moderate-penetrance genes, including TP53, PTEN, PALB2, ATM, CHEK2, and related DNA-repair genes, further contribute to hereditary breast cancer risk [11].

Advances in next-generation sequencing have enabled multigene panel testing and the development of polygenic risk scores, which integrate the cumulative effect of multiple variants to improve individual risk stratification, although their routine clinical implementation remains limited [11].

Category	Risk factors
High-penetrance genes	BRCA1, BRCA2
Moderate-penetrance genes	PALB2, TP53, PTEN, ATM, CHEK2
Demographic factors	Female sex, increasing age
Hormonal & reproductive	Early menarche, late menopause, nulliparity, late first pregnancy, limited breastfeeding
Lifestyle & metabolic	Obesity, diet, hormone replacement therapy

Table 2: Major genetic and non-genetic risk factors associated with breast cancer.

Non-genetic risk factors play an equally important role and encompass demographic, hormonal, reproductive, and lifestyle determinants. Female sex and increasing age represent the strongest epidemiological risk factors, while breast cancer incidence is also influenced by socioeconomic development. Hormonal and reproductive factors, largely reflecting cumulative lifetime exposure

to estrogens, include early menarche, late menopause, nulliparity, delayed age at first childbirth, and limited breastfeeding [11,12] (Table 2).

Additional contributors include the use of hormone replacement therapy, obesity particularly after menopause, and dietary patterns, all of which have been consistently associated with increased breast cancer risk.
Together, genetic predisposition and modifiable and non-modifiable risk factors form the basis of current risk assessment models and preventive strategies [11,12].

1.5 Therapeutic options: local and systemic therapy

Therapeutic strategies for breast cancer are individualized according to disease stage, tumor biology, and patient-related factors, with distinct goals in non-metastatic versus metastatic settings.
In early and locally advanced disease, treatment aims to achieve locoregional control and reduce the risk of distant recurrence, whereas in metastatic breast cancer the primary objectives are prolongation of survival and symptom palliation [13-15].

Local-regional therapy represents the cornerstone of management for early-stage breast cancer and includes surgical resection of the primary tumor with assessment of axillary lymph nodes, often followed by adjuvant radiotherapy. Surgical options include mastectomy and breast-conserving surgery (BCS), with multiple randomized trials demonstrating equivalent long-term survival when BCS is combined with radiotherapy in appropriately selected patients. The choice between surgical approaches depends on tumor size and extent, radiological findings, eligibility for radiotherapy, and patient preference [13-15].

Systemic therapy consists of drugs capable of reaching cancer cells throughout the body and may be administered in the preoperative (neoadjuvant), postoperative (adjuvant), or both settings to eradicate micrometastatic disease or downstage tumors prior to surgery.
The selection of systemic treatment is guided by molecular subtype and includes endocrine therapy, targeted therapy, and chemotherapy [15].

Endocrine therapy represents the primary systemic treatment for hormone receptor-positive breast cancer and acts by counteracting estrogen-driven tumor growth; it typically consists of

oral anti-estrogen agents administered for at least five years, with extended treatment considered in patients at higher risk of recurrence.

Tamoxifen can be used in both premenopausal and postmenopausal patients and functions as a selective estrogen receptor modulator by competitively inhibiting estrogen binding to the estrogen receptor, whereas aromatase inhibitors reduce systemic estrogen levels by suppressing peripheral estrogen synthesis and are preferentially used in postmenopausal women.

In advanced disease, the addition of CDK4/6 inhibitors to endocrine therapy has significantly improved clinical outcomes by delaying or overcoming endocrine resistance [15].

Targeted therapy has profoundly improved outcomes in HER2-positive breast cancer, for which recommended agents include trastuzumab, pertuzumab, trastuzumab emtansine, and neratinib. Trastuzumab and pertuzumab are humanized monoclonal antibodies with complementary mechanisms of action: trastuzumab inhibits HER2 receptor cleavage and downstream signaling, while pertuzumab prevents HER2 dimerization, particularly with EGFR family members, resulting in enhanced blockade of oncogenic pathways and induction of antibody-dependent cell-mediated cytotoxicity.

Despite advances in endocrine and targeted treatments, chemotherapy remains a fundamental component of breast cancer management, particularly in patients with stage I-III disease and as the main systemic option for triple-negative breast cancer.

Anthracyclines and taxanes are commonly used first-line chemotherapeutic agents; anthracyclines intercalate into DNA and inhibit topoisomerase II, whereas taxanes bind to microtubules and prevent their depolymerization, ultimately disrupting mitosis and inducing apoptotic cell death [13-15].

1.5.1 Neoadjuvant chemotherapy (NAC) and pathological complete response (pCR)

In early-stage non-metastatic breast cancer, surgery is traditionally considered the primary therapeutic approach; however, it may not represent the optimal initial strategy for all patients, particularly those with stage II-III disease [13].

In this setting, neoadjuvant chemotherapy (NAC), also referred to as neoadjuvant systemic therapy, offers several clinical advantages, including tumor downstaging in the breast and axilla, increased rates of breast-conserving surgery, and reduced need for complete axillary lymph node dissection without compromising long-term survival outcomes.

In addition, NAC provides an in vivo assessment of tumor sensitivity to systemic therapy, enabling treatment personalization and facilitating post-neoadjuvant therapeutic decision-making [13,14].

The principal clinical objective of NAC is the achievement of pathological complete response (pCR), defined as the absence of residual invasive disease in both the breast and axillary lymph nodes (ypT0/Tis ypN0), according to current AIOM and international guidelines.

Achievement of pCR is strongly associated with improved event-free survival, disease-free survival, and overall survival, and its prognostic significance is particularly pronounced in triple-negative breast cancer (TNBC) and hormone receptor–negative/HER2-positive subtypes [13-15].

Standard NAC regimens typically consist of sequential anthracycline and taxane-based chemotherapy; however, subtype-specific intensification strategies have demonstrated additional benefit.

In HER2-positive breast cancer, dual HER2 blockade with trastuzumab and pertuzumab significantly increases pCR rates compared with chemotherapy alone, while in TNBC, the addition of platinum agents such as carboplatin has shown efficacy in improving pCR, particularly in patients harboring BRCA1/2 mutations, albeit at the cost of increased toxicity.

Consequently, NAC is currently endorsed by the National Comprehensive Cancer Network (NCCN) and the St. Gallen International Expert Consensus Panel as the preferred standard of care for patients with HER2-positive or triple-negative breast cancer [13-15].

1.6 Immune system in cancer

The immune system plays a central role in cancer development, progression, and response to therapy by continuously interacting with tumor cells and the surrounding microenvironment [16].

Under physiological conditions, immune surveillance enables the recognition and elimination of transformed cells through innate and adaptive immune mechanisms; however, tumors can evade immune control by exploiting immune regulatory pathways, leading to immune tolerance and disease progression [16].

This dynamic interaction between cancer and the immune system is described by the concept of cancer immunoediting, which encompasses three phases: elimination, equilibrium, and escape [16]. During elimination, innate and adaptive immune effector cells recognize and destroy emerging tumor cells; in the equilibrium phase, residual cancer cells are contained but not eradicated, leading to immune-mediated tumor dormancy; finally, in the escape phase, selective immune pressure favors

tumor variants capable of evading immune recognition or actively suppressing antitumor immunity, resulting in clinically apparent disease. [16] (Figure 4).

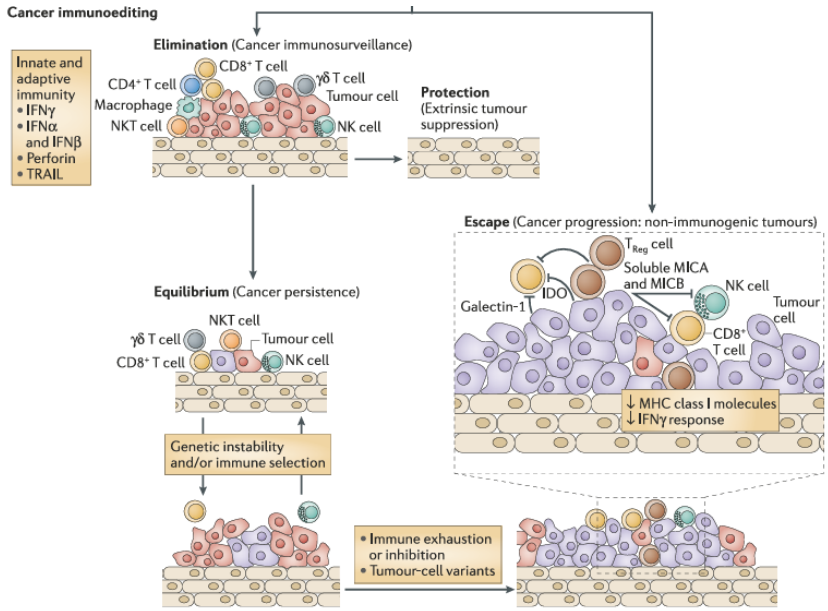


Figure 4: Schematic representation of cancer immunoediting. The sequential phases are elimination, equilibrium, and escape, highlighting the dynamic interactions between tumor cells and innate and adaptive immune responses. *Adapted from* Dunn G, Koebel C, Schreiber R. *Interferons, immunity and cancer immunoediting.* Nat Rev Immunol. 2006; 6:836-848 [34].

Within the tumor microenvironment, immune cells such as T lymphocytes, B lymphocytes, natural killer cells, macrophages, and dendritic cells exert both antitumor and protumor effects, depending on their functional state and spatial organization [17,18].

Chronic inflammation, immune checkpoint activation, and the expansion of immunosuppressive cell populations contribute to tumor progression by limiting effective immune responses [46,47]. At the same time, the presence, composition, and functional orientation of tumor-infiltrating immune cells have emerged as important prognostic and predictive biomarkers across multiple cancer types, including breast cancer.

Importantly, anticancer therapies profoundly influence the immune system. Conventional treatments such as chemotherapy and radiotherapy can induce immunogenic cell death, enhance antigen presentation, and reshape immune cell populations, thereby modulating antitumor immunity beyond their direct cytotoxic effects [17].

More recently, the clinical success of immunotherapies, particularly immune checkpoint inhibitors, has highlighted the therapeutic potential of restoring or enhancing immune-mediated tumor control.

Together, these observations underscore the critical role of the immune system not only in tumor initiation and progression but also as a key determinant of therapeutic efficacy, providing the rationale for investigating specific immune cell subsets, such as B cells, and their functional roles in breast cancer.

1.7 Circulating B cells: biology, functional plasticity and dual role in tumor immunity

B lymphocytes are key components of the adaptive immune system and originate from hematopoietic stem cells in the bone marrow, where they undergo a tightly regulated developmental program characterized by immunoglobulin gene rearrangement and selection for functional antigen receptors [19,20].

Following maturation, naïve B cells circulate through peripheral lymphoid organs such as lymph nodes, spleen, and mucosa-associated lymphoid tissues, where they participate in immune surveillance and antigen-driven immune responses [19]. Upon antigen encounter, B cells can differentiate into antibody-secreting plasma cells or long-lived memory B cells, thereby contributing to humoral immunity and immunological memory [20].

Beyond antibody production, B cells exert a broad range of immune functions that include antigen presentation to T cells via major histocompatibility complex class II (MHC II), regulation of immune responses through cytokine secretion, and participation in the organization of lymphoid structures. Depending on their activation state and microenvironmental cues, B cells can produce pro-inflammatory cytokines that enhance immune activation or immunoregulatory mediators, such as interleukin-10, that limit excessive inflammation and maintain immune homeostasis [20]. This functional plasticity allows B cells to shape both innate and adaptive immune responses under physiological conditions.

In the context of cancer, the same biological versatility that underpins normal B-cell function suggests a dual role in tumor immunity. B cells may contribute to antitumor immune responses by promoting antigen presentation, antibody-mediated cytotoxicity, and T-cell activation, while under certain conditions they may also support tumor progression by fostering immunosuppression or chronic inflammation [21,22].

The balance between these opposing activities is strongly influenced by tumor type, disease stage, and the local immune microenvironment, providing the rationale for investigating B-cell behavior and function in breast cancer and in response to anticancer therapies.

1.7.1 B cells in breast cancer

B cells represent a prominent and functionally diverse immune population within the breast cancer tumor microenvironment, where they are detected in both intratumoral regions and organized lymphoid aggregates in the tumor stroma [23].

Their presence has been increasingly recognized as a relevant component of the immune contexture of breast cancer, with accumulating evidence suggesting that B-cell infiltration influences disease progression and clinical outcome in a subtype-dependent manner [23,24].

In particular, B cells are frequently enriched within tertiary lymphoid structures (TLS), ectopic lymphoid aggregates resembling secondary lymphoid organs, which are associated with enhanced local immune activation and favorable prognosis in several solid tumors, including breast cancer [23,24].

Functionally, B cells in breast cancer can exert antitumor activity through multiple mechanisms, including antigen presentation to CD4+ T cells, production of tumor-reactive antibodies, and secretion of pro-inflammatory cytokines that support cytotoxic T-cell responses. High densities of tumor-infiltrating B cells and plasma cells, as well as the presence of TLS, have been correlated with improved survival and increased response to systemic therapies, particularly in triple-negative and HER2-positive breast cancer subtypes, which are characterized by higher immunogenicity [24,25]. These findings support a role for B cells as active contributors to effective antitumor immunity rather than passive bystanders.

Conversely, B cells may also promote tumor progression under specific conditions. Distinct subsets, including regulatory B cells (Bregs), can suppress antitumor immune responses through the production of immunosuppressive cytokines such as interleukin-10 and transforming growth factor-β, as well as by inhibiting effector T-cell and natural killer cell functions [25]. Additionally, B-cell-derived factors may contribute to chronic inflammation and immune tolerance within the tumor microenvironment, thereby facilitating tumor growth and immune escape [26].

The coexistence of antitumor and protumor B-cell activities underscores the functional heterogeneity of this immune compartment in breast cancer and highlights the importance of contextual and spatial factors in determining B-cell-mediated immune outcomes (Figure 5).

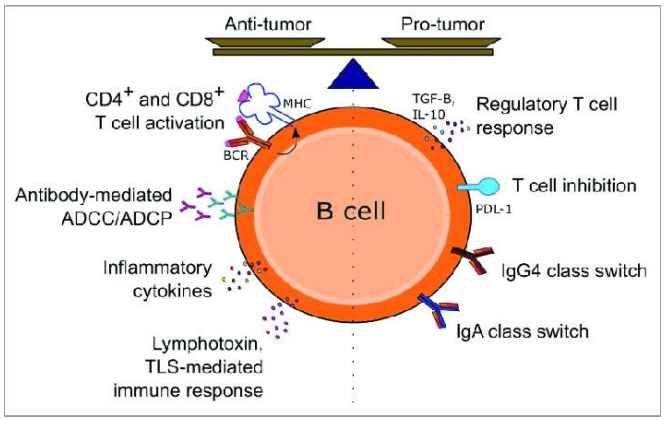


Figure 5: Pro-and antitumor functions of tumor-infiltrating B cells. *Adopted from Chiaruttini, G., Mele, S., Opzoomer, J., Crescioli, S., Ilieva, K. M., Lacy, K. E., & Karagiannis, S. N. (2017). B cells and the humoral response in melanoma: The overlooked players of the tumor microenvironment. OncoImmunology [35].*

1.7.2 Therapy induced immune modulation

Anticancer therapies, particularly neoadjuvant chemotherapy (NAC), profoundly remodel the immune landscape of breast tumors by altering immune cell composition, activation status, and functional orientation within the tumor microenvironment [24,27].

Beyond their direct cytotoxic effects, chemotherapeutic agents can induce immunogenic cell death, enhance tumor antigen release, and promote immune cell recruitment and activation, thereby shaping antitumor immune responses that contribute to treatment efficacy and pathological complete response (pCR) [24]. Consequently, the qualitative and quantitative balance of immune cell subsets emerging during therapy has gained increasing relevance as a determinant of clinical outcome.

Among B-cell populations, class-switched memory B cells, defined by a CD27+IgD- phenotype, represent an antigen-experienced subset capable of rapid antibody production, efficient antigen presentation, and sustained support of T-cell-mediated immunity [24,28]. An enrichment of switched memory B cells following NAC is indicative of active humoral immune responses and has been associated with favorable immune contextures, particularly in immunogenic breast cancer subtypes such as triple-negative and HER2-positive disease [24,28].

In parallel, effective antitumor immunity requires the presence of cytotoxic effector cells, including natural killer (NK) cells characterized by CD56 and CD16 expression, which mediate direct tumor cell killing through antibody-dependent cellular cytotoxicity (ADCC) and perforin-granzyme pathways [27,29]. Chemotherapy-induced modulation of NK-cell activity can enhance tumor clearance, especially in the presence of tumor-specific antibodies generated by activated B cells [29].

Conversely, regulatory B cells (Bregs), commonly identified by a CD24+CD27+ phenotype, exert immunosuppressive effects through the production of cytokines such as interleukin-10 and transforming growth factor-β, leading to inhibition of cytotoxic T-cell and NK-cell functions [30]. An expansion of Bregs during or after therapy may counteract effective antitumor immunity and has been linked to impaired immune-mediated tumor control [30].

Therefore, a favorable immune profile associated with pCR is hypothesized to involve a shift toward increased proportions of switched memory B cells and cytotoxic NK cells, accompanied by a relative reduction of regulatory B-cell populations. This balance promotes sustained immune activation, efficient tumor cell elimination, and enhanced responsiveness to systemic therapy, underscoring the importance of therapy-induced immune remodeling as a contributor to treatment success.

Despite growing evidence supporting the role of immune cell populations in cancer progression and treatment response, the clinical significance of circulating B-cell subsets and their relationship with cytotoxic innate immune cells during neoadjuvant chemotherapy remains incompletely understood. Further characterization of these populations may contribute to the identification of novel immune biomarkers associated with therapeutic response.

In this context, the identification of circulating immune biomarkers associated with treatment response may improve the understanding of systemic immune dynamics during neoadjuvant chemotherapy and support patient stratification strategies.

2. AIM OF THE STUDY

Accumulating evidence indicates that the composition and functional orientation of circulating immune cell subsets play a critical role in shaping antitumor immunity and influencing response to systemic anticancer therapies. In breast cancer, treatment-induced immune modulation has emerged as a key determinant of pathological complete response (pCR) and long-term clinical outcome, particularly in patients treated with neoadjuvant chemotherapy (NAC). However, the contribution of specific B-cell subsets and their interaction with cytotoxic effector cells in this setting remains incompletely characterized.

This non-interventional observational study aims to characterize therapy-associated changes in selected circulating immune cell populations and to evaluate their potential role as predictive and prognostic biomarkers in patients with operable breast cancer. Particular attention is given to the relationship between the balance of effector and regulatory immune subsets and pathological complete response, as well as clinical outcome.

Overall, the aim of this study is to determine whether a favorable immune profile, characterized by enrichment of effector immune cells and relative reduction of regulatory B cells, is associated with enhanced antitumor immunity, increased likelihood of achieving pCR, and improved prognosis in breast cancer patients treated with neoadjuvant chemotherapy.

To address this objective, we:

• Characterized circulating class-switched memory B cells (CD27+IgD-), cytotoxic NK cells (CD56dimCD16+), and regulatory B cells (CD24+CD27+) by flow cytometry following immunophenotypic staining.

• Quantified and compared the relative frequencies of these immune cell populations in breast cancer patients pre- and post- neoadjuvant chemotherapy, evaluating differences between TNBC and HER2+ subtypes.

• Evaluated the ratio between effector immune populations (switched memory B cells and cytotoxic NK cells) and regulatory B cells as a composite immune parameter.

• Assessed the association between immune cell distribution and ratios with pathological complete response.

• Investigated whether patient age was associated with the distribution of circulating immune cell subsets, therapy-induced immune modulation, and pathological complete response.

3. MATERIALS AND METHODS

3.1 Patients' enrollment and sample collection

Peripheral blood samples were obtained from 39 patients eligible for neoadjuvant chemotherapy (NAC) at two defined time points: prior to the initiation of systemic treatment and after completion of chemotherapy, immediately before surgical intervention.

Patient inclusion occurred only after written informed consent was obtained and all eligibility criteria were satisfied.

Inclusion criteria comprised:

- Age ≥ 18 years;
- Histologically confirmed diagnosis of breast cancer;
- Eligibility for neoadjuvant chemotherapy;
- Absence of clinically significant adverse events during treatment administration;
- Ability to understand the study procedures and provide informed consent.

Exclusion criteria included:

- Age < 18 years;
- Ineligibility for neoadjuvant chemotherapy;
- Evidence of metastatic disease;
- Inability to comprehend and/or sign informed consent documentation.

For each participant, a total of 25 mL of peripheral whole blood was collected into EDTA-containing tubes (BD Biosciences, Milan, Italy) at the Oncology Department of the Azienda Ospedaliero-Universitaria Maggiore della Carità, University of Eastern Piedmont, Novara. Blood samples were transported and processed within 4 hours of collection at the Center for Translational Research and Autoimmune & Allergic Disease (CAAD), University of Eastern Piedmont, Novara.

3.2 Peripheral blood mononuclear cells (PBMCs) collection

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll-Paque (Lympholyte-H, Cedarlane, Netherlands). Briefly, 7.5-10 mL of whole blood were transferred into a 50 mL Falcon tube. The original collection tube was rinsed with 3 mL of 0.9% NaCl solution to recover residual blood, and physiological solution was added to reach a final volume of 30 mL. The diluted blood was gently mixed.

Subsequently, 15 mL of Ficoll solution were carefully layered at the bottom of the tube using a 10 mL pipette, taking care to avoid mixing with the diluted blood. Samples were centrifuged at 2200 rpm for 20 minutes with acceleration set to 7 and no brake (deceleration 0).

After centrifugation, the mononuclear cell layer located at the plasma-Ficoll interphase was carefully collected and transferred to a new 50 mL tube. Cells were washed with physiological solution up to 50 mL and centrifuged at 1600 rpm for 7 minutes (acceleration and deceleration 9). The supernatant was discarded, and the washing step was repeated. If residual erythrocyte contamination was observed, red blood cell lysis was performed using lysis buffer (1:1 volume ratio) for up to 5 minutes, followed by an additional wash (Figure 6).

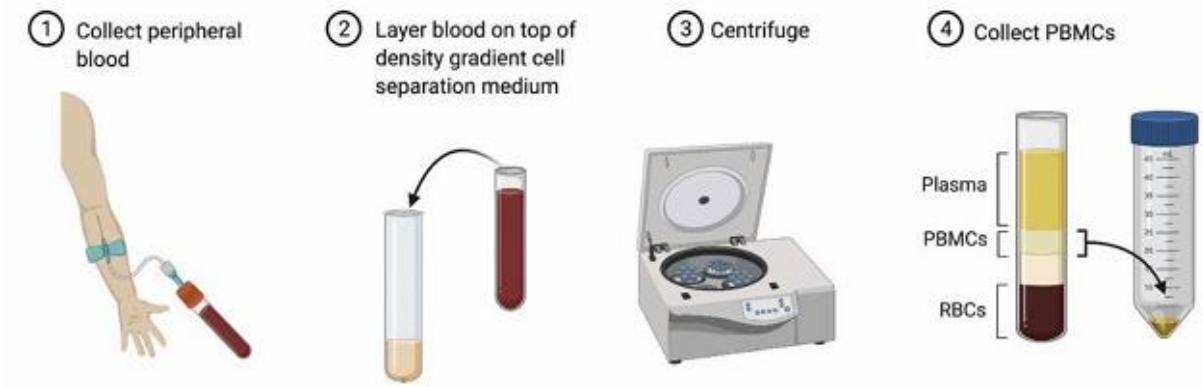


Figure 6: Collection of PMBC. Adapted from *Turocy J, Williams Z. Fertility and Sterility 2021* [36].

Finally, the cell pellet was resuspended in an appropriate volume of physiological solution depending on pellet size. Cell counting was performed using a Bürker chamber after diluting 10 µL of PBMC suspension in 90 µL of TURK solution.

3.3 B-NK cell staining

200 ul of whole blood were stained and incubated for 20 min at room temperature with monoclonal antibodies against B and NK markers (all provided by BD Bioscience, Milan, IT; Table 3).

ANTIBODY	FLUOROPHORE	VOLUME (ul)
CD45	BUV395	0,5
CD3	BV786	1
IgM	BB515	1
CD20	BV750	1
HLA-DR	BV605	1,5
CD56	APC	10
CD16	PE-Cy7	0,3
CD19	BV480	1
CD24	PE	10
CD11c	BUV661	1
CD123	PE-Cy5	10
CD27	BV421	1
IgD	PECF594	1
CD38	BB700	1

Table 3: Anti-human antibodies used for staining procedure.

Following incubation, the remaining reticulocytes were lysed with a 1x lysis buffer (BD biosciences, Milan, IT). Finally, stained cells were analyzed for B-NK cell phenotyping by FACS, using the latest updated version of FlowJo software.

Circulating CD19+CD20+ subpopulations were classified and quantified according to Velounias RL, et al. (2023) [37] as follows:

- Naïve B cells: CD27-IgD+
- Unswitched memory B cells: CD27+IgD+
- Switched memory B cells: CD27+ IgD-
- Double negative (DN) B cells: CD27- IgD-
- Transitional B cells: CD24+ CD38+
- Regulatory B cells: CD24+ CD27+
- Cytotoxic NK cells: CD56dim CD16+
- Regulatory NK: CD56+ CD16-.

3.4 Statistical analysis

Continuous variables were assessed for normality using the Shapiro-Wilk test. Normally distributed data were expressed as mean ± standard deviation (SD), whereas non-normally distributed data were

reported as median and interquartile range (IQR). Categorical variables were summarized as counts and percentages.

Comparisons between two independent groups (e.g., pCR vs non-pCR) were performed using the Mann-Whitney U test for non-parametric data. Paired comparisons between baseline and post-NAC samples were analyzed using the Wilcoxon signed-rank test.

All statistical tests were two-sided, and a p-value < 0.05 ($\alpha = 0.05$) was considered statistically significant. Statistical analyses were performed using R software and GraphPad Prism (version 8).

4. RESULTS

4.1 Patients' baseline characteristics

The project has included thirty-nine patients to the current date, and Table 4 resumed their characteristics.

Characteristics	Study population = 39
Sex	
Female	39 (100%)
Male	0 (0%)
Median age (years)	54 (29-81)
Molecular subtype	
TNBC	18 (42,2%)
HER2+	9 (23,08%)
ER+	10 (25,64%)
pCR	
Yes	18 (46,15%)
No	15 (38,5%)
Not indicated	6 (15,4%)

Table 4: The characteristics of enrolled patients.

All enrolled patients were female (study population = 39) with a mean age of 54 years (range 29-81).

Three groups were created based on the molecular subtype: ER+, HER2+ and TNBC. The most present subtype was TNBC (n = 18; 42,2%), followed by ER+ (n = 10; 25,64%) and HER2+ (n = 9; 23,08%).

4.2 Pathological complete response (pCR) after neoadjuvant chemotherapy (NAC)

To the current date, 39 patients completed NAC (100%) and all of them underwent surgery.

Out of these patients, 18 achieved pCR (46,15%), 15 had RD (38,5%) and 6 have either not yet undergone re-evaluation or not data are available.

pCR has been observed differently in the three molecular subtypes as follows:

- Of the TNBC patients, 9 achieved pCR (50% of TNBC; 50% of total pcR).
- Of the HER2+ patients, 8 achieved pCR (88,89% of HER2+; 44,4% of total pcR).
- Of the ER+ patients, 1 achieved pCR (10% of ER+; 5,6% of total pcR).

4.3 Evaluation of switched memory B cells, regulatory B cells and cytotoxic NK cells by FACS

4.3.1 Gating strategy and characterization by flow cytometry

Our gating strategy began with the exclusion of doublets by forward scatter area versus forward scatter height (FSC-A vs FSC-H), followed by the selection of viable cells using the viability dye in combination with side scatter (FSC-A). Lymphocytes were identified based on FSC-A and SSC-A characteristics and subsequently gated as CD45+ leukocytes (Figure 7).

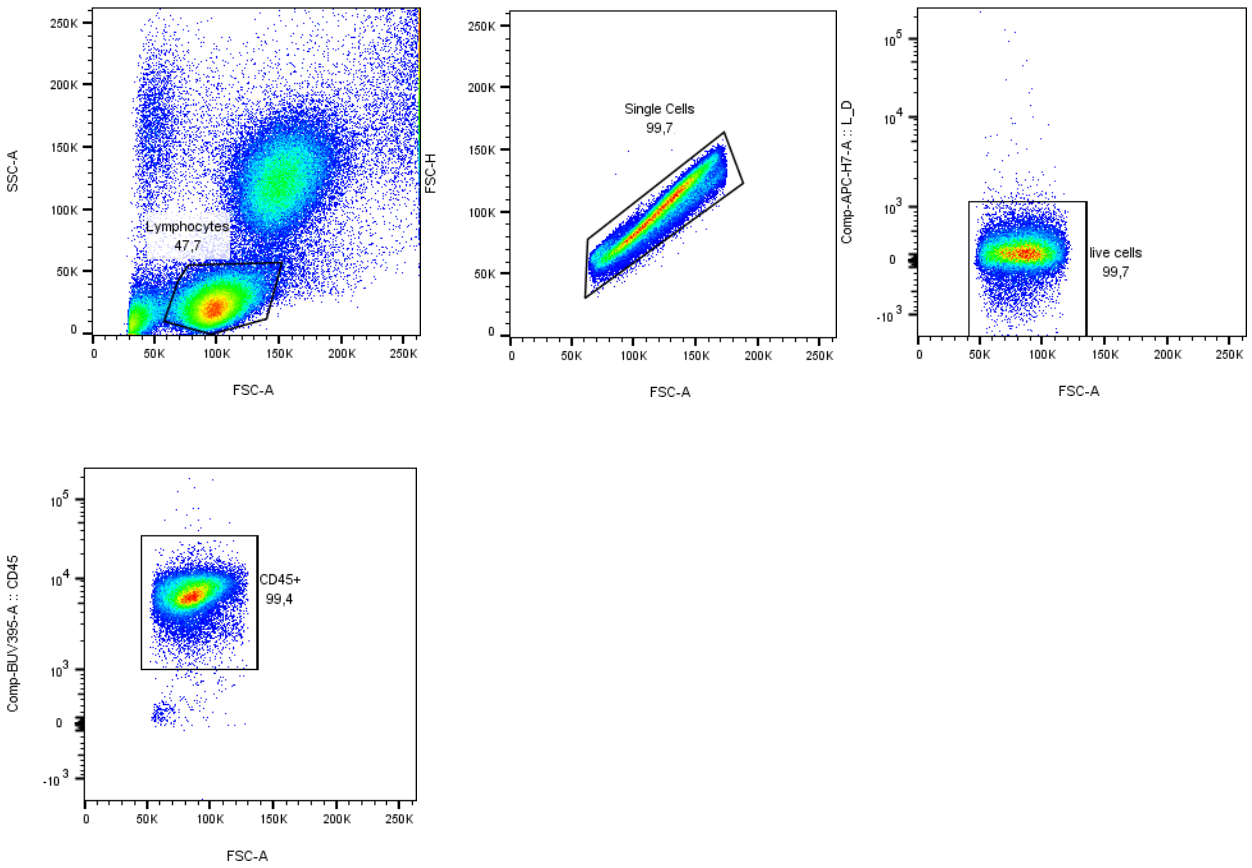


Figure 7: Representative flow cytometry gating strategy used for the identification of leukocyte populations. Doublets and dead cells were excluded, followed by the selection of lymphocytes and CD45+ leukocytes. The resulting CD45+ population was used for downstream analysis of NK-cell and B-cell subsets.

From the CD45+ population, two major cell compartments were analyzed. Natural killer (NK) cells were identified within the CD3- population and further characterized according to CD56 and CD16

expression. Regulatory NK cells were defined as CD56+CD16-, cytotoxic NK cells as CD56dimCD16+, and dysfunctional NK cells as CD56-CD16+ (Figure 8).

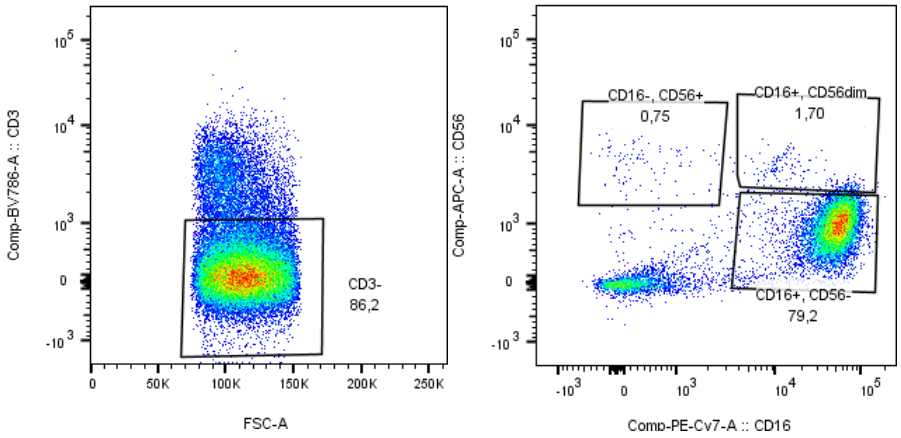
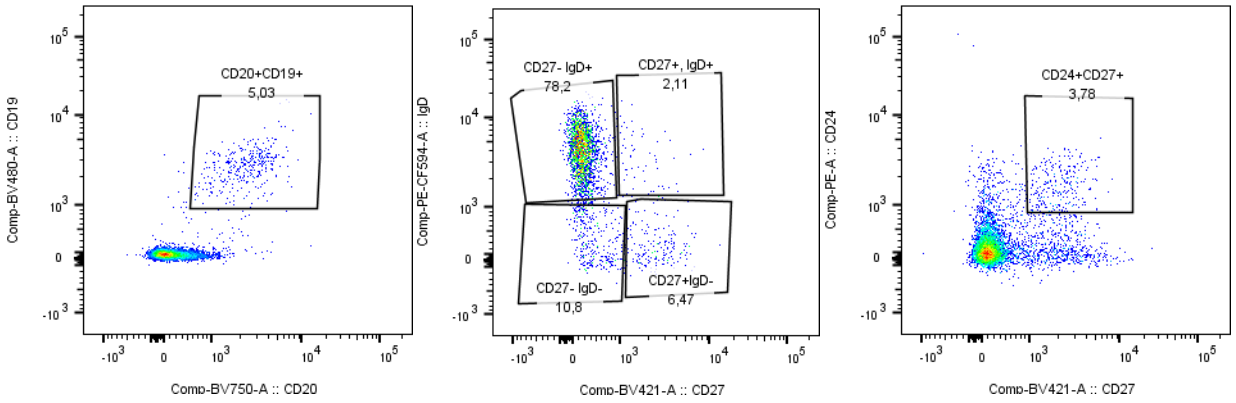


Figure 8: Representative gating strategy for NK-cell subsets. Starting from the CD45+ population, CD3- cells were identified and classified as CD56+CD16-, CD56dimCD16+, and CD56-CD16+.

B-cell populations were identified as CD19+CD20+ lymphocytes and further subdivided according to the expression of CD24, CD27, CD38, and IgD. Regulatory B cells (Bregs) were defined as CD24+CD27+, while total memory B cells were identified as CD27+ cells. Additional B-cell subsets included plasmablasts (CD27+CD38+), non-switched memory B cells (CD27+IgD+), switched memory B cells (CD27+IgD-), naïve B cells (CD27-IgD+), exhausted B cells (CD27-IgD-), and transitional B cells (CD38+CD24+) (Figure 9).



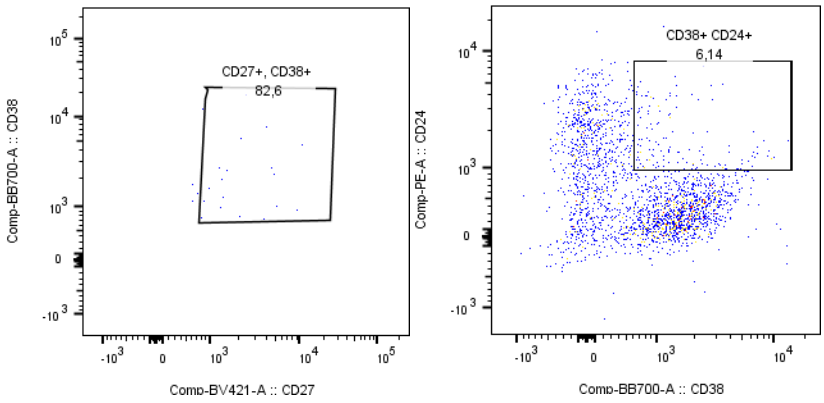


Figure 9: Representative gating strategy for B-cell subsets. B cells were identified as CD19+CD20+ lymphocytes and further characterized into CD27-IgD+, CD27+IgD+, CD27-IgD-, CD27+IgD-, CD24+CD27+, CD27+CD38+, and CD38+CD24+.

To investigate therapy-induced immune changes associated with pathological complete response (pCR) in breast cancer patients, particular attention was focused on the frequency of switched memory B cells (CD27+IgD-), regulatory B cells (CD24+CD27+), and cytotoxic NK cells (CD56dimCD16+). The frequency of these populations was assessed at baseline and after treatment and subsequently compared between patients achieving pCR and those with residual disease.

4.3.2 CD27+IgD-, CD56dimCD16+, and CD24+CD27+ comparison pre and post-treatment

Paired pre and post-treatment frequencies of CD27+IgD-, CD56dimCD16+, and CD24+CD27+ were compared using the Wilcoxon signed-rank test. No statistically significant differences were observed between baseline and post-treatment samples for any of the analyzed populations within the study cohort (Table 5, Figure 10).

In addition, a ratio was calculated to estimate the balance between effector and regulatory immune populations, defined as [(CD27+IgD-) + (CD56dimCD16+)] / (CD24+CD27+). This parameter, hereafter referred to as the "ratio", was determined for both pre-treatment and post-treatment samples and analyzed alongside the individual cell populations. The paired Wilcoxon test for the ratio was significant (Table 5, Figure 10).

variable	median_pre	median_post	median_change	p_value
CD27+,IgD-	18,2	23,7	6,73	0,0900
CD24+,CD27+	11,4	7,88	-2,96	0,2221
CD16+,CD56dim	7,79	9,35	1,11	0,6302
Ratio	2,05	3,74	1,13	0,0077

Table 5: Summary of the Wilcoxon signed-rank test values comparing the populations pre and post-treatment.

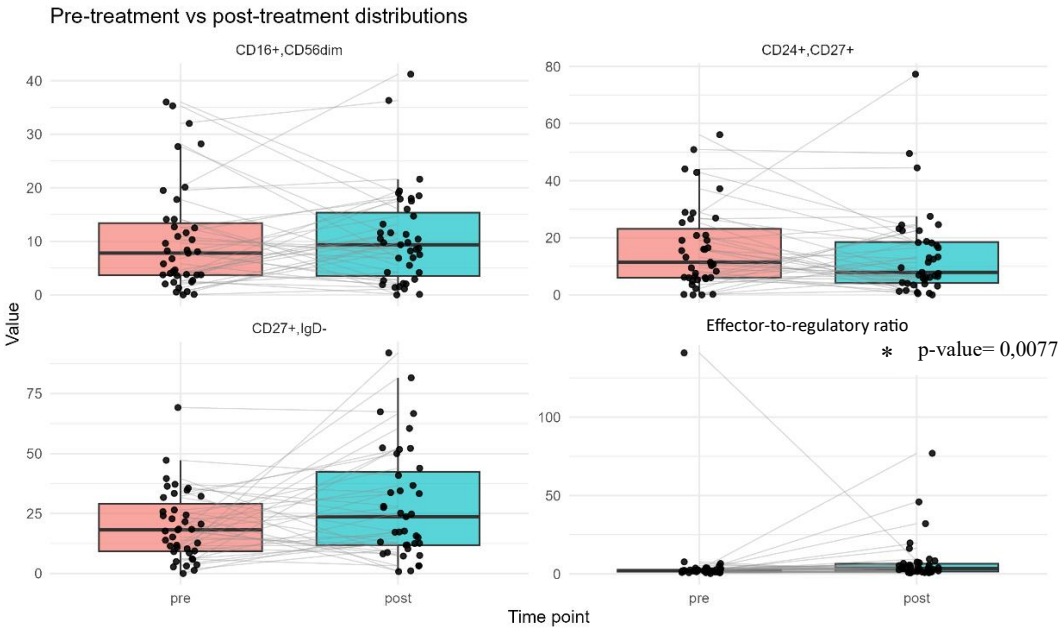


Figure 10: Representative plots of paired pre and post-treatment frequencies of CD27+IgD-, CD56dimCD16+, CD24+CD27+, and effector-to-regulatory ratio (Wilcoxon signed-rank test).

4.3.3 CD27+IgD-, CD56dimCD16+, and CD24+CD27+ comparison in TNBC vs HER2+ patients

To investigate whether immune cell frequencies differed between breast cancer subtypes, the baseline frequencies of switched memory B cells (CD27+IgD-), regulatory B cells (CD24+CD27+), and cytotoxic NK cells (CD56dimCD16+) were compared between patients with triple-negative breast cancer (TNBC) and HER2-positive breast cancer using the Mann-Whitney U test. No statistically significant differences were observed between the two groups for any of the analyzed cell populations at baseline, indicating comparable immune profiles prior to treatment (Table 6, Figure 11).

Subsequently, treatment-induced changes were assessed by calculating delta (Δ) values for each immune cell population. Comparisons of Δ values between TNBC and HER2+ patients using the Mann-Whitney U test also revealed no statistically significant differences. After adjustment for multiple testing, none of the comparisons remained statistically significant, suggesting that neither baseline frequencies nor treatment-related changes in these immune cell subsets differed according to breast cancer subtype in the study cohort (Table 6, Figure 11).

variable	group	n	mean	sd	median	q1
CD27+,IgD- baseline	HER2	10	18,65	14,4141	14,55	11,58
Δ CD27+,IgD-	HER2	10	2,32	20,3554	1,05	-5,55
CD24+,CD27+ baseline	HER2	10	16,18	14,3343	8,52	6,05
Δ CD24+,CD27+	HER2	10	-3,24	7,3279	-0,12	-4,41
CD16+,CD56dim baseline	HER2	10	13,31	12,7885	8,18	3,85
Δ CD16+,CD56dim	HER2	10	-2,70	11,3685	0,53	-12,21
CD27+,IgD- baseline	TNBC	18	18,47	17,4802	14,55	5,93
Δ CD27+,IgD-	TNBC	18	13,05	29,1399	9,00	-2,87
CD24+,CD27+ baseline	TNBC	18	14,02	12,2668	11,05	5,45
Δ CD24+,CD27+	TNBC	18	1,08	18,3178	-3,38	-13,09
CD16+,CD56dim baseline	TNBC	18	9,05	9,3915	6,32	2,49
Δ CD16+,CD56dim	TNBC	18	2,81	10,1104	2,71	-2,57

variable	comparison	n	n_TNBC	n_HER2+	median_TNBC
CD27+,IgD- baseline	Baseline TNBC vs HER2+	28	18	10	14,55
Δ CD27+,IgD-	Δ TNBC vs HER2+	28	18	10	8,995
CD24+,CD27+ baseline	Baseline TNBC vs HER2+	28	18	10	11,05
Δ CD24+,CD27+	Δ TNBC vs HER2+	28	18	10	-3,38
CD16+,CD56dim baseline	Baseline TNBC vs HER2+	28	18	10	6,32
Δ CD16+,CD56dim	Δ TNBC vs HER2+	28	18	10	2,705

Table 6: Descriptive and summary tables for populations comparison in TNBC vs HER2+ subtypes.

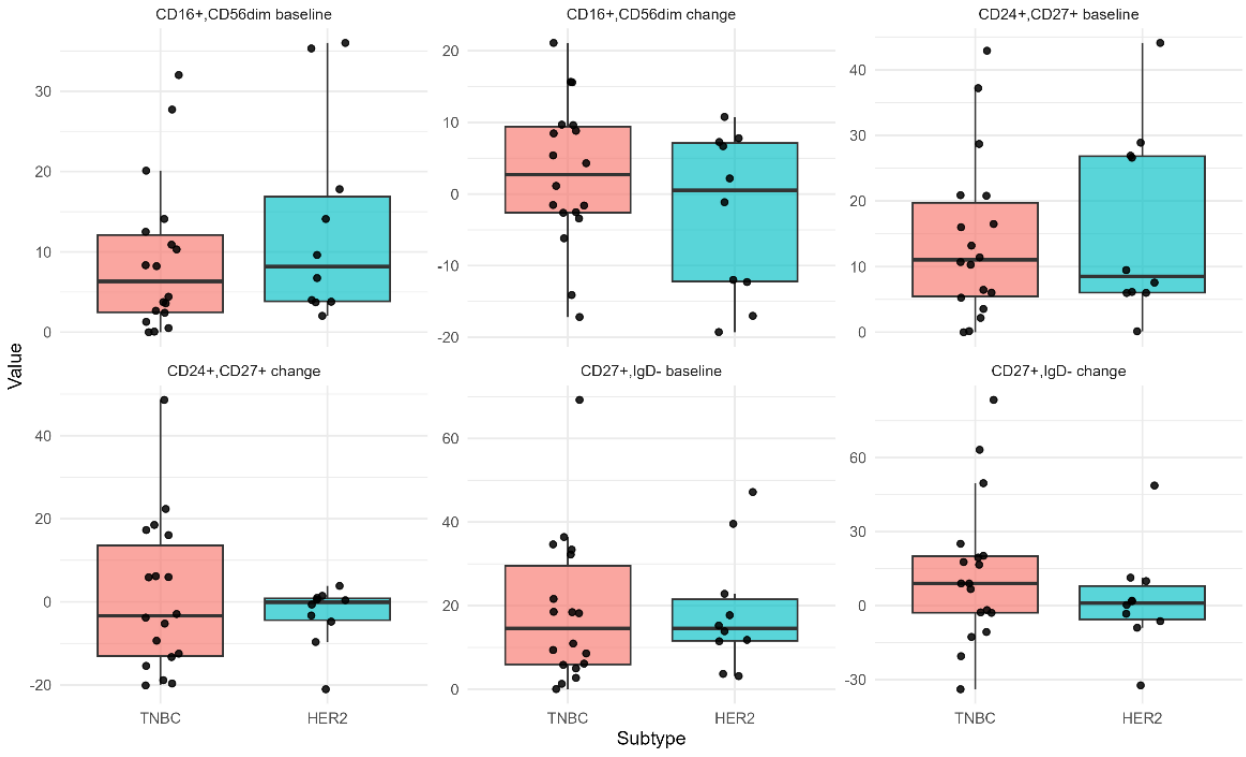


Figure 11: Representative box plots of populations comparison in TNBC vs HER2+ subtypes.

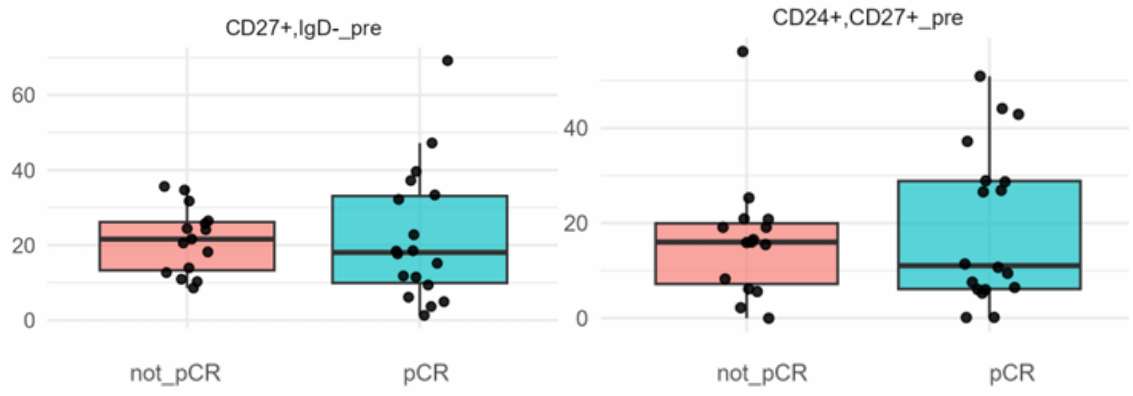
4.3.4 CD27+IgD-, CD56dimCD16+, and CD24+CD27+ baseline value and their association with pCR achievement

To investigate whether baseline immune profiles were associated with treatment response, the frequencies of switched memory B cells (CD27+IgD-), cytotoxic NK cells (CD56dimCD16+), regulatory B cells (CD24+CD27+), and the effector-to-regulatory ratio were compared between patients achieving pathological complete response (pCR) and those with residual disease (RD) using the Mann-Whitney U test. No statistically significant differences were observed for any of the analyzed parameters, suggesting that baseline frequencies of these immune cell populations were not associated with treatment response in the study cohort (Table 7, Figure 12,13).

Subsequently, therapy-induced changes were evaluated by calculating the delta (Δ) values, defined as the difference between post-treatment and baseline frequencies for each population and for the effector-to-regulatory ratio. These Δ values were also compared between pCR and RD patients using the Mann-Whitney U test. No statistically significant differences were detected (Table 7, Figure 12,13).

variable	median_not_pCR	median_pCR	p_value
CD27+,IgD- baseline	21,6	18,05	0,6776
CD24+,CD27+ baseline	16	11,05	0,7312
CD16+,CD56dim baseline	9,62	5,7	0,4159
Ratio baseline	2,27	1,97	0,6622
Δ CD27+,IgD-	-2,74	5,495	0,3201
Δ CD24+,CD27+	-9,45	-1,02	0,1869
Δ CD16+,CD56dim	-0,84	6,035	0,6776
Δ Ratio	1,53	0,56	0,1138

Table 7: Summary of the Mann-Whitney U test comparing baseline and delta (Δ) values to pCR/RD.



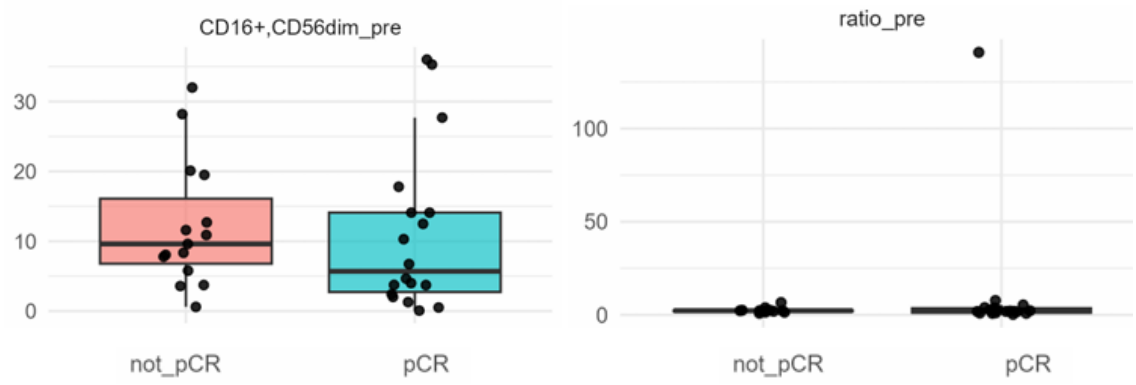


Figure 12: Representative box plots for baseline values compared between patients achieving pCR and not pCR (Mann-Whitney U test).

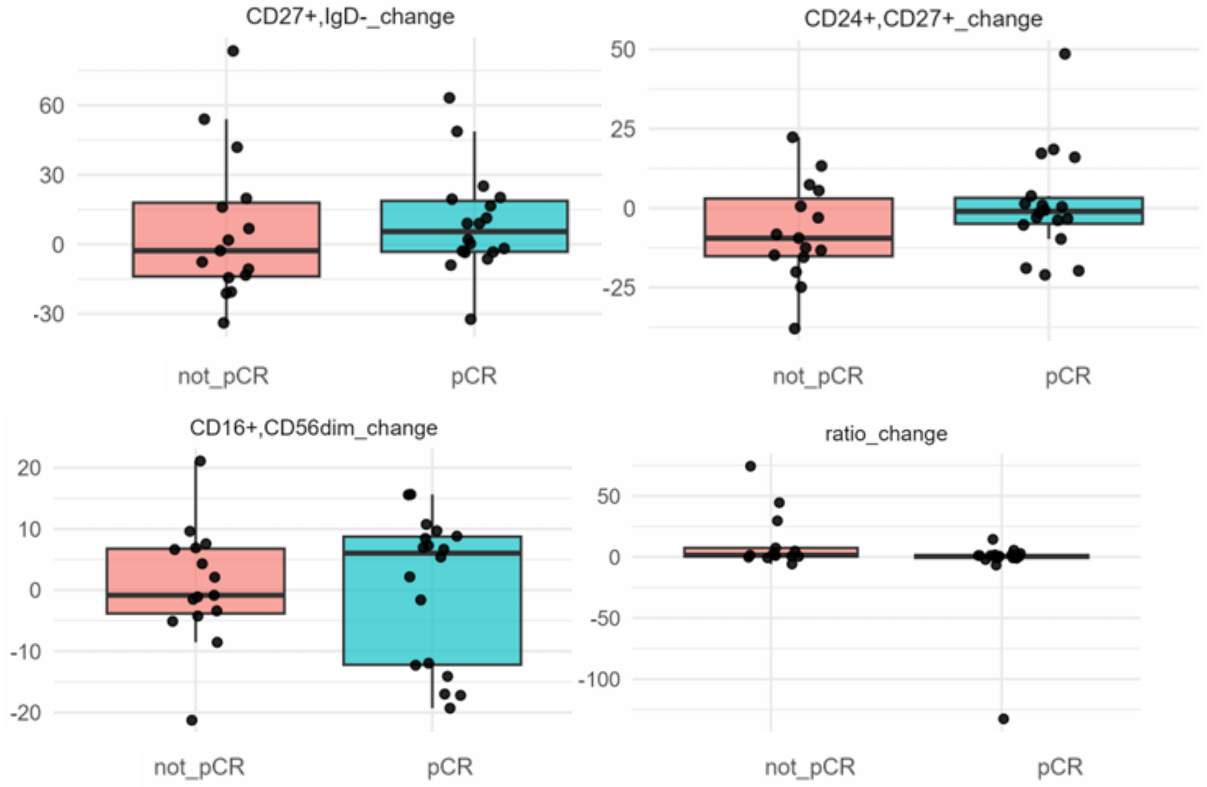


Figure 13: Representative box plots for delta (Δ, called "change" in the figure) values compared between patients achieving pCR and not pCR (Mann-Whitney U test).

4.3.5 Therapy-induced changes correlation with patients' age

Given the wide age range of the 39 patients included in the study cohort, an additional analysis was performed to investigate whether age was associated with therapy-induced changes in the investigated immune cell populations. Spearman's rank correlation test was used to assess the relationship between patient age and the Δ values (post-treatment minus baseline) of switched memory B cells (CD27+IgD-

), cytotoxic NK cells (CD56dimCD16+), regulatory B cells (CD24+CD27+), and the effector-to-regulatory ratio.

No statistically significant correlations were observed for any of the analyzed parameters (Table 8, Figure 14).

variable	rho	p_value	p_adjust
CD27+,IgD- baseline	-0,2078	0,2043	0,7490
CD27+,IgD- post	-0,1661	0,3121	0,7490
Δ CD27+,IgD-	0,0045	0,9785	0,9807
CD24+,CD27+ baseline	-0,2715	0,0945	0,7490
CD24+,CD27+ post	-0,0625	0,7054	0,8554
Δ CD24+,CD27+	0,2154	0,1879	0,7490
CD16+,CD56dim baseline	-0,0040	0,9807	0,9807
CD16+,CD56dim post	-0,1697	0,3016	0,7490
Δ CD16+,CD56dim	-0,0781	0,6365	0,8554
Ratio baseline	0,1443	0,3942	0,7884
Ratio post	-0,0820	0,6244	0,8554
Δ Ratio	-0,0635	0,7129	0,8554

Table 8: Summary of the Spearman's rank correlation test between the Δ values and age.

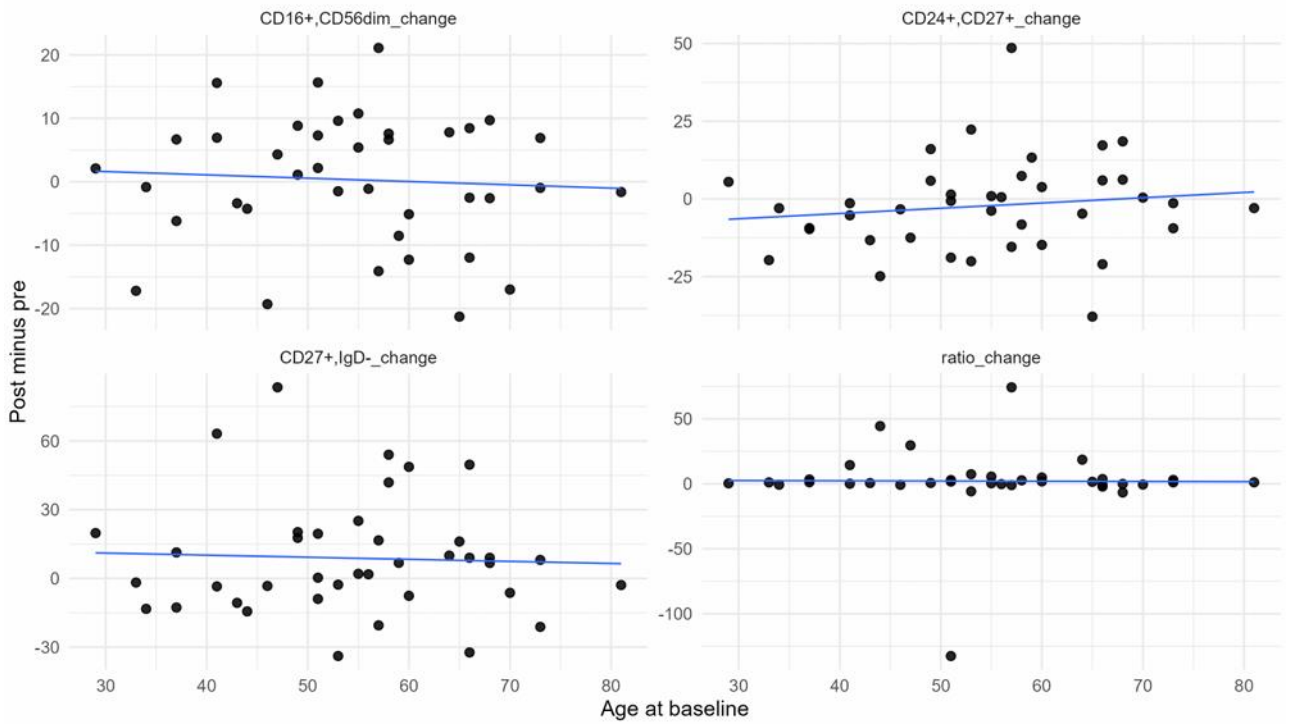


Figure 14: Representative box plots for delta (Δ, called "change" in the figure) values correlation with age (Spearman's rank correlation test).

Finally, to evaluate whether patient age was associated with treatment response, age distributions were compared between patients achieving pathological complete response (pCR) and those with residual disease (RD) using the Mann-Whitney U test. No statistically significant differences were observed between the two groups, indicating that age was not associated with treatment outcome in the study cohort (Table 9, Figure 15).

variable	n	median_not_pCR	median_pCR	p_value
Age	33	56	53	0.9567

Table 9: Summary of the Mann-Whitney test to evaluate association between age and treatment response.

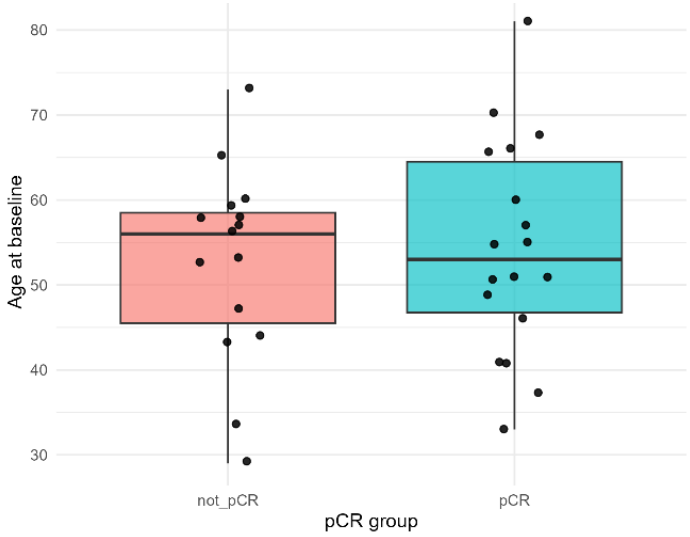


Figure 15: Representative box plots for age correlation between pCR and not pCR patients.

5. DISCUSSION

Breast cancer is a biologically heterogeneous disease and remains one of the most frequently diagnosed malignancies among women worldwide. In patients with early-stage and locally advanced disease, systemic treatment administered before surgery has become an integral component of therapeutic management. Neoadjuvant chemotherapy is currently considered the preferred standard of care for many patients with HER2+ and triple-negative breast cancer, as it promotes tumor downstaging, increases the likelihood of breast-conserving surgery, and provides important information regarding tumor sensitivity to treatment. The principal objective of neoadjuvant chemotherapy is the achievement of pathological complete response, defined as the absence of residual invasive disease in both the breast and axillary lymph nodes at the time of surgery. Achievement of pathological complete response is associated with improved clinical outcomes, including longer disease-free and overall survival, particularly in the more immunogenic breast cancer subtypes. Consequently, the identification of biological factors associated with treatment response has become an area of increasing interest, with particular attention directed toward the role of the immune system and its contribution to therapeutic efficacy.

B lymphocytes exhibit marked functional heterogeneity within tumor-associated immune responses. Antigen-experienced effector populations, such as class-switched memory B cells, together with cytotoxic natural killer (NK) cells, can support antitumor immunity by enhancing antigen presentation, antibody-dependent cellular cytotoxicity, and T-cell activation. In contrast, regulatory B cells exert immunosuppressive functions that may impair effective immune-mediated tumor control and limit therapeutic efficacy. The balance between these opposing immune compartments has therefore been hypothesized to influence treatment response and clinical outcome in patients receiving neoadjuvant chemotherapy (NAC).

The present study investigated selected circulating immune cell populations in patients with operable breast cancer undergoing neoadjuvant chemotherapy, with particular focus on switched memory B cells (CD27+IgD-), regulatory B cells (CD24+CD27+), cytotoxic NK cells (CD56dimCD16+), and a composite effector-to-regulatory immune ratio. These populations were selected based on their established roles in the regulation of antitumor immune responses. Switched memory B cells represent antigen-experienced B lymphocytes that have undergone class-switch recombination and are involved in long-term humoral immunity, antigen presentation, and the maintenance of immunological memory, all mechanisms that may contribute to effective antitumor responses. In contrast, regulatory B cells constitute a functionally distinct B-cell subset with immunosuppressive

properties that can limit immune activation and promote immune tolerance. Cytotoxic NK cells were included because they represent a major innate effector population capable of directly eliminating malignant cells and mediating antibody-dependent cellular cytotoxicity [24,28,29,30]. Given the potentially opposing functions of effector and regulatory immune compartments, an effector-to-regulatory ratio was additionally evaluated to provide an integrated measure of the balance between immune populations potentially associated with tumor control. The objective was therefore to evaluate whether the distribution of these immune subsets, either at baseline or following treatment, was associated with pathological complete response.

Within the study cohort, pCR was achieved in 46,15% of patients who completed NAC. Consistent with established clinical observations, pCR rates differed across molecular subtypes, with the highest frequency observed among HER2-positive patients, followed by triple-negative breast cancer (TNBC), whereas hormone receptor-positive disease showed the lowest proportion of complete responses. These findings reflect the known heterogeneity of treatment sensitivity among breast cancer subtypes and confirm the relevance of pCR as a clinically meaningful endpoint in the neoadjuvant setting.

The longitudinal analysis of immune cell populations demonstrated that NAC did not induce statistically significant changes in the individual frequencies of switched memory B cells, regulatory B cells, or cytotoxic NK cells when pre-treatment and post-treatment samples were compared. However, the composite effector-to-regulatory ratio showed a statistically significant difference between the two time points. This observation suggests that, although the individual immune populations did not undergo detectable quantitative modifications within the cohort as a whole, NAC may influence the overall balance between effector and regulatory immune compartments.

Given the distinct biological and clinical characteristics of TNBC and HER2-positive breast cancer, subtype-specific differences in immune composition or treatment-induced immune modulation might have been expected [38,39]. However, no significant differences were observed in the baseline frequencies of switched memory B cells, regulatory B cells, or cytotoxic NK cells between the two subtypes, and NAC-induced changes in these populations were similarly comparable. While these findings suggest that the peripheral immune parameters analyzed were not substantially influenced by breast cancer subtype in the present cohort, they should be interpreted with caution due to the limited sample size. The relatively small number of patients in each subgroup may have reduced the statistical power to detect modest but potentially biologically meaningful differences. This is particularly relevant given the well-established immunological distinctions between TNBC and

HER2-positive disease, especially within the tumor microenvironment. Therefore, although no subtype-specific associations were identified in this study, larger and more balanced cohorts are needed to validate these findings and to determine whether subtle differences in circulating immune cell populations or their treatment-induced dynamics may contribute to the distinct clinical behavior of these breast cancer subtypes. Such investigations could provide valuable insights into the interplay between systemic immunity, tumor biology, and response to neoadjuvant chemotherapy.

To determine whether these immune parameters were associated with treatment efficacy, baseline frequencies and therapy-induced changes were compared between patients achieving pCR and those with residual disease. No statistically significant differences were observed for switched memory B cells, regulatory B cells, cytotoxic NK cells, or for the effector-to-regulatory ratio. Similarly, analysis of delta (Δ) values revealed no significant association between treatment-induced immune changes and pathological response.

The absence of significant associations between the investigated circulating immune subsets and pathological response should be interpreted within the context of the present study. Although switched memory B cells, regulatory B cells, and cytotoxic NK cells have been implicated in the regulation of antitumor immunity, the present findings suggest that the frequencies of these circulating populations alone may not be sufficient to predict pathological complete response. One possible explanation is that treatment response is influenced by a complex interplay of multiple immune and tumor-related factors that may not be fully captured by the evaluation of selected peripheral blood populations. Furthermore, the functional activity, spatial distribution, and interactions of immune cells within the tumor microenvironment may have a greater impact on therapeutic efficacy than their circulating frequencies. Therefore, while these populations were selected based on their established immunological relevance, their distribution in peripheral blood was not associated with pathological complete response in the present cohort.

An additional analysis was performed to investigate the potential influence of age on immune modulation and treatment response. This aspect was explored because aging is associated with progressive changes in both innate and adaptive immunity, a phenomenon commonly referred to as immunosenescence. Previous studies have reported age-related alterations in B-cell homeostasis, immune responsiveness, and antitumor immunity, suggesting that increasing age could potentially influence both immune cell composition and clinical outcome [40,41,42,43]. In light of these observations, we assessed whether patient age was associated with therapy-induced changes in the investigated immune populations or with pathological complete response achievement. No significant

correlations were identified between age and immune cell dynamics, and age did not differ significantly between patients achieving pathological complete response and those with residual disease. These findings indicate that, within the present cohort, age was not associated with either treatment-induced modulation of the investigated circulating immune subsets or response to neoadjuvant chemotherapy. Although the age range of the study population was broad, the absence of significant associations suggests that the immune parameters evaluated in this study were not substantially influenced by age-related effects.

The absence of significant associations between the investigated circulating immune subsets and pathological response should be interpreted within the context of the present study. Although the biological rationale supporting a role for B-cell and NK-cell populations in antitumor immunity is well established, the current data do not demonstrate a measurable relationship between the selected circulating immune parameters and pCR. Therefore, the hypothesis that a favorable immune profile characterized by increased effector populations and reduced regulatory B cells would be associated with improved response to NAC was not confirmed in this cohort.

Nevertheless, the significant variation observed in the effector-to-regulatory ratio between pre-treatment and post-treatment samples indicates that NAC may influence immune homeostasis at the systemic level. While this finding was not associated with pCR, it supports the concept that chemotherapy can modulate the immune compartment beyond its direct cytotoxic activity. Further investigation in larger patient populations may help clarify the clinical significance of these immune changes and their potential relationship with treatment outcome.

In conclusion, this study provides a characterization of circulating switched memory B cells, regulatory B cells, and cytotoxic NK cells in breast cancer patients undergoing neoadjuvant chemotherapy. Although no significant associations were identified between these immune populations and pathological complete response, the observed modification of the effector-to-regulatory ratio following treatment suggests that neoadjuvant chemotherapy is accompanied by measurable changes in systemic immune balance. The absence of significant associations between the investigated immune parameters and treatment response should also be interpreted in light of the relatively limited sample size of the study, which may have reduced the statistical power to detect subtle biological differences. Consequently, the potential predictive value of these circulating immune populations cannot be definitively excluded. Future studies involving larger and more homogeneous patient cohorts will be required to further clarify their clinical relevance. In particular, the evaluation of these immune subsets in larger populations of patients with triple-negative breast

cancer may be of particular interest, given the recognized immunological involvement of this breast cancer subtype and its generally higher sensitivity to immune-mediated mechanisms. Such investigations may help to better define the role of circulating immune biomarkers in predicting response to neoadjuvant chemotherapy.

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