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The University of Eastern Piedmont "Amedeo Avogadro"

School of Medicine

Department of Health Sciences

Master's degree in medical biotechnology

Master's Degree Thesis:

"Exploring Immune Cell Subpopulations as Biomarkers for Immunotherapy Response in Triple Negative Breast Cancer"

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ABSTRACT

The rationale of the study: Triple-negative breast cancer (TNBC) represents a subtype of breast cancer characterized by the absence of estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (HER2) expression. TNBC is associated with aggressive behavior, limited treatment options, and poor prognosis compared to other breast cancer subtypes. The lack of targeted therapies for TNBC underscores the urgent need for alternative treatment approaches. Immunotherapy has emerged as a promising strategy in the field of oncology, offering the potential to harness the patient's immune system to target and eliminate cancer cells. While immunotherapy has shown remarkable success in certain cancer types, its efficacy in TNBC remains variable, with some patients experiencing significant benefits while others show limited or no response. Understanding the immune landscape in TNBC is crucial for optimizing immunotherapy outcomes. By characterizing the immune context in early and metastatic TNBC patients through longitudinal analysis of peripheral blood immune profiles, this study aims to identify potential biomarkers, immune signatures, and predictive factors associated with treatment response and resistance. By elucidating the dynamic interaction between the immune system and TNBC, this research seeks to enhance personalized treatment strategies, improve patient outcomes, and pave the way for developing targeted immunotherapeutic approaches tailored to the specific immune profiles of TNBC patients.

Planning of the study: The study aims to analyze the immune context in early and metastatic triple-negative breast cancer (TNBC) using peripheral blood immune profiles. It uses multiparameter flow cytometry to phenotype circulating T and B cell subsets in TNBC patients. The research aims to identify potential immune biomarkers associated with immunochemotherapy outcomes in TNBC. The study also assesses immunological factors linked to treatment responses, evaluates the predictive role of circulating immune cells as a liquid biopsy tool, and investigates the impact of immune cell dynamics on TNBC progression and therapy outcomes. The findings can inform personalized treatment approaches and enhance immunotherapy efficacy.

Results: The study analyzed circulating immune cells in 20 early TNBC patients and 4 metastatic TNBC patients at baseline using multiparameter flow cytometry. Significant differences in Treg and dendritic cell levels were observed compared to healthy individuals, indicating potential immune dysregulation in TNBC. No significant relationship was found between baseline immune cells and pathologic complete response in early TNBC patients, suggesting that other factors may influence treatment outcomes in this population. However, higher levels of effector memory T cells and T-helper 2 cells were related to lymph node involvement, indicating a potential association between specific immune cell subsets and clinicopathological features in TNBC. Variations in T cell subsets were also noted to relate to primary tumor size, highlighting the dynamic nature of the immune response in TNBC and its potential impact on disease progression.

Conclusion: These findings provide valuable insights into the immune landscape of TNBC and its implications for immunotherapy stratification and monitoring. Understanding the immune landscape is crucial for tailoring personalized treatment approaches and improving outcomes.

1. INTRODUCTION

1.1 Breast Cancer Epidemiology and Worldwide Overview

According to the World Health Organization's International Agency for Research on Cancer (IARC), breast cancer continues to be one of the most common cancers worldwide. In 2020:

- There were an estimated 2.3 million new cases of breast cancer globally, making it the most frequently diagnosed cancer in women worldwide.
- Breast cancer was the leading cause of cancer death in less developed countries and the second leading cause of cancer death in more developed countries.
- There were an estimated 685,000 breast cancer deaths globally in 2020, making it the fifth most common cause of death from cancer overall[2].

In the US specifically, statistics from the American Cancer Society (ACS) show:

- In 2022, an estimated 287,850 new cases of invasive breast cancer will be diagnosed in women.
- Around 43,250 new cases of non-invasive (in situ) breast cancer will be diagnosed.
- An estimated 43,250 women will die from breast cancer in 2022.

So, in summary, breast cancer remains one of the leading causes of cancer death in women worldwide, according to recent statistics from the WHO/IARC and ACS. Early detection and treatment are critical to reducing mortality.[3]

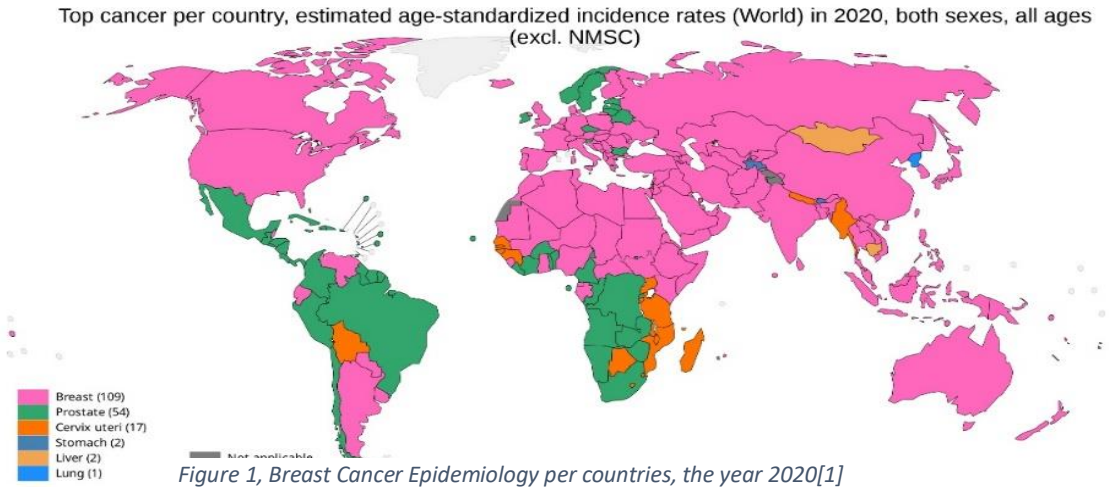


Figure 1, Breast Cancer Epidemiology per countries, the year 2020[1]

1.2 Breast Cancer Staging, Subtypes, and Treatments

Breast cancer is assigned a stage based on the size and spread of the tumor at the time of diagnosis. The TNM system (Tumor-Node-Metastasis system) is the most widely used approach for breast cancer staging, which indicates the extent of the disease and helps determine prognosis and treatment

recommendations[4].**T** describes the size and local extent of the primary tumor, with higher values indicating larger or more invasive tumors, **N** denotes the degree of regional lymph node involvement. Higher N values signify greater nodal spread, and **M** represents the presence of distant metastasis.Combinations of T, N, and M categories make up formal stage groupings (stage I - IV) reflecting overall disease progression, with stage IV as a metastatic disease.[5] Understanding TNM classifications can inform clinical expectations, selection of neoadjuvant/adjuvant therapy, and overall prognosis across breast cancer subtypes[6]. Specific implications may differ for TNBC compared to hormone-receptor-positive disease. [5]

Breast cancer remains the most prevalent form globally, but its perception has evolved due to the identification of molecular characteristics such as immunohistochemical markers such as Estrogen Receptor (ER), Progesterone Receptor (PR), Human Epidermal Growth Factor Receptor-2 (HER2), and proliferation marker protein Ki-67 (MKI67), genomic features like BRCA1, BRCA2, and PIK3CA, as well as immunomarkers including Tumor-Infiltrating Lymphocytes(TILs) and Programmed cell Death receptor Ligand-1(PD-L1).[7] Biomarker combinations serve as the framework for diagnostic algorithms that are becoming more complicated. The presence of ER, PR, Her2 status, Ki67 protein, and tumor grade was utilized to define five fundamental molecular subtypes of breast cancer: luminal A, luminal B Her2+/luminal B Her2−, basal/triple-negative, normal-like, and Her2-enriched. The state of each subtype is briefly summarized in (Figure 2)[8].

1.2.1 Triple Negative Breast Cancer(TNBC) and metastatic Triple Negative Breast Cancer(mTNBC)

TNBCs comprise ten to twenty percent of invasive (BCs). They exhibit more aggressive clinical and biological behavior than other BC types. TNBCs are moderate to high-grade cancers with few treatment options and a poor prognosis. The subgroup of TNBCs primarily affects women under 40 years old. Managing TNBCs is among the most challenging compared to other types of BC, as they are more difficult to treat and have a higher risk of recurrence. The prognosis for most TNBCs is dismal, as they are infiltrative carcinomas that invade the surrounding tissue around the primary tumor site.[9]TNBC is the only molecular subtype of BC lacking progesterone and Estrogen Receptors and lacking HER2 protein overexpression. Clinical assays have identified TNBC with HER2 expression being negative or amplified, PR expression below 1%, and ER expression below 1%. Also, the expression of Ki67 is high.[9] BRCA1/2 genes are the primary drivers of TNBC susceptibility. Noteworthy genetic mutations, such as those in TP53 and BRCA1, have been extensively studied about TNBC. Additionally, pathogenic mutations in BRIP1, BARD1, RAD51C, and PALB2 are more prevalent in TNBC than in other subtypes of breast cancer.[9]

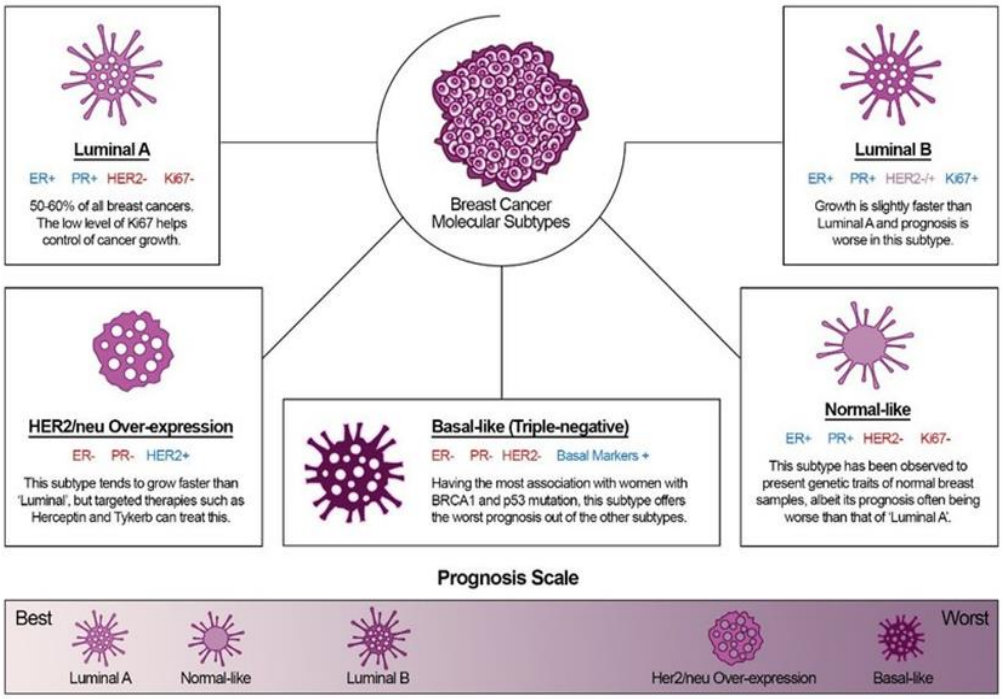


Figure 2 provides an overview of five fundamental molecular subtypes of breast cancer, categorized based on the biomarker profile showing the presence or absence of ER, PR, HER2, and Ki67. The major takeaway here is understanding how these biological markers are used to define the specific intrinsic subtypes.[10]

While BRCA1 and BRCA2 mutations collectively only account for a minority of breast cancer cases, approximately 70% of women who inherit a harmful BRCA1 gene mutation will develop breast cancer in their lifetime. Additionally, 16-23% of breast cancers diagnosed in carriers of BRCA2 mutations are classified as TNBC, indicating an association between BRCA2 and this more aggressive subtype.[9]Lehmann et al. discovered six subtypes of TNBC. They also identified the specific signaling pathways that each subtype may have a high concentration [13]. (as outlined in Table 1)

Type	Main Molecular Characteristics
Basal-like 1 (BL1)	Enriched in: Cell cycle and proliferation genes (AURKA, AURKB, CENPA, BUB1, TTK, CCNA2, PRC1, MYC, NRAS, PLK1, BIRC5) DNA damage response genes (CHEK1, FANCA, FANCG, RAD54BP, RAD51, NBN, EXO1, MSH2, MCM10, RAD21, MDC1) High Ki-67 mRNA expression
Basal-like 2 (BL2)	Enriched in: Growth factor signaling genes (EGF, NGF, MET, Wnt/β-catenin, IGF1R pathways) Growth factor receptor genes (EGFR, MET, EPHA2) Myoepithelial markers (TP63 and MME or CD10)
Immunomodulatory (IM)	Enriched for gene ontologies in immune cell processes, including: Immune cell signaling (TH1/TH2, NK cell, BCR signaling, DC, T-cell receptor signaling pathway) Cytokine signaling (cytokine, IL-12, IL-7 pathway) Immune signal transduction (NFKB, TNF, JAK/STAT pathway)
Mesenchymal (M)	Enriched in: Cell motility (regulation of actin by Rho) ECM receptor interaction Cell differentiation pathways (Wnt/β-catenin, ALK, TGF-β signaling)
Mesenchymal stem-like (MSL)	Similar to M type. Also enriched in: Angiogenesis genes (VEGFR2, TEK, TIE1, EPAS1) Growth factor signaling pathways (including adipocytokine signaling, EGFR, PDGF, G-protein coupled receptor, ERK1/2)
Luminal androgen receptor (LAR)	Enriched in: Signaling pathway of androgen receptor (including FASN, APOD, CLDN8, DHCR24, ALCAM, FKBP5, PIP, SPDEF)

Table 1 outlines the Lehmann TNBC subtypes and the associated enriched gene expression pathways. The key conclusion is that there is significant molecular heterogeneity within TNBC tumors, allowing for classification into subtypes that may respond differently to therapies targeting the dominant signaling pathway.[13]

Currently, TNBC treatment options are limited, and the primary systemic therapy is chemotherapy. Doctors often use chemotherapy before and after surgery to reduce the size of the tumor and prevent any potential recurrence. Patients with early-stage TNBC may receive radiation therapy to eliminate any remaining cancer cells after surgery. It is essential to note that hormonal and HER2-targeted therapies, which are effective in treating other forms of breast cancer, are not helpful for TNBC patients because there are no available receptors for these treatments to target. [7]

Physicians can classify and guide treatment for metastatic triple-negative breast cancer (mTNBC) based on European Society for Medical Oncology(ESMO) guidelines. These guidelines evaluate physiological markers such as PD-L1 expression and BRCA gene mutations to categorize mTNBC at the molecular level and determine appropriate therapeutic strategies.[10] Patients with mTNBC have a poor prognosis, limited treatment alternatives, and resistance to chemotherapy, necessitating the development of novel treatment strategies. One promising approach involves combining ICIs with novel immunotherapy agents or targeted therapies. Clinical trials are underway, involving diverse combinations of PARP inhibitors, AKTi, and MEK inhibitors in several cohorts of patients with mTNBC. Patients with germline BRCA mutations have demonstrated the efficacy of PARP inhibitors. Researchers must conduct further studies to determine whether combination therapies can enhance this effectiveness.[11] (Figure 3)

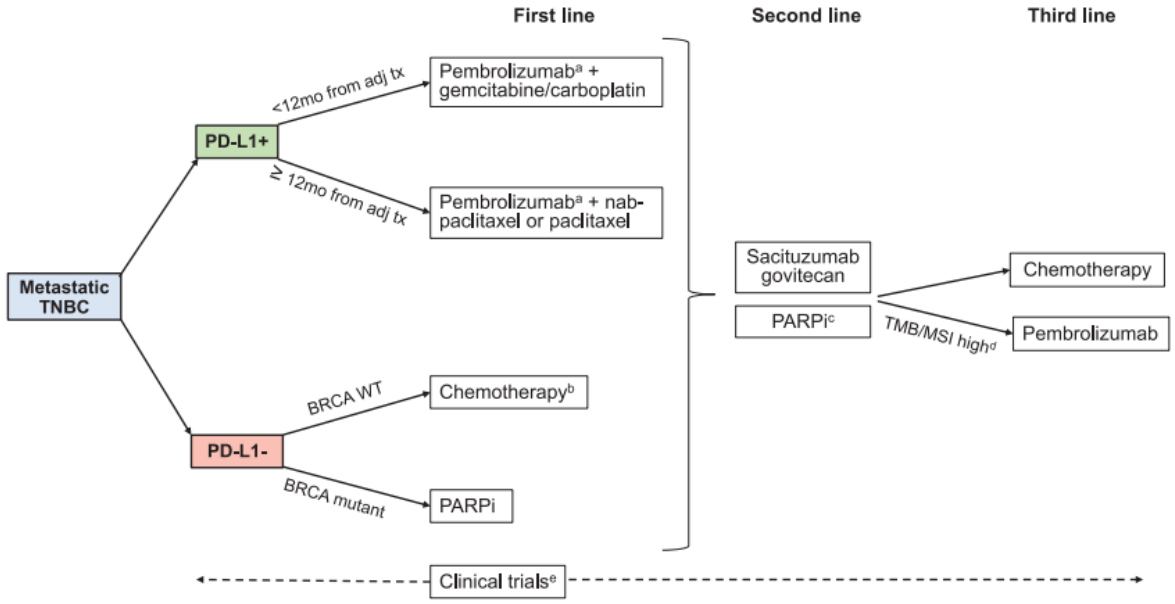


Figure 3 Treatment paradigm for metastatic triple-negative breast cancer (TNBC).[19]

1.3 Immunotherapy Treatment for Breast Cancer and TNBCs

Cancer immunotherapy has been an essential subject of study since the nineteenth century, with immunotherapies utilized for a variety of solid tumors, including breast cancer, melanoma, lung cancer, and kidney cancer. Immunotherapy is any technique for treating cancer that increases innate and adaptive immune responses. Immune checkpoint inhibitors (ICIs), cancer vaccines, oncolytic viruses, and adoptive immune cell therapies are examples of these[12]. Unlike radiation and chemotherapy, which try to inhibit tumor cell growth and survival directly, immunotherapies target the tumor indirectly by increasing antitumor immune responses that occur spontaneously in many patients.[13]

1.3.1 Immune checkpoint inhibitors (ICIs) in breast cancer and TNBCs

ICIs have significantly altered the therapeutic environment for numerous neoplasms. They have shown much promise in treating both early-stage and metastatic triple-negative breast cancer when used with chemotherapy. There is still a lot to learn, and finding new biomarkers is essential for improving the effectiveness of ICI, finding patients who cannot take them, and figuring out how harmful they are.[14] In typical circumstances, experts assert that the immune system regulates the response to pathogens, prevents hyperactive reactions, and restricts tissue injury and autoimmune activity through an inhibitory checkpoint pathway. Several immune checkpoint molecules regulate this pathway: programmed cell death protein 1 (PD1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). By interacting with PD-L1, PD-L2, and CD80/CD86, these molecules inhibit the functionality of T cells. However, tumor cells use this mechanism to create a microenvironment that suppresses the immune system. Nevertheless, experts suggest that anti-PD-1, anti-PD-L1, and anti-CTLA-4 monoclonal antibodies can stop the inhibition of the immune system and fight cancer cells directly.[14] TNBC is the subtype with the highest immunogenicity. Blocking immune checkpoints in TNBC is hard because basal-like and high-grade tumors have the most mutations. Response rates to anti-PD1 and anti-PDL1 monoclonal antibodies have been scant. Chemotherapy enhances the expression of PD-L1 and increases the immunogenicity of tumors. Anti-PD-1 or anti-PD-L1 antibodies combined with immune-compatible cytotoxic medications constitute most clinical trials.[14]

The development of immune checkpoint inhibitors (ICIs) that target T-cell co-inhibitory receptors such as (CTLA-4) and (PD-1)/(PD-L1) has transformed the treatment of cancer over the last decade. Subsequently, antibodies blocking the PD-1/PD-L1 axis, including pembrolizumab, nivolumab, atezolizumab, and durvalumab, were rapidly introduced and showed significant clinical activity across various cancer types. Anti-PD-1 drugs such as pembrolizumab and nivolumab have been widely adopted in clinical practice. They are now commonly used either as single agents or in combination with chemotherapy for the treatment of approximately 50 different forms of advanced cancer. The success of ICIs has revolutionized cancer treatment and catalyzed an explosive expansion of immunotherapy research.[15]

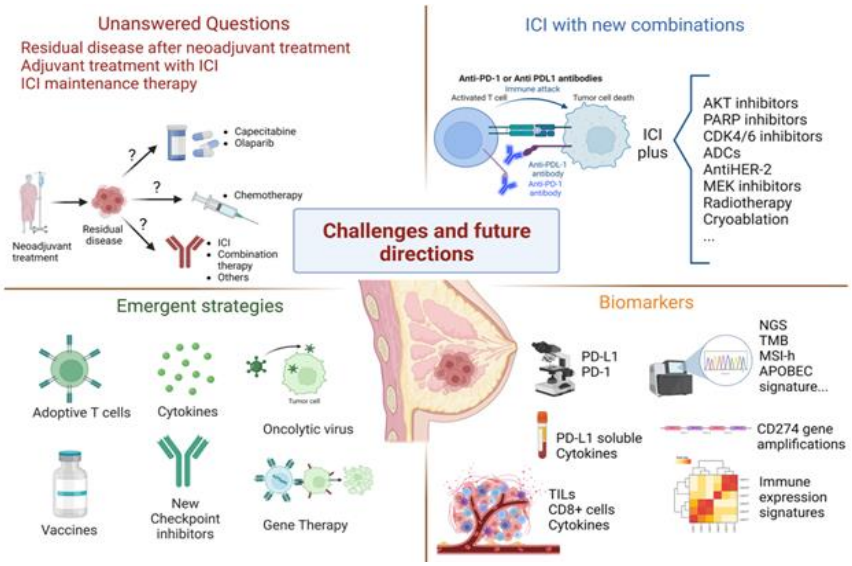
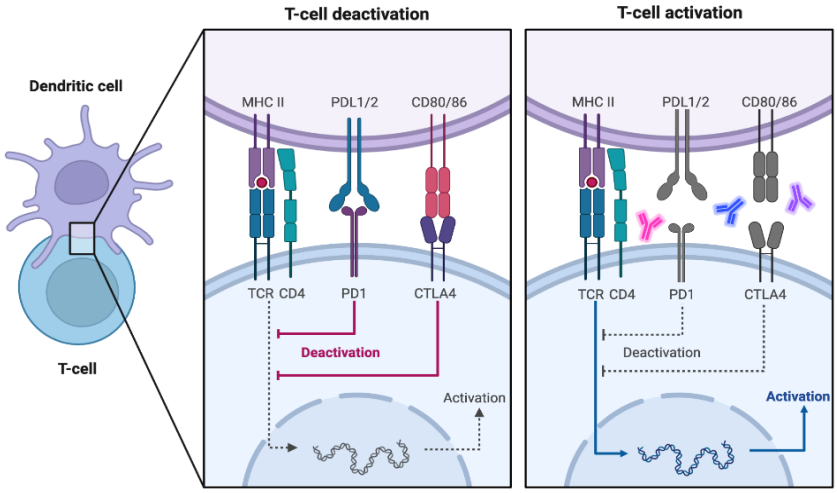


Figure 4 Current challenges and novel immunotherapy approaches in breast cancer.[22]

1.3.2 PD-L1 as a biomarker and biological target

Immune cells, including dendritic cells (DCs), T cells, B cells, natural killers (NK), macrophages, and monocytes, express PD-1, an immune checkpoint, on their surface. It can inhibit both adaptive and innate immune responses. Its gene is located on chromosome 9p24.1 and encodes for a 33 kDa protein. It comprises immunoglobulin V-like and C-like domains outside considering of cells, a hydrophobic transmembrane region, and a 30-amino acid tail in the cytoplasm. PD-1 controls when antigen tolerance starts and when the immune response ends. It is necessary for maintaining immune tolerance and stopping autoimmunity in a healthy body. PD-1 ligand (PD-L1) is a transmembrane protein in immune cells (DCs, B cells, T cells, and macrophages) and tumor cells. It functions as an "adaptive immune mechanism" that cancer cells might employ to evade the immune system's antitumor responses. In normal conditions, the binding of the PD-L1 ligand to its receptor, PD-1, maintains homeostasis by reducing T-cell expanded repertoire, suppressing cytokine production, and activating cell intrinsic tolerogenic signals to prevent autoimmunity. Instead, the tumor uses the same immunosuppressive effect in the context of tumor cells to avoid detection by the immune system. PD-1/PD-L1 signaling malfunctions can disrupt cellular processes such as cell proliferation, antigen presentation, and cell survival (Figure 4). It is possible to guess how well anti-PD-L1/anti-PD-1 therapy will help people with TNBC by looking at the amount of PD-L1 protein in tumor cells and immune cells that attack them.[16, 17]

According to what was already said, PD-1/PD-L1 ligation is a signaling pathway that helps tumors grow by stopping T-cells and letting cancer cells avoid immune surveillance. A variety of signals can have an impact on the regulation of PD-1/PD-L1 expression in cancer cells. For instance,(1) the PI3K/AKT pathway, which facilitates increased external signaling and decreases PTEN downregulation, can be activated to promote PD-L1 expression; (2) the MAPK signaling pathway, which is associated with the PD-1/PD-L1 axis and is responsible for converting extracellular signals into intracellular responses; (3) The JAK-STAT signaling pathway is a critical mechanism through which extracellular signals regulate gene expression, including PD-L1 expression; (4) An aberrant WNT signaling pathway may inhibit cancer immune monitoring and facilitate immune suppression through a crosstalk mechanism between PD-L1 expression and WNT activity; (5) The NF-κB signaling pathway is responsible for mediating the expression of PD-L1 induced by INF-α; (6) The hedgehog signaling pathway, which promotes PD-1/PD-L1 axis expression and whose inhibition could induce lymphocyte antitumor activity.[16]



T-cell activation is a crucial immune response process, involving antigen recognition by TCRs and dendritic cells. Tolerance is regulated by molecules like PD-1 and CTLA-4.[25]

The protein PD-L1 plays a vital role in tumor immune evasion by binding to its receptor PD-1 on T cells and inhibiting their activity. PD-L1 is expressed in approximately 20% of TNBC cases. It can be defined on tumor cells and infiltrating immune cells within the tumor microenvironment.[16]Several studies have investigated PD-L1 expression levels in primary breast tumors and corresponding lymph node metastases. One such study analyzed 101 patients with TNBC and found significant heterogeneity in PD-L1 expression between primary tumors and paired lymph node metastases (Li et al., 2018). This study found that PD-L1 expression was more frequently detected in the lymph node metastases (LNM) compared to the primary tumors, with PD-L1 positivity seen in 59.41% of LNM versus 38.61% of primary tumors. Furthermore, the levels of PD-L1 expression were significantly higher in both the tumor-infiltrating lymphocytes and tumor cells of the LNM compared to the primary tumors.[16] There are several possible explanations for this observed difference. Firstly, the lymph nodes represent one of the first sites of metastasis for breast cancer cells. The process of metastasis involves cancer cells undergoing epithelial-to-mesenchymal transition, making them more stem-cell-like and invasive. This transition has been linked to increased PD-L1 expression.[25] The lymph node microenvironment also contains a higher immune cell concentration than the primary tumor bed. Studies have shown that cytokines secreted by these immune cells, such as IFN-gamma, can induce PD-L1 expression on tumor cells as a protective mechanism. Therefore, cancer cells traveling to lymph nodes may encounter an immune landscape that selects for those with elevated PD-L1 levels.[25] In summary, the increased PD-L1 expression observed specifically in LNM compared to primary tumors could result from cancer cells undergoing alterations that promote metastatic ability and render them more resistant to immune attack at secondary tumor sites. This has implications for using lymph node tissues to identify TNBC patients most likely to respond to PD-1/PD-L1 targeted therapies[25]

1.4 Immune system and Breast cancer

The immune system consists of two main branches: the innate immune response and the adaptive immune response. The innate response provides rapid initial protection through physical and chemical barriers and cellular responses that do not require immunologic memory or specificity. However, these reactions are non-specific and can sometimes damage healthy tissues. In comparison, the adaptive response relies on antigen-specific lymphocytes and immunological memory to enable highly targeted immune protection with repeated exposures. However, it lacks complete specificity and can potentially harm non-infected healthy cells.[18]

Lymphoid cells, which include natural killer T (NK-T) cells and innate lymphoid cells (ILCs), are traditional effectors of innate immunity. Among them, NK cells are a particularly significant population that recognizes their ligands without any restriction by MHC. On the other hand, T and B lymphocytes comprise the lymphoid cells responsible for adaptive immunity. Human NK cells are a diverse population of cytotoxic cells divided into two primary subsets: CD56dim/CD16brigt and CD56bright/CD16dim/neg, depending on the expression densities of CD16 and CD56. While both NK populations are capable of cytotoxicity and cytokine secretion, they exhibit distinct differences in both appearance and function, much like CD8 and CD4 T cells. The CD56dim/CD16brigt NK cells, which constitute around 90% of the NK cells in peripheral blood, demonstrate strong spontaneous cytotoxic activity as well as antibody-dependent cell cytotoxicity (ADCC). NK cells are particularly effective at recognizing and lysing target cells from the hematopoietic system, such as immune cells, red/white blood cells, and stem/progenitor cells involved in blood/immune cell production. In contrast, CD56bright/CD16dim/neg NK cells can generate immunoregulatory cytokines, specifically interferon and tumor necrosis factors.[19, 20] B and T lymphocytes are present in secondary lymphoid tissues such as lymph nodes, spleen, tonsils, and mucosa-associated lymphoid tissue. These tissues offer a suitable environment for encountering specific antigens and harbor proficient antigen-presenting cells. The production of cytokines and the presence of a well-organized group of adhesion molecules assist in the movement of cells through the tissue. B cells differentiate from progenitor cells in the bone

marrow, while T cells migrate to the thymus as thymocytes. Both cell types generate antigen-specific receptors through a unique process involving random DNA rearrangement and splicing. [26]

Cancer cells and immune cells compete for nutrients, leading to nutrient deficiencies and acidosis, which impairs the function of immune cells. Metabolites such as glutamine, fatty acids, amino acids, and cholesterol can affect immune cells. Cancer metabolism influences the expression of immune molecules such as lactate, PGE2, and arginine. Targeting metabolic pathways in cancer cells, such as inhibiting lactate production, could be a potential therapeutic strategy that enhances the antitumor immune response and improves T cell functionality.[21] Understanding the cancer-immunity cycle is crucial to comprehend the sequence of events in anticancer immune responses. Each stage, including those outside the cancer and immune system, can limit optimal immunity. The tumor microenvironment (TME) is vital in facilitating the anticancer response. T cells in the TME simulate systemic events through a series of reactions. However, disruptions in metabolism, inhibitory immune cells, and T-cell dysfunction may impede the cancer-immunity cycle.[22] The cancer-immunity cycle involves various stimulatory and inhibitory factors that influence the success or failure of each step. Some elements can be either stimulatory or inhibitory. These include tumor-associated antigens (TAAs), endogenous retrovirus proteins (ERVs), interferon genes (STING), ATP (ATP), the tumor microenvironment (TME), interferon (IFN), damage-associated molecular patterns (DAMPs), toll-like receptors (TLRs), tumor necrosis factor (TNF), chemokine ligands and receptors (CCL, CXCR, TCR), pMHC (MIC), PD-L1, CTLA-4, LFA, ICAM, VLA, VEGF, MMP, LAIR, TGF, CAF, Tregs, MDSC, TREM, VSIG, PGE, B2M, LAG-3, and TIM.[27] (Figure 7).

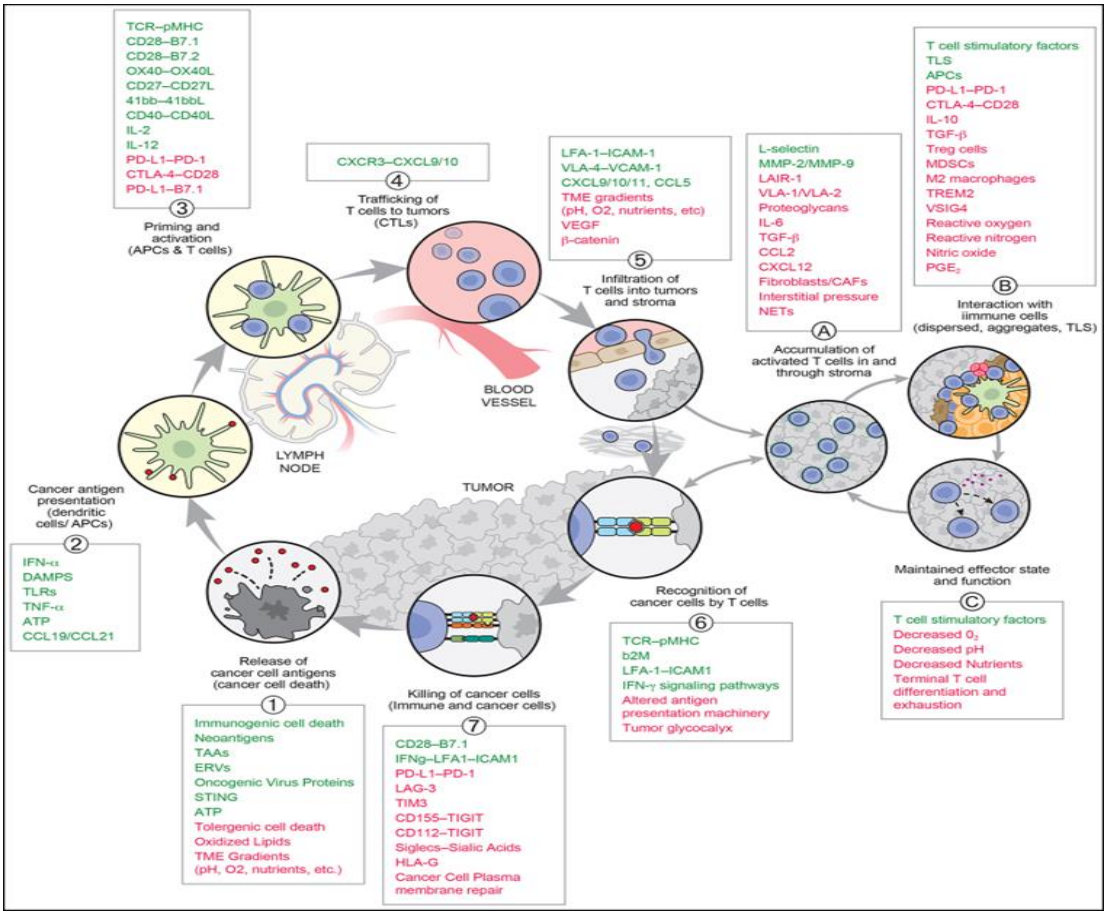


Figure 7 The cancer-immunity cycle with stimulatory and inhibitory factors.[29]

1.4.1 T lymphocytes and Breast cancer

T lymphocytes, immune cells, have a T cell receptor (TCR) that identifies antigens from the MHC gene family. Allies include CD8+ cytotoxic lymphocytes (CTL) and CD4+ T helper (Th) cells. The cells release peptides from MHCI or MHCII. Th1 releases IFN-γ and TNF-α, while Th2 releases IL-4, IL-5, and IL-13. Cytokines or TCR stimulation activates natural killer T (NKT) cells and γδ T cells, which control CD8+ T cell responses [23]. T lymphocytes play a crucial role in controlling pro- and anti-tumor immunity by mediating inflammatory processes in the tumor microenvironment. Most leukocyte subsets have either pro- or anti-tumor activities, highlighting the importance of cellular crosstalk in shaping the tumor microenvironment. Tregs, rediscovered as CD4+CD25+ Tregs, are particularly interesting. Tregs can develop naturally in the thymus or be induced by transforming growth factor (TGF)-β exposure, using immune suppression mechanisms. Both natural and induced Tregs can limit anti-tumor immunity by suppressing effector T cell responses in lymphoid tissues and tumors. Evaluating the role of specific lymphocyte subsets like Tregs in tumorigenesis and balancing pro- and anti-tumor immunity is of interest[23].

IL-17 is a cytokine produced by Th17 cells, a subset of CD4+ T cells characterized by having IL-17, IL-17F, IL-21, and IL-22. These cytokines promote inflammation and tissue damage.[24] In cancer, IL-17 can have both pro-tumorigenic and anti-tumorigenic effects. For instance, IL-17 can stimulate the production of vascular endothelial growth factor (VEGF), promoting tumor growth and angiogenesis. Additionally, IL-17 can recruit myeloid-derived suppressor cells (MDSCs) to the tumor microenvironment, further suppressing anti-tumor immune responses[25]. Conversely, IL-17 can also have anti-tumorigenic effects by activating cytotoxic T cells and natural killer (NK) cells. The balance between these opposing effects likely depends on the tumor type and stage of development[25]. Moreover, T helper cells are critical in developing CD4+ and CD8+ T lymphocytes. During thymic development, these cells commit to a specific lineage, and their activation requires the interaction of MHCII, co-stimulatory molecules, and cytokine-dependent signaling. Interestingly, individuals with ovarian, prostate, and gastric cancers have been found to have higher T-H17 cells in their peripheral blood.[24] NKT cells play a crucial role in determining the immune system's response by producing cytokines like IFNγ, TNFα, IL-4, and IL-13. Type I NKT cells recognize α-GalCer, while type II express various αβ TCR chains and are activated by undefined glycolipids. The effects of NKT cells depend on intermediate cells like DCs, which are unresponsive to IL-13. Adoptive transfer of NKT cells varies based on their CD4+ or CD4- status and tissue used for separation[23].

Tumor-infiltrating lymphocytes (TILs) are immune cells that move into the tumor microenvironment. They help the body fight against cancer and can provide insight into the prognosis and potential treatment of breast cancer.[26]The assessment of TILs in breast cancer tissue samples has been shown to have prognostic and predictive value. High levels of TILs have been associated with improved response to chemotherapy and better overall survival in some subtypes of breast cancer. However, using TILs as a biomarker to guide treatment decisions is still investigational and requires further validation in prospective clinical trials(Figure 8).[26] High TIL levels in pre-treatment biopsy are associated with higher rates of pathological complete responses (pCRs) to neoadjuvant chemotherapy (NAC)[27]. Furthermore, patients with high TILs post-NAC may benefit from strategies designed to enhance the immune response against the tumor [28]

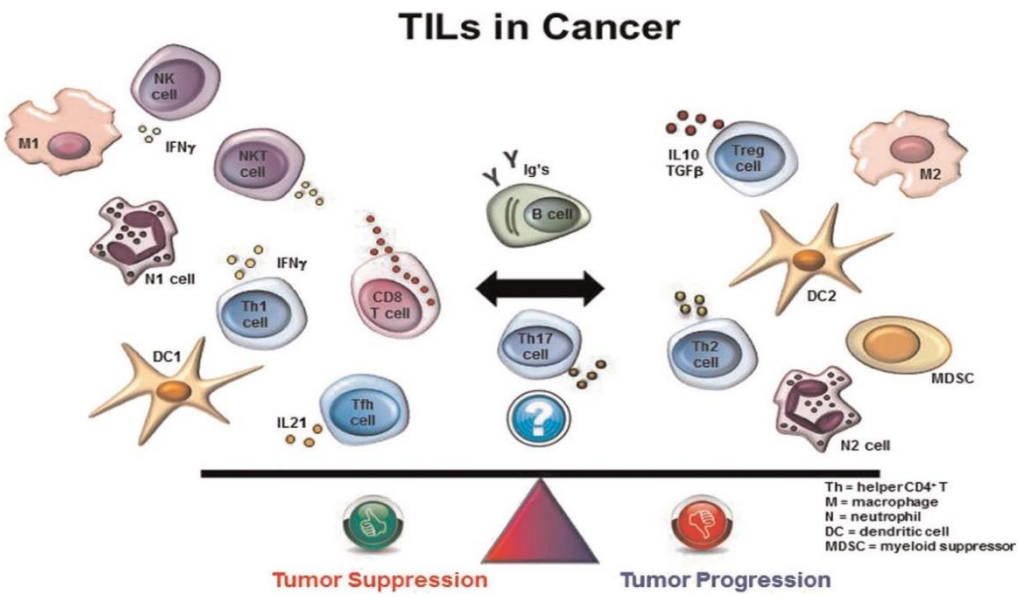


Figure 8 The image illustrates the communication between different leukocyte groups and their primary role in promoting pro- or anti-tumor activities. These groups include myeloid lineage leukocytes, tumor-associated macrophages that can have either protumorigenic (M2) or antitumorigenic (M1) properties, helper T-cell subsets, cytotoxic T cells, regulatory T cells, B cells, dendritic cells, and myeloid-derived suppressor cells. These cells are crucial in shaping the microenvironment through the substances they produce, which determine immune-mediated anti- or protumor activities.[35]

Research indicates that patients with a higher peripheral CD4+/CD8+ ratio show better treatment efficacy for TNBC. T-cell subsets in peripheral blood predict and prognosticate TNBC treatment response, especially in chemotherapy combined with immunotherapy. Understanding the relationship between hematologic parameters, treatment response, and immune-related markers is crucial. Detecting peripheral blood lymphocyte subsets may help stratify TNBC patients for chemotherapy and immune therapies[29].

1.4.2 B Lymphocyte and Breast Cancer

B lymphocytes, also known as B cells, contribute to anti-tumor immunity through several mechanisms. They produce antibodies that target and eliminate tumor cells or block oncogenic pathways.[30] Additionally, B cells present antigens to helper T cells, stimulating downstream cytotoxic T lymphocyte responses.[31]. B cell-derived cytokines like IL-10 and TGF-β can modulate the tumor microenvironment with context-dependent pro- or anti-tumor consequences.[32] Tumor-infiltrating B cells have been extensively studied in breast cancer, with their presence correlating with clinical outcomes. In ER+ subtypes, increased B cell infiltration is associated with improved prognosis, However, in TNBC and HER2+ disease, this correlation is reversed, with higher B cell infiltration indicating poorer outcomes[33]. Further research has identified a potential role for B cells in generating effective anti-tumor immunity at the tumor site[37]. Recent research has highlighted the diverse functionality of intratumoral B cells in breast cancer, with memory B and plasma cells appearing more immunosuppressive, and naïve and germinal center B cells associating with activated T cell responses[34]. This suggests that the balance of B cell phenotypes in the tumor niche is crucial, and further research is needed to identify druggable targets that can shift this balance[34]. B cell and B cell-related pathways have been identified as potential targets for novel cancer treatments, with a focus on their roles in activating immune responses and forming tertiary lymphoid structures[35]. Tumor-infiltrating B cells in breast cancer have a complex role, with both positive and negative effects on prognosis and response to therapy ([36] [37]). Studies have identified increased expression of inhibitory immune checkpoint proteins like PD-1 on peripheral memory B cells of breast cancer patients, suggesting a potential role in disease progression[36]. Furthermore, CDK4/6 inhibitors have been shown to promote anti-tumor immunity through the induction of T-cell memory, potentially enhancing the efficacy of immunotherapies[38]. The identification of inhibitory immune checkpoints in breast cancer stem cells, and their potential

regulation by stemness-related pathways presents a promising avenue for precision immunotherapy[39]. (Figure 9) provides an overview of the complex immunological interactions occurring within the tumor microenvironment of breast cancer[40].

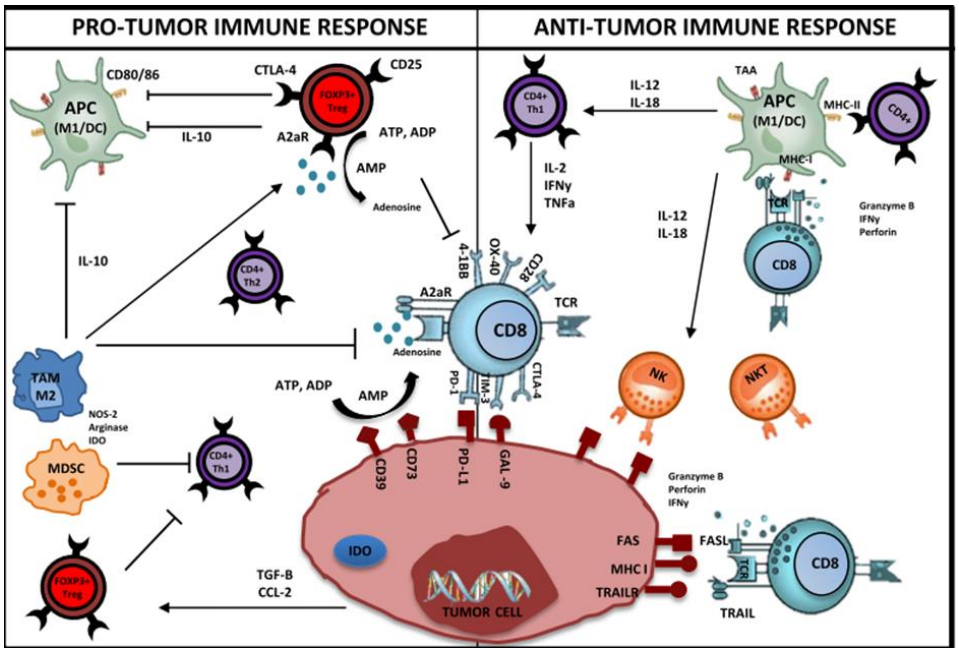


Figure 9 illustrates the intricate immunological interactions within the tumor microenvironment of breast cancer, highlighting how immune cell types and signaling pathways coordinate to promote or suppress anti-tumor immune responses. Effective cell-mediated cytotoxicity requires CD4+ helper T cells and CD8+ cytotoxic T lymphocytes to recognize tumor cells, causing the release of perforin, granzymes, and proinflammatory cytokines. However, several inhibitory mechanisms exist to attenuate this response, including immune checkpoint signaling, immunosuppressive regulatory T cells, and metabolites.[48]

1.5 Toxicity and immune-related adverse events

Immune checkpoint inhibitors (ICIs) show promise in treating TNBC, but their use requires careful management due to unique immune-related adverse events (irAEs) [([41],[42])]. These irAEs, which can range from mild to fatal, include colitis, pneumonitis, hepatitis, and endocrinopathies[43]. However, the reporting of irAEs in clinical trials is often incomplete, highlighting the need for a standardized reporting method[44]. A systematic review by Chen (2015) highlighted the need for standardized reporting of immune-related adverse events (irAEs) in immune checkpoint inhibitor (ICI) clinical trials, The study found that the quality of reporting of (irAEs) in (ICI) clinical trial publications is suboptimal. The incidence of grade 3/4 adverse events was higher for inhibitors against CTLA-4 compared with other immune checkpoints. The study suggests the need for a standardized reporting method for irAEs to enable a more precise evaluation of the therapeutic risk-benefit ratio of ICIs[44]. Morehouse (2019) found that early occurrence of irAEs may predict survival benefits in patients treated with ICIs[45]. Kalinich (2021) identified younger age and treatment with combination ICI therapy as risk factors for severe irAEs requiring hospitalization, The incidence of irAEs requiring hospitalization in patients receiving ICI therapy was 3.5% overall, with varying rates for different types of ICIs. Combination therapy was associated with the highest incidence of irAEs requiring hospitalization[46]. Velasco (2017) conducted a meta-analysis that revealed a higher incidence of grade 3-4 irAEs with CTLA-4 inhibition compared to PD-1/PD-L1 blockade. These findings underscore the importance of early detection and management of irAEs, as well as the need for further research to identify patient and tumor factors that predispose towards life-threatening irAEs[47]. Immune

checkpoint therapies are associated with a small but significant increase in the risk of developing immune-related adverse events and high-grade gastrointestinal and liver toxicities. Fatal immune-related adverse events are rare, but early recognition of adverse events is crucial for preventing future complications[47].

Pembrolizumab treatment in TNBC trials has been associated with a range of adverse events, including fatigue, rash, arthralgia, diarrhea, hepatitis, and endocrine dysfunction[48]. These events are thought to be driven by the activation of specific immune cell subsets, such as T cells and natural killer cells[48]. The management of these immune-related adverse events (irAEs) is crucial, and vigilantly monitoring them can allow for prompt intervention to mitigate their impact on patient well-being and treatment outcomes[48]. The unique spectrum of irAEs, including dermatologic toxicities, requires tailored supportive care guidelines[49]. The evolving field of immunotherapy and related toxicities necessitates continuous updates in knowledge and management strategies[50].

1.6 Liquid Biopsy

Liquid biopsy, a non-invasive method for analyzing tumor components, has shown promise in the management of breast, lung, and colorectal cancers[51]. It can provide real-time information on tumor heterogeneity and evolution, as well as predict resistance mechanisms[51, 52]. These findings highlight the potential of liquid biopsy in providing valuable insights into cancer biology and its applications in cancer surveillance, treatment response monitoring, and early detection[52]. In TNBC, liquid biopsy has demonstrated concordance with traditional biopsies and can predict resistance mechanisms[51]. The analysis of circulating tumor cells (CTCs) at a single-cell level has been precious in revealing tumor heterogeneity [53, 54]. Liquid biopsy, a method for analyzing circulating tumor cells and DNA, has emerged as a powerful tool for characterizing the immune context of cancers[55]. The use of liquid biopsy in cancer screening, patient stratification, and treatment monitoring has been highlighted, with a focus on the analysis of circulating tumor cells and DNA[56]. The clinical applications of liquid biopsy, including its potential in precision oncology, have been discussed, with a particular emphasis on the analysis of circulating tumor DNA and cells[57]. The clinical applications of circulating tumor DNA as a liquid biopsy have also been reviewed, with a focus on its potential to detect and monitor genomic alterations in cancer patients[58].

A range of immune cell populations in the blood have been identified as potential biomarkers for predicting and monitoring responses to (ICI) therapies. These include lymphocytes (B, T, and NK cells), myeloid cells, and dendritic cells [59, 60]. For instance, high levels of CXCL13+ T cells have been associated with effective responses to anti-PD-L1 therapies in TNBC[61]. Furthermore, the presence of specific myeloid cell subsets has been linked to response to anti-PD(L)1 and anti-CTLA-4 treatment, suggesting their potential as predictive biomarkers[62]. Antibodies targeting CTLA-4 and PD1/PD-L1 show efficacy in various malignancies, but response rates are variable and can cause immune-related adverse events, highlighting the potential of blood-immune cell populations as biomarkers for ICI therapies [63]. Standardized and optimized flow cytometry panels have been developed for deep immunophenotyping of immune cell populations, providing a powerful tool for immune blood cell monitoring in clinical trials [64, 65].

2. OBJECTIVE OF THE THESIS

The principal aim of this study is to characterize and compare the immune context in early and metastatic triple-negative breast cancer (TNBC) through longitudinal analysis of peripheral blood immune profiles.

Specifically, the objectives are:

1. To characterize circulating T and B lymphocyte subsets in early and metastatic TNBC patient cohorts by multiparametric flow cytometry.
2. To correlate changes in immune cell profiles over time with disease progression and clinicopathological characteristics.
3. To assess immunological factors associated with immunochemotherapy outcomes in TNBC by comparing baseline and progression time points.
4. To assess the predictive role of circulating immune cells as a liquid biopsy tool in early and metastatic TNBC patients under immune checkpoint inhibition.

This multi-parameter immune monitoring approach may provide insights into dynamic tumor-immunity interplay not fully captured by static biopsies alone. The overarching goal is to optimize immunotherapy for TNBC by identifying predictive immune signatures.

3. MATERIALS AND METHODS

3.1 Study design and patients

The Immune Response Individualization Study (IRIS) project is a multi-centered, observational study that involves 18 centers in total, distributed all around Italy. The thesis focuses on the patients only enrolled from Novara Hospital (Ospedale Maggiore della Carità di Novara). The inclusion and exclusion criteria, defined in the protocol version 2.0 – 15/11/2021, are the following:

• Inclusion criteria

− Patients with Metastatic Triple Negative Breast Cancer (mTNBC), are candidates to receive Immune Checkpoint Inhibitors (ICI) therapy (Atezolizumab + Nab-paclitaxel) as 1st line treatment. Moreover, patients with early TNBC, are candidates to receive Pembrolizumab + Paclitaxel + carboplatin, following clinical practice guidelines.

− Patients with TNBC at every stage of the disease, treated following clinical practice Guidelines

− Ability to understand and sign an informed consent

− Patients of both sexes, ≥18 years old

− Formalin-fixed, Paraffin-Embedded (FFPE) tumor tissue from a primitive lesion or biopsy availability

− Measurable/non-measurable, valuable disease

• Exclusion criteria

− Inability to understand and sign an informed consent

− Patients ≤18 years old

− Unavailability of FFPE tumor tissue

3.2 PBMCs isolation

PBMCs were isolated from the blood samples of the patients enrolled in the study through the Ficoll-density separation technique. 10 mL of blood EDTA was diluted with 20 mL of physiological solution (NaCl 0,9%) in a 50 mL falcon tube, then 15 mL of Ficoll (Lympholyte-H, Cedarlane, and ND) was added. The tube was centrifuged for 20 minutes at 2200 rpm, with 7 and 0 values for acceleration (AC) and deceleration (DC), respectively. Thanks to this setting, cells were divided by density gradient. The PBMC ring was collected from the interphase between diluted plasma and separation medium and then washed to remove all the remaining Ficoll. Then, the obtained cells were counted in a Burker's chamber, diluted 1:10 with TURK solution (Carlo Erba reagents, MI).

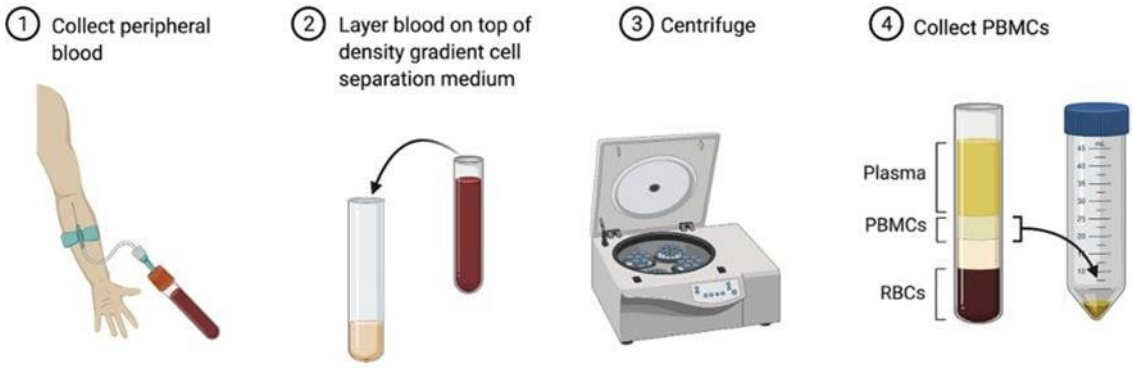


Figure 10 PBMC collection through density centrifugation gradient. (1) Peripheral blood is collected from the patients using two EDTA tubes and one Na-citrate tube. (2) Blood is diluted with a physiological solution and is put on top of the density gradient separation medium to allow Ficoll stratification, thus creating two distinct layers. (3) The sample is centrifuged at 800 ×g for 20 minutes, with acceleration 7 and brake 0. (4) four layers can be distinguished after centrifugation. From the bottom to the top: red blood cells, Ficoll, PBMCs and diluted plasma. The PBMCs ring of interest is then collected in another Falcon tube.

Then, according to the number of cells obtained, those were divided into different vials for cryopreservation. The cryopreservation media comprised 10% DMSO (Mediatech, MI) and 90% FBS (Microgem, NA).

3.3 B, T, and NK staining

50µl of EDTA whole blood was used for the unstained FACS tube, whereas 400µl was used for the B/T FACS tube. Only the B/T tube was stained with Live and Dead (LD – FV780) antibody (Ab) for 15 minutes in room temperature dark conditions. The blood in the B/T tube was split into two FACS tubes, one for T staining and the other for NK/B. The following two mixes were prepared and used for the two tubes:

NK/B	Fluorophore	T	Fluorophore
Staining Buffer		Staining Buffer	
CD45	BUV395	CD45	BUV395
HLA-DR	BUV605	HLA-DR	BUV605
CD3	BV786	CD3	BV786
CD16	PE-CY7	CD4	BUV737
CD19	BV480	CD8	BUV496
IgM	BB515	CD45RA	BUV563
CD56	APC	CD197	BUV711
CD20	BV750	CD127	APC-R700
CD24	PE	CD25	PE-CF594
CD27	BV421	CD194	BV480
IgD	PE-CF594	CD28	BV650
CD38	BB700	CCR6/CD196	APC
CD11C	BUV661	CXCR3/CD183	PE-CY7
CD123	PE-CY5	CD45RO	BUV805

After 15 minutes of incubation with those antibodies at room temperature and dark conditions, a lysing wash was done with Lysis Buffer 1X (BD et al.) to eliminate the red blood cells. The pellet was resuspended in 200µl of Staining Buffer (2% FBS, 48% PBS 1X) and acquired using the cytometer BD Symphony (BD et al.) Data were analyzed using FACSDiva v 6.1.3 (BD) software. BD Bioscience, Milan, provided all the Abs.

3.4 Flow cytometry analysis

A panel of well-known membrane markers for the lymphocyte subpopulations was prepared. The gating strategy and the evaluation of non-specific fluorescence were determined by fluorescence minus one (FMO) control combined with the appropriate isotype control. 70% of power lasers were used, and the threshold was set up on FSC at 25000. Acquisition of the samples was performed by BD FACSymphony™ A5, a powerful machine equipped with five lasers, able to detect up to 30 colors. Final analyses were performed by using FACSDiva™ Software (version 9.0.2).

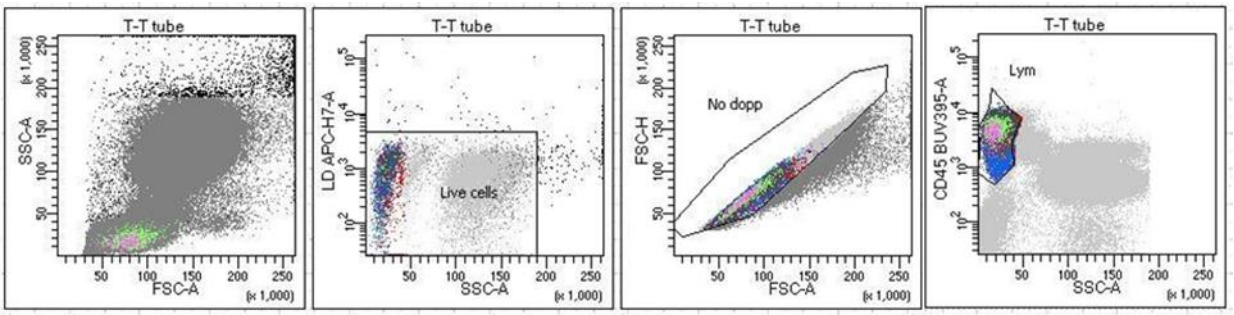


Figure 11, . Lymphocyte gating strategy. To properly select the events conducible to lymphocytes, we used four plots. From left to right: FSC-A vs. SSC-A as first, SSC-A vs. LD-APC-H7-A to gate the live cells, FSC-A vs. FSC-H was used to remove the duplets, and finally, the lymphocytes were gated in the plot SSC-A vs. CD45-BUV395-A bright.

The Lymphocyte gating strategy is illustrated in Figure 11 above. We gated the lymphocytes in both panels using the forward scatter area (FSC-A) and the side scatter area (SSC-A). Then, we excluded the death cells through SSC-A and LD-APC-H7-A plots, and finally, we removed doublets with the parameters FSC-A and FSC-H (forward scatter height). We looked at the CD45 expression in the plot CD45-BUV395-A vs. SSC-A to further select lymphocytes. Starting from this last population, we identified all the related subpopulations.

3.4.1 NK/B panel gating strategy

To isolate B-cells from the CD45 bright ones, a CD19-BV480-A vs CD3-BV786-A plot was created. Under this, B subpopulations were identified as follows:

- Class-switched memory
- Switched memory
- Naïve

- Transitional
- Memory
- Plasmoblasts
- Plasmacells

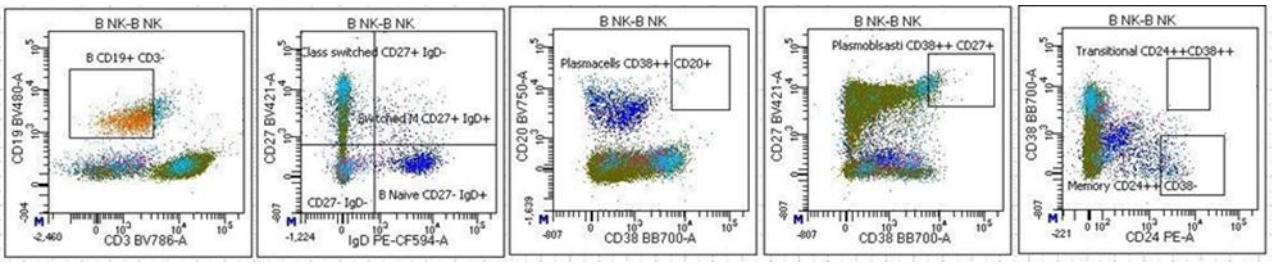
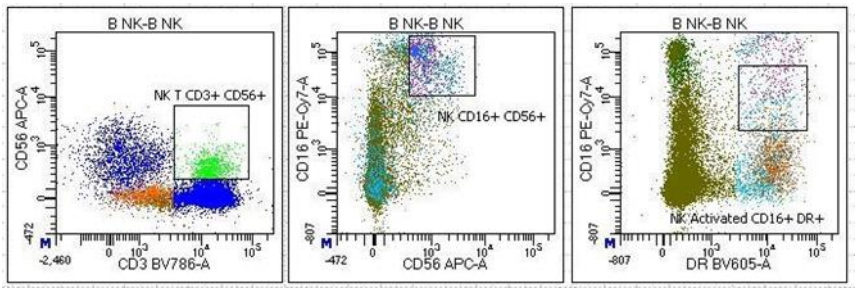


Figure 12 B-cell subpopulations. From left to right, we have CD3-BV786-A vs CD19-BV480-A. We isolate and divide class-switched, switched, and naïve B cells from this gate through the plot IgD PE-CF594-A vs CD27- BV421-A. Plasma cells can be found by crossing CD38-BB700-A and CD20-BV750-A, whereas Plasmoblasts result from the CD38-BB700-A vs. CD27-BV421-A plots. Transitional B cells are the result of CD24-PE-A vs CD38- BB700-A.

cells are CD19+/CD27+/IgD+. In the same plot, we find B naïve cells as CD19+/CD27-/IgD+. Plasma cells and Plasmoblasts are CD30 bright/CD20+ and CD38 bright/CD27+, respectively. Memory B cells are CD24 bright/CD38- and transitional B cells are CD24 bright/CD38 bright.



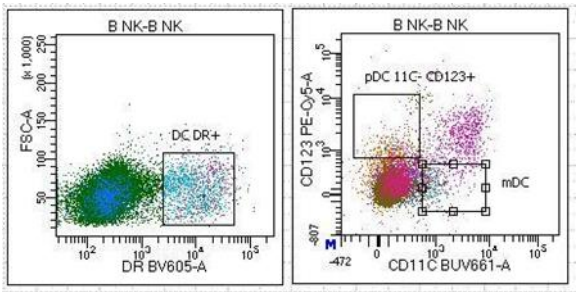


Figure 13 NK and Dendritic cells (DCs). NK-T cells (CD3+/CD56+) result from CD3-BV768-A and CD56-APC-A plots. From those events, NK-activated cells can be isolated as CD16+/HLA-DR+ by crossing HLA-DR- BV605-A and CD16-PE-Cy7-A. By using only HLA-DR-BV605-A and FSC-A, we can isolate DCs (HLA-DR+). From them, myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) can be found by crossing CD11C-BUV661-A and CD132-PE-Cy5-A.

NK and Dendritic cells (DCs) gating strategy is illustrated in Figure 13 above. NK-T cells were isolated by CD3+ and CD56+ markers. An inverted gate comprehending NOT (CD3-/CD19-) was used to isolate NK cells and then activate the NK. NK cells resulted in CD16+/CD56+. From that gate, NK-activated cells were also isolated as CD16+/CD56+/HLA-DR+.

Also, DCs were found under the NOT CD3+/NOT CD19+ plot and HLA-DR+ ones. Finally, NOT (CD16+/CD56+) and DC-HLA-DR+ plot was used to identify myeloid DCs (mDCs) and plasmacytoid DCs (pDCs), respectively, as HLA-DR+/CD123+/CD11C+ and HLA- DR+/CD123+/CD11C-.

Figure 14 shows the whole B and NK lymphocyte ladder, representing the abovementioned panels.

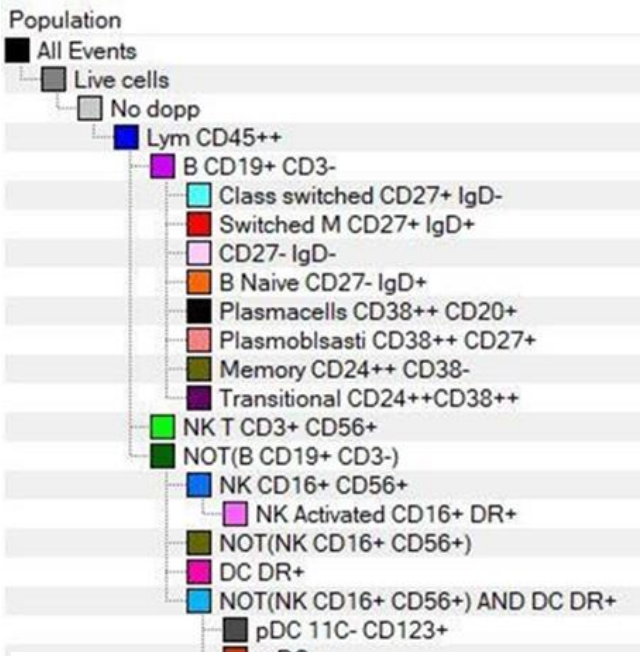


Figure 14 B lymphocytes panel hierarchy. This family tree shows the relationship between each plot and how the different populations were isolated.

3.4.2 T panel gating strategy

Starting from the plot CD45-BUV395-A vs SSC-A, T cells were divided into CD8+ and CD4+. From the CD8+ gate, we were able to distinguish the following:

- Naïve cells
- Central memory (CM) cells
- Effector memory (EM) cells
- Terminally differentiated effector memory (TEMRA) cells

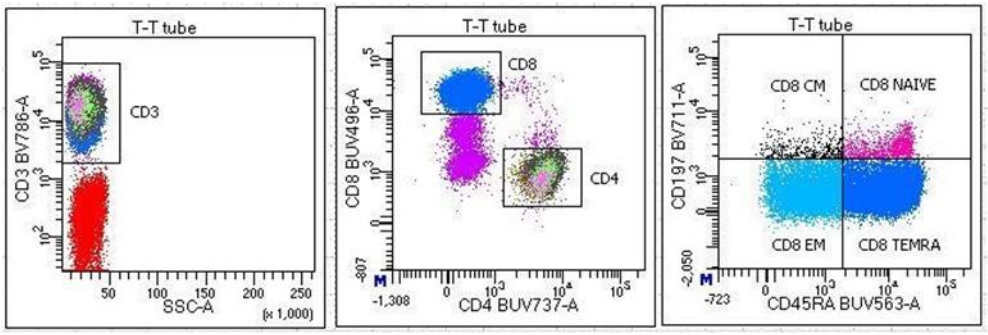


Figure 15 Isolation of CD8+ and CD4+ events and subsequent CD8+ subpopulations identification. SSC-A vs CD3-BV786-A was used to isolate CD3+ events. CD4-BUV737-1 vs CD8-BUV496-A plot identifies CD4+ and CD8+ events. CD45RA-BUV563-A vs CD197-BV711-A allows us to isolate CD8+ T lymphocyte subpopulations.

Starting from the CD3+ gate in the plot SSC-A vs CD3-BV786-A, we isolated both CD8+ and CD4+ events. Those were distinguishable in the plot CD4-BUV737-1 vs CD8-BUV496-A. Finally, by crossing CD45RA-BUV563-A and CD197-BV711-A, we distinguished CD8+ CM (CD45RA-/CD197+), CD8+ Naïve (CD45RA+/CD197+), CD8+ EM (CD45RA-/CD197-) and CD8+ TEMRA (CD45RA+/CD197-).

From CD4+ gate, we isolated:

- Naïve cells
- Central memory (CM) cells
- Effector memory (EM) cells
- Terminally differentiated effector memory (TEMRA) cells
- T regulatory cells (Tregs), both memory and naïve
- Memory cells
- T helper cells

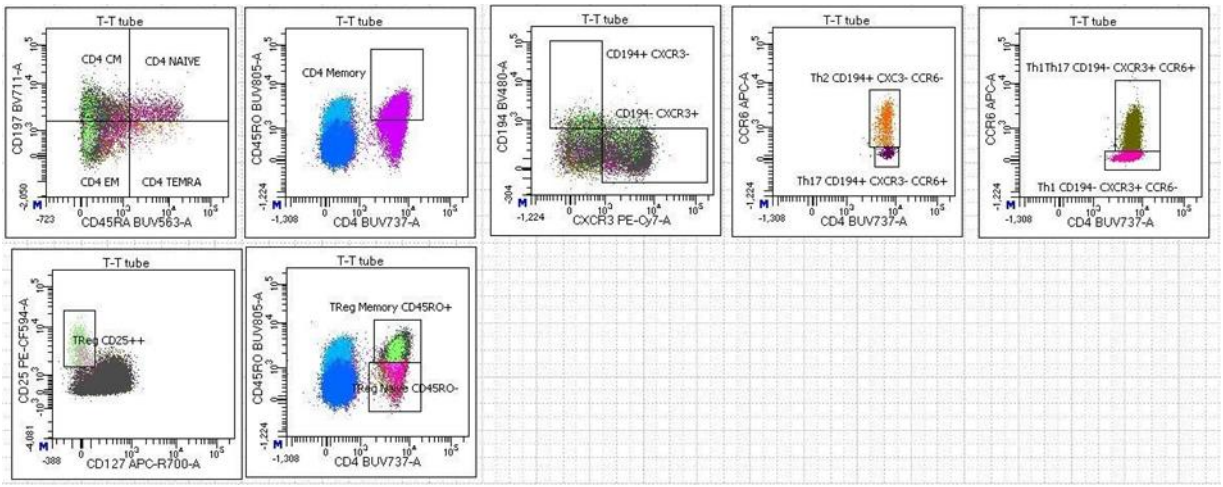


Figure 16 CD4+ T lymphocytes and Tregs. From left to right: CD45RA-BUV-563-A vs. CD197-BV711-A to subdivide the events into CD4+ CM, Naïve, ER, and TEMRA. CD4-BUV737-A vs CD45RO-BUV805-A to find the CD4 memory T cells. CD4-BUV737-A vs CCR6-APC-A to distinguish T Helper subtypes. CD127-APC-R700-A vs. CD25-PE-CF594-A to isolate Tregs. CD4-BUV737-A vs CD45RO for the TReg memory and TReg naïve.

The previous panels were set to isolate CD4+ T lymphocyte subpopulations. As first, CD45RA-BUV-563-A vs CD197-BV711-A plot was created from CD4+ events to distinguish the four main CD4+ subtypes: CD4+ CM (CD45RA-/CD197+), CD4+ Naïve (CD45RA+/CD197+), CD4+ EM (CD45RA-/CD197-) and CD4+ TEMRA (CD45RA+/CD197-).

To isolate CD4+ Memory cells (CD4+/CD45RO+), we crossed CD4-BUV737-A and CD45RO-BUV805-A.

From CD4+ events, we created a CXCR3-PE-Cy7-A vs. CD194-BV480-A plot to isolate T helper cells. Th17 (CD194+/CXCR3-/CCR6-) and Th2 (CD194+/CXCR3-/CCR6+) were found under the CXCR3-/CD194+ gate, whereas Th1Th17 (CD194-/CXCR3+/CCR6-) and Th1 (CD194-/CXCR3+/CCR6-) under the CXCR3+/CD194- gate.

Tregs (CD25 bright) were found in the plot CD127-APC-R700-A vs CD25-PE-CF594-A. Treg memory (CD25++/CD45RO+) and Treg naïve (CD25++/CD45RO-) cells can be distinguished from those events.

Figure 17 shows the whole ladder of T lymphocytes, representative of the panels described above.

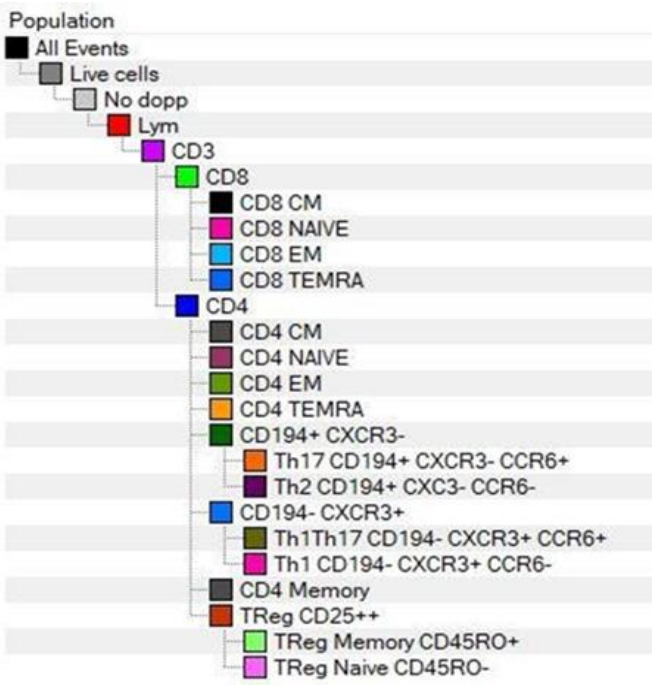


Figure 17 T lymphocytes panel hierarchy. This family tree shows the relationship between each plot and how the different populations were isolated.

3.5 Statistical analysis

The raw data were obtained as FSC files from BD FACSDiva™ Software and analyzed on FlowJo 10.9.0 Ashland, Oregon, FlowJo LLC, a subsidiary of Becton Dickinson. The baseline characteristics of the study participants were described as categorical variables and presented as values and percentages. data were summarized using the median and interquartile range (IQR). To compare continuous variables between two groups, the non-parametric Mann-Whitney U test was employed. For comparisons involving more than two groups, the Kruskal-Walli's test was used. Statistical significance was determined using a two-sided p-value threshold of less than 0.05 ($\alpha = 0.05$), indicating statistical significance with a confidence level of 95%. The association between the study variables and the pathological complete response (pCR) outcome, lymph node status, and primary tumor side were analyzed. The statistical analyses were performed in GraphPad Prism version 8.

4. RESULT

4.1 Characteristics of the patients

Patients' characteristics are summarized in Table 3. The table presents the characteristics of patients in the study, specifically focusing on **Early Triple-Negative Breast Cancer (TNBC)** and **Metastatic TNBC**. The table provides patient characteristics, including age, body mass index (BMI), family history, menopausal status, node involvement, primary tumor classification, pathological complete response (pCR), and histological grade.

For the **Early TNBC group** (consisting of 20 patients), the median age was 54 years (with a range of 36-77 years), and the average BMI was 24.28 (ranging from 18.37 to 33.65). Among these patients, 10 (50%) had a family history of cancer, while 10 (50%) did not. Regarding menopausal status, 7 (35%) were premenopausal, and 13 (65%) were postmenopausal. In terms of node involvement, 12 patients (60%) were in N0 lymph nodes, with 1 (5%) classified as N0-1, 4 (20%) as N1, 2 (10%) as N2, and 1 (5%) was not available. Additionally, 4 patients (20%) had a primary tumor classified as T1, 8 (40%) as T2, 4(20%) as T3, and 2(10%) as T4, 1(5%) was not available. Out of the 20 patients, 8 (40%) achieved a pathological complete response (pCR), with 5 (25%) not having a pCR and 7 (35%) were not available. The histological grade of the tumors was available, with 4 (20%) classified as G2 and 14 (70%) as G3, also 2(10%) were not available.

For the **Metastatic TNBC group** (consisting of 4 patients), the median age was 65 years (with a range of 47-68 years), and the average BMI was 22.24 (ranging from 20.9 to 24.55). Among these patients, 3 (75%) had a family history of cancer, while 1 (25%) did not. One patient (25%) was premenopausal, and 3 (75%) were postmenopausal. One patient (25%) was in N0, while 1 (25%) was in N1, remaining 2 (50%) patients were in N2. Tumor classification for this group included 0 patients classified as T1, 1 (25%) as T2, 2(50%) as T3, and 1 (25%) as T4. No patient in this group achieved a pCR. The histological grade of the tumors was available for 3 patients, with 3 (75%) classified as G3, 0 as G2, and 1(25%) not available. These findings provide an overview of the demographic and clinical characteristics of patients in the Early TNBC and Metastatic TNBC groups, offering insights into their age, BMI, family history, menopausal status, lymph node involvement, tumor classification, pathological response, and historical grade.

Table 3, Characteristics of the Patients

Characteristic	**Early TNBC n:20**	**Metastatic TNBC n:4**
Age		
Median(Range) _ yr.	54(36-77)	65(47-68)
BMI	24.28(18.37-33.65)	22.24(20.9-24.55)
Family History _ no (%)		
Yes	10(50%)	3(75%)
No	10(50%)	1(25%)
Menopausal Status *_ no (%)*		
Premenopausal	7(35%)	1(25%)
Postmenopausal	13(65%)	3(75%)
Node involvement *_ no(%)*		
N0	12(60%)	1(25%)
N0-1	1(5%)	
N1	4(20%)	1(25%)
N2	2(10%)	2(50%)
Not Available	1(5%)	0
Primary tumor classification *_ no(%)*		
T1	4(20%)	0
T2	8(40%)	1(25%)
T3	4(20%)	2(50%)
T4	2(10%)	1(25%)
Not Available	1(5%)	0
pCR *_*		
Yes	8(40%)	
No	5(25%)	
Not Available	7(35%)	
Historical grade _		
G2	4(20%)	0
G3	14(70%)	3(75%)
Not Available	2(10%)	1(25%)

4.2 The frequency of circulation immune cells in TNBC in Our cohort

In Table 4, T cells and their subset frequencies in our TNBC patients on CD8+ and CD4+ cells:

Table 4, T cells and their subsets in our TNBC patients. Black numbers denote normal ranges while red numbers denote frequencies out of normal range.

	on CD3		on CD4											on CD8			
	%CD4	%CD8	% CM	% NAIVE	%EM	% TEMRA	% CD4 memory	%Treg	% Treg Memory	% Th1	% Th1Th17	% Th2	% Th17	% CM	% EM	% NAIVE	% TEMRA
IRIS01	65.5	27.8	23.9	35.3	31.2	9.49	43.4	4.21	2.86	17.2	7.75	8	5.03	3.31	8.43	22.2	66.1
IRIS02	76.5	19.5	29.5	21.5	40.6	8.43	84.3	3.66	3.02	21	13.4	4.42	5.42	26.9	35.9	23.1	14.2
IRIS03	75.2	20.8	25.6	33.7	34.5	6.13	21.6	3.39	1.44	21.4	11.9	8.48	3.12	2.22	31.2	11.7	54.9
IRIS04	60.4	32	20.6	42	29.7	7.68	1.2	4.48	0.94	26.3	8.95	3.74	1.74	3.03	28.2	3.79	65
IRIS05	53.1	40.6	34.7	29.3	28.7	7.33	27.3	4.35	1.99	22.5	17.4	2.98	4.6	4.87	30.5	11.4	53.2
IRIS06	59.2	33.4	21.5	56.1	13.9	8.47	24.4	6.43	2.13	18.4	8.62	2.38	2.01	2.56	27.9	26.9	42.6
IRIS07	36.2	40.9	30.8	34.8	25	9.37	18.3	2.08	0.98	16.1	14.9	1.97	2.26	3.55	53.3	10.7	32.4
IRIS08	61.3	30.3	25.7	43	22.6	8.7	6.14	2.97	0.74	14.4	11.4	3.08	5.13	3.22	35.3	23.7	37.7
IRIS09	72	25.3	30	24.2	38.9	6.89	51.3	1.61	1.05	21.1	6.72	3.9	1.4	11.1	28.7	11	49.2
IRIS10	65.2	30.8	13.6	54.5	24.3	7.63	30	3.85	2.54	29.9	5.4	3.8	2.98	2.52	15.7	14.1	67.7
IRIS11	62.1	32.5	13.9	59.9	21.8	4.42	5.68	3.63	1.72	17.5	11.7	1.66	1.26	1.63	36.3	52.4	9.64
IRIS12	48.1	48	34	22.3	37.6	6.09	27.5	3.87	1.72	25.8	10.2	5.89	4.06	3.7	33.7	6.53	56.1
IRIS13	66.8	28.9	32.6	30.8	30.2	6.36	35.4	2.77	1.77	29	8.85	2.65	1.63	27.9	31.4	19.8	20.8
IRIS14	66.3	24.4	23.6	32.9	35.4	8.15	22.9	2.23	1.18	26.3	8.85	3.59	2.2	5.68	38.6	14.7	41
IRIS15	55.5	35	24.8	31.9	34.5	8.82	4.63	4.64	0.77	23.3	12.8	3.62	1.31	8.55	32.3	38	21.1
IRIS16	52.5	39	37.5	46.3	15.1	1.04	0.81	4.78	0.25	21.7	2.06	2.8	0.59	4.9	21.9	48.2	25.1
IRIS17	61.9	32.1	22.4	36.9	32.9	7.88	6.76	4.03	1.47	28.4	12.6	3.19	2.25	2.4	21.4	16.4	59.8
IRIS18	60.3	33.7	18.6	53.9	22.7	4.87	13	5.06	2.33	24.4	6.08	3.51	2.04	3.47	17.5	25.3	53.7
IRIS19	74.7	18.8	24.3	53.1	17.6	5	24.8	6.1	3.13	20	4.74	3.65	2.5	15.6	33.4	24.6	26.5
IRIS20	60.9	35.6	23	48.7	25.9	2.35	20.4	3.59	1.98	30.7	6.22	3.25	1.36	3.91	15.1	54.7	26.4
Median	61.6	32.05	24.6	36.1	29.2	7.48	22.25	3.86	1.72	22.1	8.9	3.55	2.225	3.625	30.85	21	41.8

We compared our Cohort with a group of European FOCIS Centers of Excellence adapted panels of the Human Immunophenotyping Consortium (HIPC) for whole blood analysis, they presented their findings on the absolute and relative frequencies of the leukocyte subpopulations in the peripheral blood of more than 300 healthy volunteers across six different European centers. In our Cohort,

In our cohort the median of Treg memory% which was 1.72 demonstrated lower circulating at the baseline compared to the normal range (Normal Range: 2-11 Median: 4)%.In Tables 5 and 6, B cells, NK cells, and Dendritic cells and their subset frequencies in our TNBC patients:

Table 5, B cells and their subsets in our TNBC patients. Black numbers denote normal ranges while red numbers denote frequencies out of normal range.

	on CD19							
	% Class Switched memory	% Switched memory	% Naive	% Plasmacells	% Plasmoblasts	% Memory	% Transitional	Memory CD27-IgD-
IRIS01	15	2.3	76.5	7.5	0.37	10.6	2.58	6.19
IRIS02	24.6	7.87	57	1.77	0.45	15.8	2.47	10.5
IRIS03	15.8	3.26	73.1	4.89	0.48	10.8	1.09	7.82
IRIS04	55.6	9.45	24.6	7.19	0.82	23	3.29	10.3
IRIS05	38.5	14.2	43.7	10.4	3.07	5.91	3.78	3.55
IRIS06	15.8	2.4	79.4	12.4	3.67	4.65	1.83	2.4
IRIS07	6.39	3.6	83.2	3.2	0.4	5.59	1.07	6.79
IRIS08	18.2	5.48	68.2	3.32	0.8	10.5	0.98	8.18
IRIS09	15.3	3.15	73.5	3.23	0.25	9.98	0.028	8.04
IRIS10	14.5	2.4	77.1	7.58	1	11.3	0.67	5.96
IRIS11	33.1	16.8	42.5	5.41	0.94	16.6	1.65	7.65
IRIS12	13.9	30.1	49.7	2.73	1.31	31.6	0.94	6.32
IRIS13	11	1.81	79.2	3.91	0.16	12.5	1.95	8
IRIS14	21.3	19.2	48.8	5.18	0.66	11.4	0.83	10.6
IRIS15	21	5.34	45.1	9.73	0.54	24.5	1.25	28.6
IRIS16	28.7	13.2	49.8	4.5	1.84	16	1.97	8.28
IRIS17	5.01	3.77	82.8	3.92	0.65	9.55	1.36	8.41
IRIS18	8.5	3.49	82.3	7.97	1.43	6.44	2.08	5.69
IRIS19	11.7	2.68	75.7	20.3	3.53	3.41	6.33	9.98
IRIS20	23.3	5.18	54.6	6.46	1.25	10.7	0.8	17
Median	15.8	4.475	70.65	5.295	0.81	10.75	1.505	8.02

Upon the same referencing standard literature, we conducted a comparative analysis of our B cells, NK cells, and dendritic cells, and the median of circulating %NK in our cohort was below the normal range. (Normal Range: 2-55, Median:12)

Table 6, NK cells, Dendritic cells, and their subsets in our TNBC patients. Black numbers denote normal ranges while red numbers denote frequencies out of normal range.

	Lym CD45+	Lym CD45	(DR+ CD3-CD19-CD16-CD56-)	
	NK-T	NK	pDC	mDC
IRIS01	7.48	0.17	10.8	60.4
IRIS02	0.68	0.14	0.32	65.6
IRIS03	3.8	0.51	9.26	32.4
IRIS04	6.45	1.85	8	11.2
IRIS05	9.32	0.77	5.41	11.3
IRIS06	5.89	0.93	4.68	27.5
IRIS07	2.45	0.55	12.7	4.23
IRIS08	2.71	0.27	37.5	13.4
IRIS09	1.98	0.48	0.81	36.6
IRIS10	2.34	0.15	5.1	19.4
IRIS11	4.23	0.67	1.69	23.7
IRIS12	1.63	0.39	0.92	45.2
IRIS13	1.47	0.76	49.8	3.2
IRIS14	2.85	0.14	2.88	8.65
IRIS15	1.53	0.24	14.1	3.36
IRIS16	3.31	0.23	5.49	8.47
IRIS17	6.97	0.59	1.79	71.9
IRIS18	3.36	0.47	0.65	66.8
IRIS19	1.84	1.55	0.91	27.7
IRIS20	1.66	0.13	2.25	45.9
Median	2.78	0.475	4.89	25.6

4.3 Analysis of circulating immune cells in early TNBC patients at baseline and pathologic complete Response (pCR)

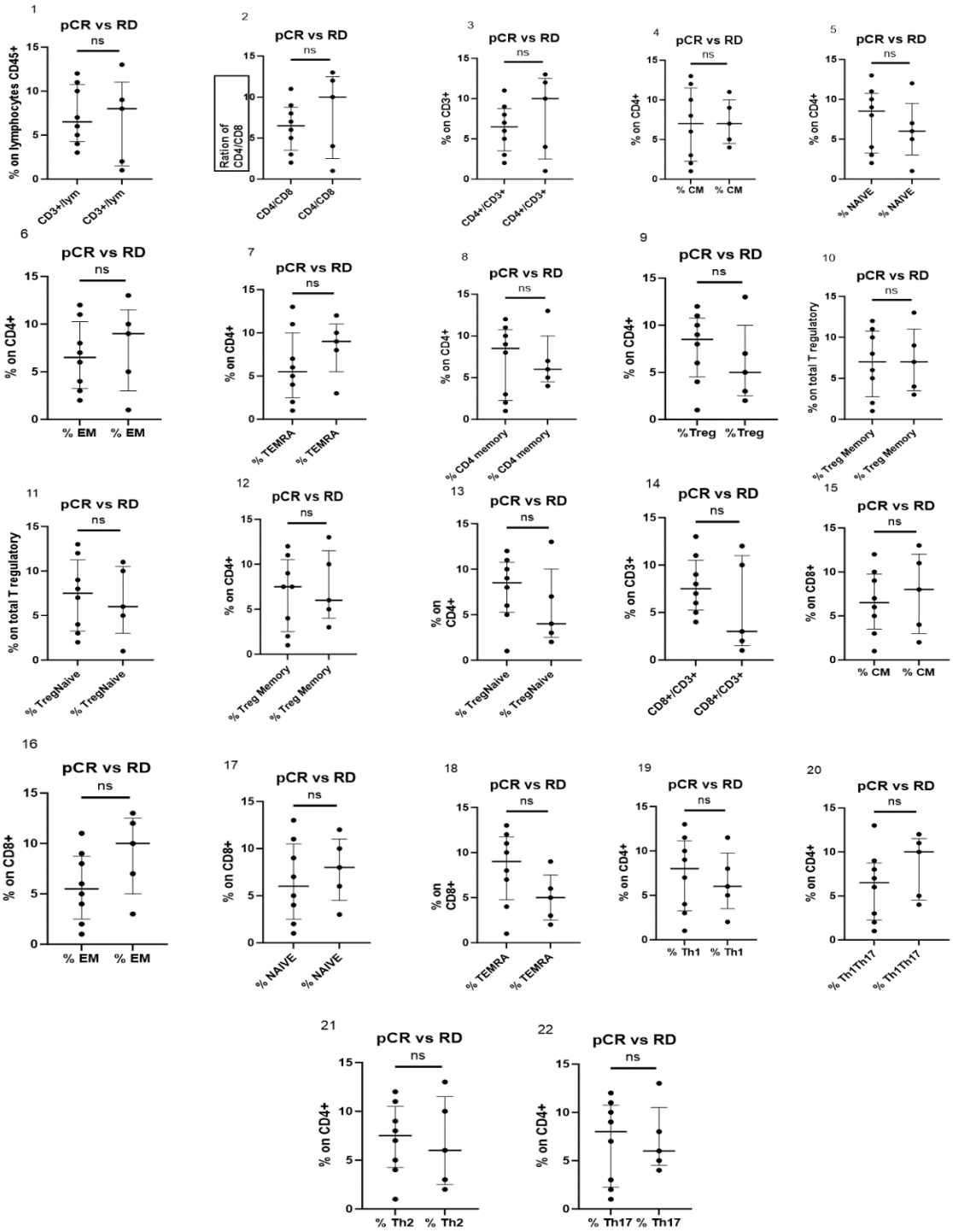


Figure 10 (A) , Analysis of T Lymphocyte Profiles in Early TNBC Patients Achieving Pathological Complete Response (pCR) Compared to Those Without pCR.

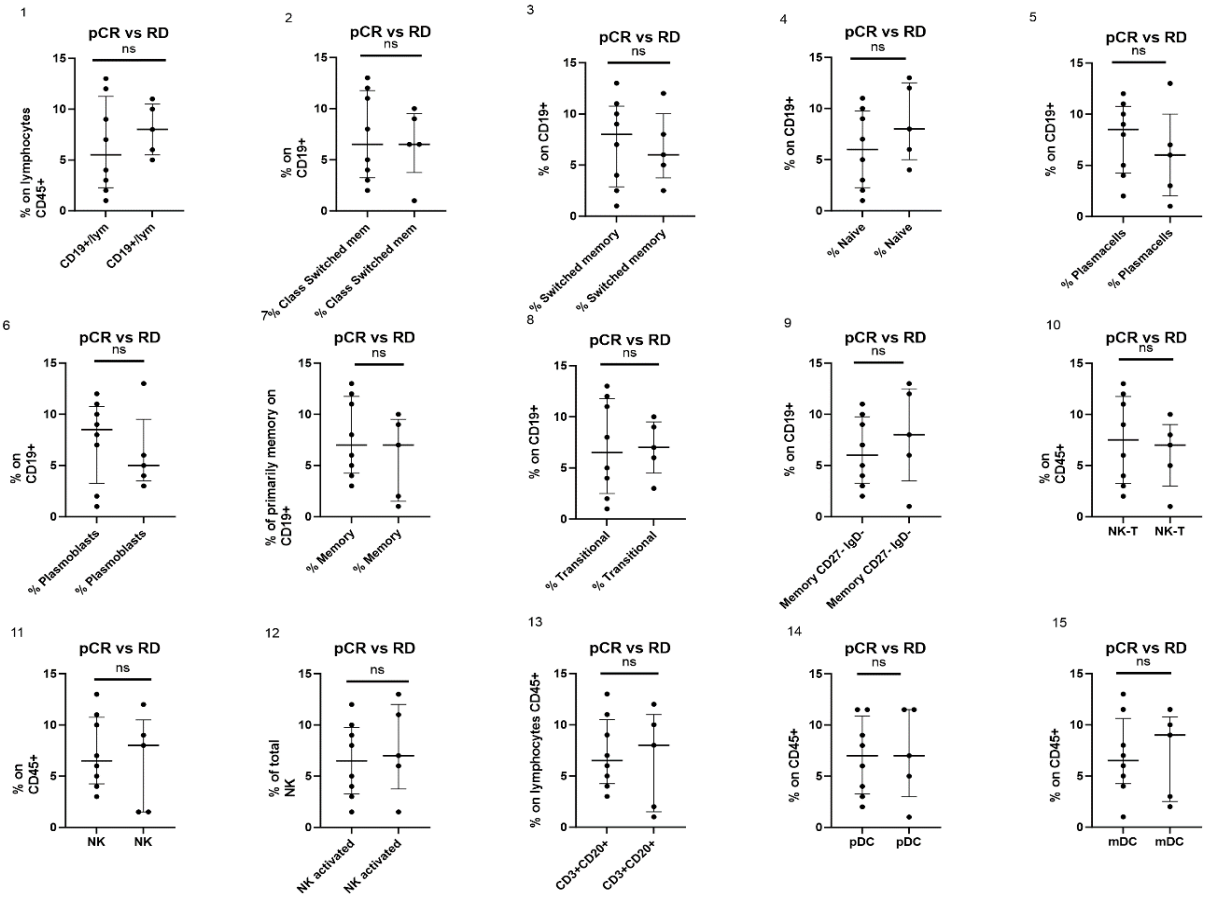


Figure 10 (B), Analysis of B Lymphocyte and NK and Dendritic cell Profiles in Early TNBC Patients Achieving Pathological Complete Response (pCR) Compared to Those Without pCR. pDC and mDC stand for plasmacytoid dendritic cells and myeloid dendritic cells, respectively.*

Our investigation focused on exploring the relationship between pathologic complete response (pCR) and T cell subsets and B cell subsets in the peripheral blood of 13 patients with early triple-negative breast cancer (TNBC). (8 pCR vs 5 no pCR) Our findings, as shown in **Figure 10(A)**, revealed that there was no significant relation between pCR and baseline circulating immune cells, which belong to the T-cell category.

We base our research and analysis on a clinical perspective, and according to **Figure 10(B)**, our findings suggest that there was no relation between pathologic complete response (pCR) and other circulating immune cell subtypes including B cells, NK cells, and dendritic cells.

4.4 Analysis of circulating Immune cells in early TNBC patients at baseline and Node Involvement status

Our findings, displayed in **Figure 11(A)**, showed a relation between the presence of cancer cells in the regional lymph nodes and circulating T cell subsets for 18 patients(12 N0 and 6 N1, N2). Graph number 6* demonstrated that CD4+ Effector Memory (EM) cells were significantly higher in the node involvement group (N1, N2) when compared to the node negativity group(N0) *(P-value: 0.0336* –

Range in N0: 13.90-35.40 _ Median:24.65 / Range in N1, N2: 25.90-40.60_ Median:32.85). also, in Graph number 21* appears that T helper 2 (Th2) cells were significantly higher in the node involvement group (N1, N2) when compared to the node negativity group(N0) groups *(p-value: 0.0032 _ Range in N0: 1.660-3.800 _ Median: 3.135/ Range in N1, N2: 3.250-8.480 _ Median: 5.155).*

Figure 11(B) shows the relation between the presence of cancer cells in the regional lymph nodes and the subsets of B cell, NK, and Dendritic cells circulating immune cells. Results indicated that there is no significant difference between node involvement and node negativity groups.

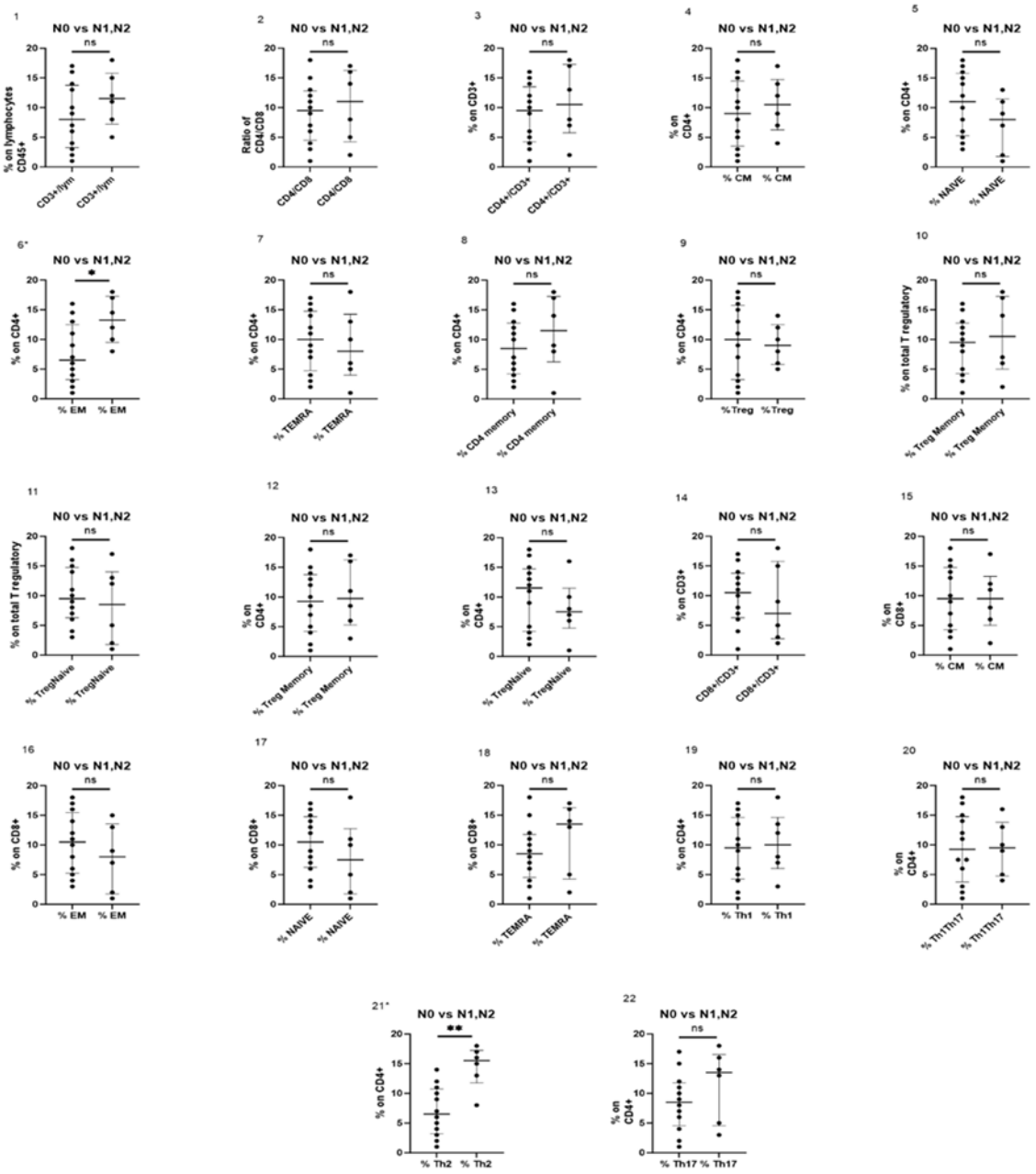


Figure 11 (A), T Lymphocyte Profiles in Early TNBC Patients Compared to Node Involvement stages N0 vs N1 and N2 positivity. THE P VALUE FOR 6 was 0.0336<0.05/ AND 21*was 0.0032<0.05.*

A low p-value (<0.05) indicates an unlikely chance of occurrence under the null hypothesis, leading to its rejection in favour of the alternative hypothesis.

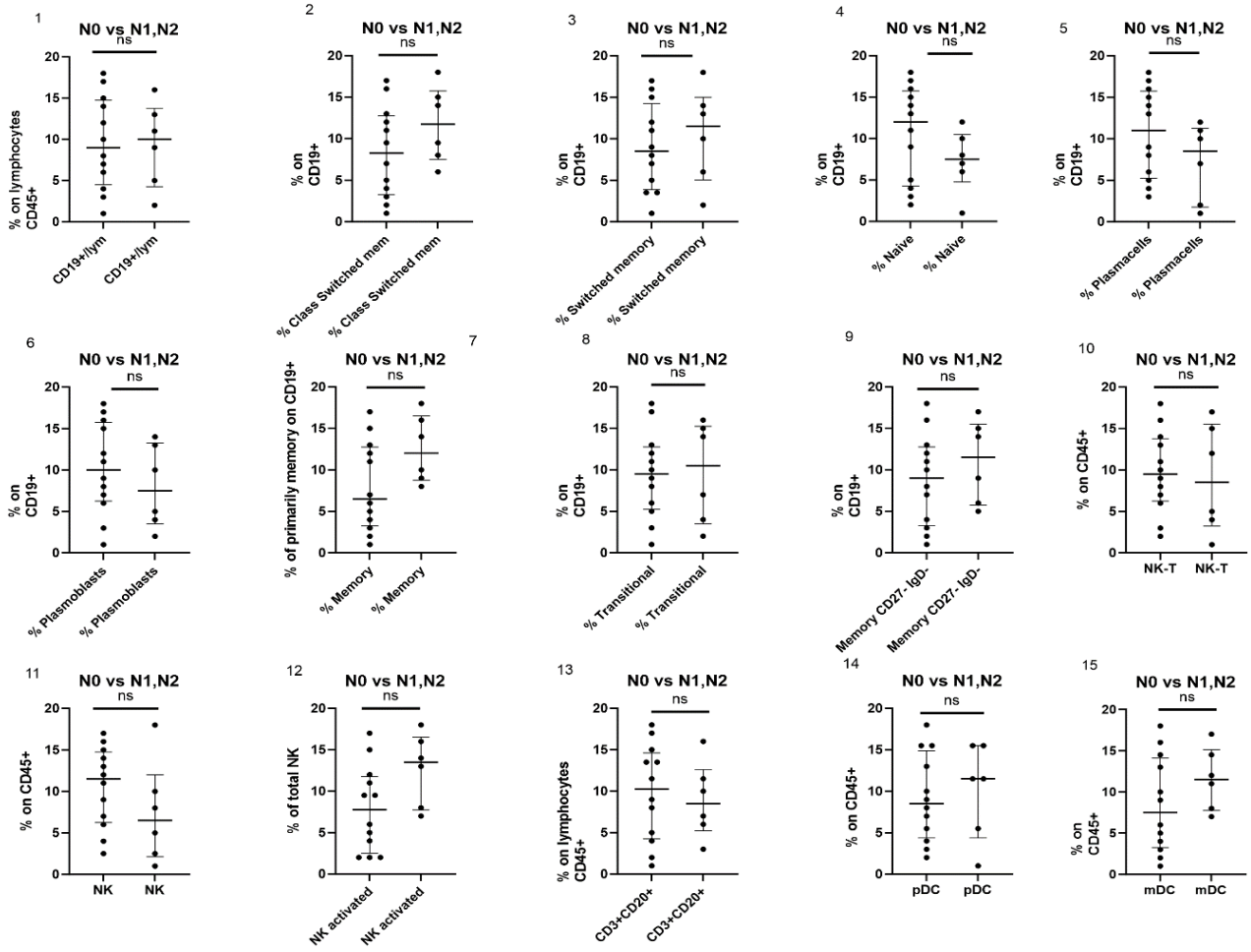


*Figure 11 (B), B Lymphocyte, NK cells and Dendritic cell Profiles in early TNBC Patients Compared to Node Involvement. (N0 vs N1 and N2), *pDC and mDC stand for plasmacytoid dendritic cells and myeloid dendritic cells, respectively.*

4.5 Analysis of Circulating Immune Cells in early TNBC patients and Tumor size (T1, T2, T3)

We analyzed baseline circulating T lymphocytes and found some significant differences among T1, T2, and T3 stages, for 16 patients (4 in T1, 8 in T2, and 4 in T3). as shown in **Figure 12(A)**, We used a non-parametric one-way ANOVA test "Kruskall-Wallis" to analyze the data. Graph 7* indicates that there is a significant increase in CD4+ Terminally differentiated Effector Memory T cells (TEMRA) cells in the T2 stage compared to T3 *(P-value: 0.0025, Range in T2: 5000-9.490, Mean in T2: 8.016)*. Similarly, Graph 17* shows that CD8+ Naive cells are significantly higher in the T3 stage compared to Naive T1 *(P-value: 0.0208, Range in T3: 19.80-54.70, Mean: 43.78)*. In Graph 18*, we see a significant difference between T1 and T3 stages in CD8+ Terminally differentiated Effector Memory T cells (TEMRA) cells *(P-value:0.0076, Range in T1: 53.70-65.00, Mean: 57.43)*. Graph 21* demonstrates that CD4+ T helper2 (Th2) cells have a significant difference between T3 vs T1 and T2 and are lower than them *(P-value: 0.0319, Range in T3: 1.660-3.250, and Mean: 2.590)*. Finally, in Graph 22*, we show a significant difference between CD4+ T helper17 (Th17) T3 and T2 status. *(P-value: 0.0155, Range in T3: 0.5900-1.630, and Mean: 1.210)*. No other significant differences were observed between the immune cells and T1, T2, and T3. The relationship between the size and extent of the primary tumor and the presence or activity of immune cells in the bloodstream is examined through the relation between T

staging and circulating immune cells. The purpose of this study is to identify differences in immune cell profiles and functional responses based on tumor size.

Figure 12(B) reveals the relation between B cells, NK cells, and Dendritic cell profiles among T1, T2, and T3 status. Our findings show a relation or significant differences in these profiles.

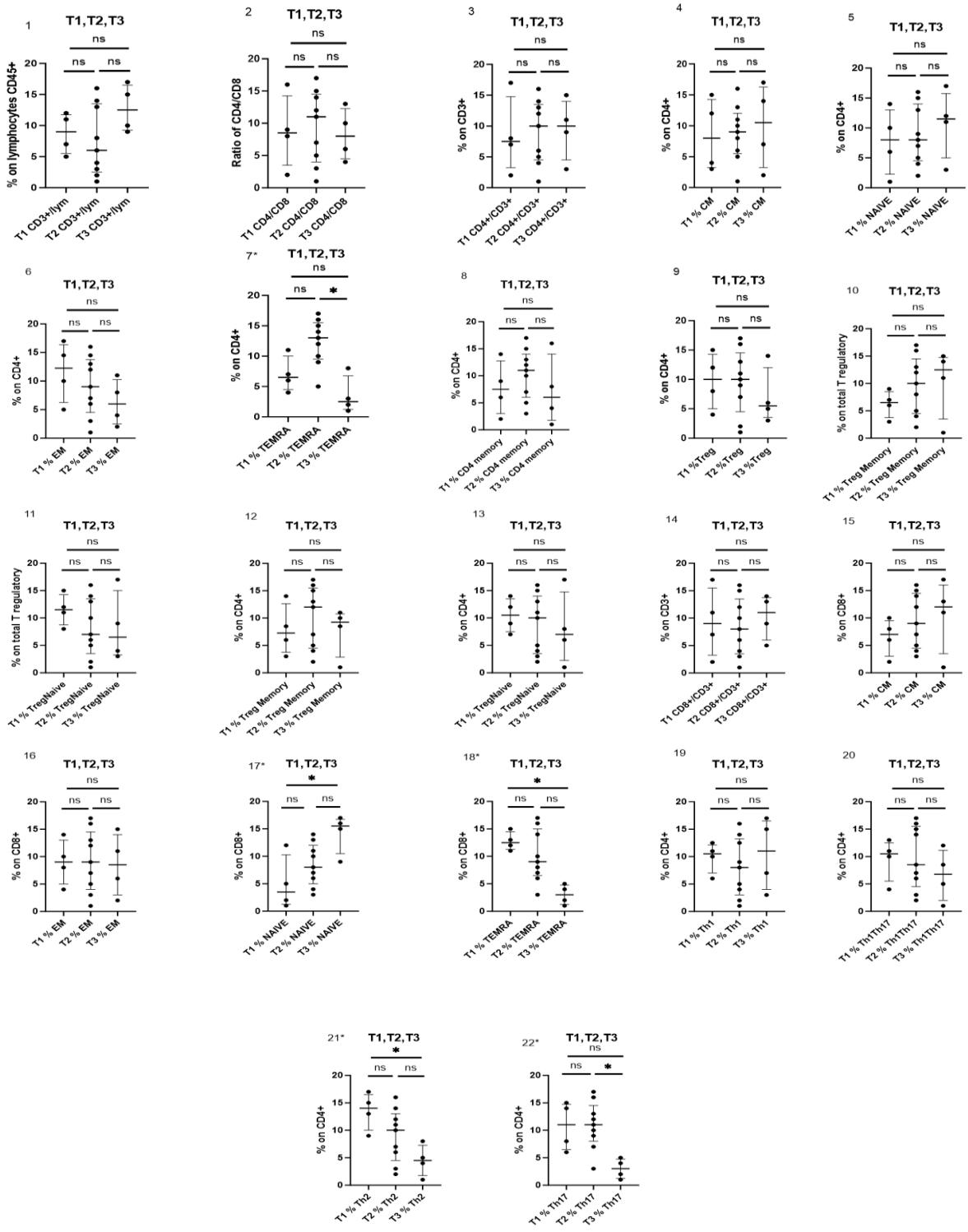


Figure 12 (A), T Lymphocyte Profiles in early TNBC Patients Compared in T status. (T1 vs T2 and T3).

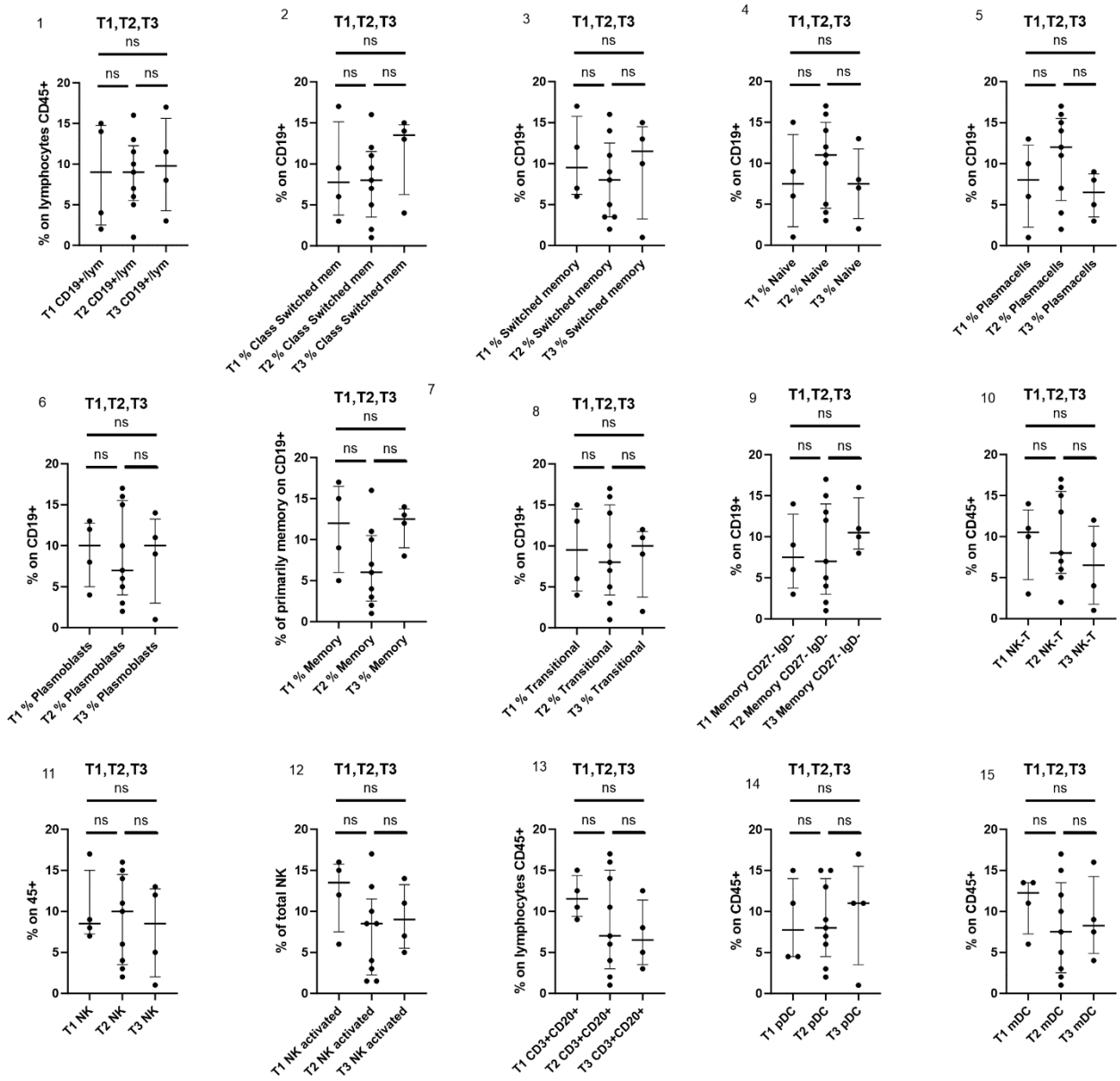


Figure 12(B), B Lymphocytes, NK, and Dendritic Profiles in early TNBC Patients Compared in T status. (T1 vs T2 and T3).

5. Discussion

Breast cancer is the most diagnosed type and the leading cause of death for cancer in women worldwide[3]. Triple-negative breast Cancer(TNBC) is a subtype of breast cancer characterized by the absence of estrogen, progesterone, and human epidermal growth factor receptor 2. It accounts for more than 15-20% of all breast cancers and is a focus of research due to its therapeutic challenges and highly invasive behavior. TNBC is difficult to treat as it has low responsiveness to available therapies. Conventional treatments are typically used since specific treatment options for TNBC are not widely available, but these often result in relapse[66]. Although the mortality rate of metastatic triple-negative breast cancer (mTNBC) has decreased over time due to improved early detection rates and the use of immune checkpoint inhibitor (ICI) therapies as the standard treatment, these therapies have also demonstrated remarkable transformative effects in other cancer types, like advanced melanoma[67], and NSCLC[68], but the same transformative effect has not been observed in TNBC[69].

In clinical settings, individuals with metastatic triple-negative breast cancer (mTNBC) who test positive for PD-L1 are commonly treated using a combination therapy involving two drugs: Nab-Paclitaxel and Atezolizumab. Atezolizumab is a monoclonal antibody that specifically targets PD-L1, a protein involved in immune regulation. In normal circumstances, the interaction between PD-1 and PD-L1 helps maintain the balance of the immune system by limiting the proliferation of T-cells, thereby preventing autoimmune reactions or excessive immune responses. However, in the context of tumor development, this interaction becomes a key mechanism for the tumor to evade the immune system[70]. In TNBC patients, PD-L1 is primarily expressed on tumor-infiltrating immune cells, which can block anticancer immune responses[71]. Inhibiting PD-1 and PD-L1 could be an effective treatment option. Tumor cells express PD-L1 on their surfaces, soluble forms, and exosomes. This last component contributes to anti-PD-L1 treatment resistance by attaching to antibodies and lowering their efficacy. Clinical trials are looking into the safety and efficacy of PD-L1 inhibition in combination with chemotherapy, as well as its usage as a prognostic and predictive marker in breast cancer, which has been connected to poor clinicopathologic characteristics and outcomes[72]. however, using them requires careful monitoring because of certain immune-related adverse events (irAEs)[53].

Cancer risk is influenced by tumor antigens, which are specific to each tumor. White blood cell activity involves receptor activation, inhibition, and cytokine release[28]. Tumor-infiltrating lymphocytes (TILs) support immune defenses against cancer, providing insight into survival and therapies for breast cancer. However, their use as biomarkers is still in its early stages[32]. Research shows that patients with TNBC with a higher peripheral CD4+/CD8+ ratio respond better to treatment. Peripheral blood T-cell subsets predict treatment outcomes, especially when combined with immunotherapy and chemotherapy. Understanding these interactions can aid in treatment stratification[33]. B cells support anti-tumor immunity in many ways. They generate antibodies that disrupt oncogenic pathways or specifically target and eradicate tumor cells[34]. Furthermore, helper T cells get antigens from B cells, which activates subsequent cytotoxic T lymphocyte responses[35].

Here, we report on an observational, cohort, prospective, multicenter, and translational study that aims to find new predictive markers of immuno-chemotherapy response in patients with TNBC, candidates for anti-PD-L1 first-line therapy. Our analysis suggests that several factors may explain the lower Treg percentages observed in TNBC patients compared to healthy individuals. reduced Treg infiltration into the tumor microenvironment could indicate a systemic decrease in Treg levels, potentially resulting from a less immunosuppressive tumor microenvironment in TNBC. moreover, Tregs modulate gene expression within the tumor microenvironment, and lower Treg levels in TNBC could lead to less modulation of gene expression favoring tumor growth. Additionally, the relationship

between Treg levels and immune therapy response could impact TNBC patients' therapy response. Overall, these differences in Treg levels may have implications for tumor classification, progression, and therapy response in TNBC. It highlights the potential for novel treatment strategies targeting Tregs in this subtype of breast cancer[73].In addition, one potential reason for the lower median circulating %memory Treg cells levels seen in our TNBC patient cohort at baseline is that these immune cells may be trafficking to and accumulating in tumor tissues. It has been established that %memory Treg cells possessing an effector memory phenotype, characterized as CD4+CD25+CD127-CD45RO+, readily migrate out of circulation and into non-lymphoid organs secondary stimulation. As tumor tissues effectively act as sites of chronic inflammation and antigenic stimulation, %memory Treg cells are likely migrating into the breast tumor microenvironment. Within tumor sites, %memory Treg cells can then exert potent effects through mechanisms. Therefore, while still playing an important role in dampening anti-tumor immunity locally, this trafficking may explain the relatively lower levels of circulating %memory Treg cells observed in our cohort of TNBC patients at baseline[74].

Also, in our TNBC patient's baseline compared to healthy individuals 14 patients demonstrated lower circulating levels of plasmacytoid dendritic cells (pDCs) (5 Patients) and myeloid dendritic cells (mDCs) (9pz), which could be due to several reasons, as suggested by Paek, S.H. et al, one can be Migration to tumor site as DCs which play a crucial role in initiating and regulating immune responses against tumors. In the context of TNBC, DCs may migrate from the bloodstream to the tumor site, leading to decreased levels of circulation. Differ among breast cancer subtypes, mainly based on estrogen receptor (ER) and human epidermal growth factor receptor 2 (HER-2) status. ER-positive breast cancer patients had higher DC levels, while HER-2-positive patients had higher DC levels in the circulation. These differences may be due to the recruitment of DCs into the tumor microenvironment, potentially depleting them from peripheral blood. However, there was no significant relation between specific DC immunophenotypes and patient prognosis[75].

Our research focused on examining the potential relation between baseline circulating immune cells and pathologic complete response (pCR) in early TNBC patients. Our findings indicate that there is no significant relation between pCR and B and T cell subsets in the peripheral blood. Given our small sample size of only 13 patients, we cannot yet draw any definitive conclusions based on our research. Because subgroup analyses of immune cell subsets require large patient cohorts.

As part of our research, we investigated the relationship between Node involvement status and baseline circulating Immune cells in early TNBC patients. Specifically, we found that circulating CD4+ Effector Memory (EM) cells were significantly higher in the node involvement group (N1, N2) when compared to the node negativity group (N0). Additionally, we observed that T helper 2 (Th2) cells were significantly higher in the node involvement group (N1, N2) when compared to the node negativity group (N0) group, although they were within the normal range circulating baseline. This finding aligns with the concept discussed in an article by Sallusto et al, that memory T lymphocytes contain distinct populations of central memory (TCM) and effector memory (TEM) cells characterized by distinct homing capacity and effector function. effector memory T cells (TEM) mediate immediate effector functions and migrate to inflamed peripheral tissues, while central memory T cells (TCM) are home to T cell areas of secondary lymphoid organs and have little or no effector function[76]. Our observation of higher levels of CD4+ Effector Memory (EM) cells in the node involvement N1 and N2 groups suggests an increased differentiation of T central memory (CM cells) into T Effector memory (EM cells) because the lymph node metastasis may lead to higher stimulation and direct contact from the tumor which may lead to further circulating of Effector Memory, which is associated with essential effector function of eradicating the tumor[76]. Similarly, the higher levels of T helper 2 (Th2) cells in the node involvement N1 and N2 group align with the concept that the direct encounter of Naïve T cells with the tumor metastasis in the lymph node leads to differentiation into Th1 or Th2 and that may be one

of the reasons for having higher circulating Th2[99]. While in the circulating B cell, NK cells, and Dendritic cells we found no relation between the Node involvement status.

We observed a relation between T lymphocytes among individuals in T1, T2, and T3 stages. Notably, these variations were observed within the normal range circulation, we observed a decrease in the frequency of CD8+ and CD4+ Terminally differentiated Effector Memory T cells (TEMRA) in the T3 stage compared toT1 and T2. Conversely, CD8+ Naive cells were significantly lower in the T1 stage compared to the T3 stage in other words circulating naïve CD8+ cells were positively related to the tumor size but circulating TEMRA cells were negatively related to tumor size. Additionally, observed less circulation between CD4+ T helper2 (Th2) cells in T3 versus T1 and T2, and a decrease in the relation between CD4+ T helper17 (Th17) at T3 versus T1 and T2 levels.

Jameson, S.C. et al, suggested that TEMRA cells due to repeated boosting/stimulation of the immune response lead to a more sustained representation of TEM cells in the circulation, explaining their higher frequency at T1 and T2 compared to T3[77]. The drop in frequency of TEMRA cells from T1/T2 to T3 observed in our study could be explained by the terminal differentiation and lack of self-renewal ability of TEMRA cells, as well as less recent stimulation/boosting at the T3 time point providing less support for their maintenance through differentiation from other memory populations or cytokines. As tumors grow, they can suppress naive CD8+ T cells through mechanisms like downregulating MHC expression, increasing immune checkpoint ligands, and secreting immunosuppressive cytokines. Larger tumors deplete the pool of circulating naive CD8+ T cells, causing lower levels of circulation in the T1 stage compared to the T3 stage. This trend is attributed to larger tumors' ability to evade immune detection through various immune evasion strategies[78]. In our study, observed lower levels of CD4+ Th2 and Th17 cells in the T3 tumor stage compared to T1 and T2, DeNardo, D.G., et al suggest that tumors grow from early T1 to later T3 stages, they shape their immune microenvironment in an immunosuppressive manner. Larger tumors induce more immunosuppressive cytokines like TGF-β and IL-10, suppressing the differentiation of Th2 and Th17 effector lineages and impairing Th2 and Th17 cell proliferation and functions. This may explain why these cell types have a decreased relation with tumor stage at later T3 stages[79].

In conclusion, our observational study provides insights into potential predictive immune biomarkers for response to immuno-chemotherapy in triple-negative breast cancer patients. We observed differences in circulating immune cell levels among early TNBC patients that could help explain the immunological characteristics and treatment responses seen in this breast cancer subtype. The purpose of this study is to use multiparametric flow cytometry to assess the immunological environment in patients with early-stage and metastatic triple-negative breast cancer. It seeks to determine immunological variables, evaluate the predictive potential of circulating immune cells as a liquid biopsy tool, and correlate changes in immune cells with the advancement of disease. While our study was limited by a small sample size, baseline levels of specific immune cell subsets may help predict outcomes of immuno-chemotherapy in TNBC. We will continue to enroll more patients and follow up with these patients to assess any potential biomarkers. Further studies are required to validate any future findings. In addition, future research should investigate continuous immune monitoring and examination of tumor tissue to gain a better understanding of the mechanisms involved. Our findings provide valuable insights into the complicated immunological environment of triple-negative breast cancer and its impact on treatment methods targeting the immune system.

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"Each soul is unique, a one-time treasure,

Precious in the eyes of the Creator.

No two people walk the same life path,

No two have the same joy or sorrow.

Your soul print is as unique as your fingerprint,

No one must walk your path but you.

Follow your wisdom, speak your truth,

Let your light shine for all to see.

For in your soul-journey, fulfillment you'll find,

By being the person, you're meant to be."

Rumi (13th-century Persian poet)